Doctoral Thesis

Quantitative analysis of the morphology of neurons in cat visual cortex

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Quantitative analysis of the morphology of neurons in cat visual cortex

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
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Doctor of Natural Sciences

presented by

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2000
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Abstract

A prominent feature of cortical cells ('neurons') is the formation of complex axonal and dendritic trees that innervate the three dimensional cortical space to communicate with other cortical neurons. In this work certain aspects of the trees were investigated and described quantitatively for individual neurons.

The first chapter introduces the basic framework upon which this work should be seen. In a historical overview it is shown that the current understanding of our brain is that of a highly complex network of neurons which are specialised to process and exchange signals with many thousands of other neurons in the network. It is argued that the complicated connectivity pattern, involving about $10^{12}$ neurons and $10^{15}$ contacts between neurons ('synapses') can be largely understood by the study of the three dimensional structure of the dendritic and axonal trees of a relatively small sample of neurons. The main argument is that the cortical network can be viewed as the multiple repetition of a small number of neurons with stereotyped axonal and dendritic trees which are interconnected according to some few principles. For a better understanding of the cortical network it is therefore relevant to describe thesis small number of 'generic' neurons accurately and to learn more about the principles that governs their interconnections.

For our analysis we used neurons of cat primary visual cortex which had been recovered over several years from in vivo experiments. The second chapter outlines in detail the experimental methods that were used to obtain these neurons. The receptive field properties of the neurons were determined with intracellular recordings. Subsequently the neurons were labelled with horseradish peroxidase ('HRP'), so that the complete dendritic and axonal branching patterns could be reconstructed in three dimensions with the aid of a computerised light microscope. Thus we were in the quite rare situation of knowing the receptive field properties of the neurons together with their three dimensional dendritic and axonal structures.

In the third chapter we focused on dendritic trees and investigated to what extent their shape can contribute to the formation of receptive field properties. Livingstone (1998) suggested recently that asymmetries in the tangential spread of the dendrites of a neuron could be responsible for the emergence of direction selectivity. This model makes the strong prediction that the direction of the asymmetry ('dendritic bias') of the tangential spread of the dendrite is correlated with the preferred stimulus direction of the neuron. We quantified the dendritic bias for 30 neurons and showed that no such correlation exists. It is argued that direction selectivity is more likely to emerge from a collective computation of many neurons in a network.
In the fourth chapter the axonal trees were more closely investigated. We were primarily interested in the three dimensional distribution of the small swellings ('boutons') that are formed on the axonal branches and can be recognised at the light microscope level. Boutons are of interest because the presynaptic terminals, which can only be recognised in the electron microscope, are formed at these sites. The boutons of a neuron are not homogeneously distributed in cortical space, but tended to be located in a particular cortical layer and within a layer they tended to be segregated into clusters. We therefore applied an algorithm that determined the bouton clusters of 32 neurons in an objective way. The clusters of each neuron were then studied and described by a mixture of three dimensional normal distributions. The distributions were used to estimate the number of boutons that each cell type contributes to the different cortical layers. It was further tested how far the horizontal distribution of the bouton locations are determined by the receptive field properties of the cell. In addition to the formation of clusters, which agrees well with the hypothesis that cells of similar receptive field properties are connected, the location of the layer 6 pyramidal cells tended to be aligned on an axis that is perpendicular to the preferred stimulus orientation of the cell.

The three dimensional distribution of the bouton cloud provides useful information about the location of contacted cells within the cortex. On a smaller scale the bouton distributions along the axonal branches becomes relevant. This one dimensional distribution, which is studied in chapter 5, can reveal principles of synapse formation between the an axonal branch and the closely apposed neurons along that branch. There are regions on the axonal tree for which the axonal branches form only few synapses with the cells in the local environment. The boutons tend to be located towards the 'leaves' of the axonal tree. The distribution of the distances between neighbouring boutons in this region allows one to think that the bouton placement along a branch could follow a Poisson process (i.e. the boutons are placed randomly along the branch). However, long distances appear to occur more frequently than one would expect from a simple Poisson process. The average interbouton distance could be dramatically different even for cells in the same cell class. We observed that in general the average interbouton distance increases with the percentage of spines that are contacted by the neuron.

The last chapter summarises the results.
Zusammenfassung


Im dritten Kapitel konzentrieren wir uns auf die Dendritenbäume und untersuchen, inwieweit deren Form zur Bildung von Eigenschaften der rezeptiven Felder beiträgt. Livingstone (1998) schlug kürzlich vor, dass Asymmetrien in der tangentialen Verteilung der Dendritenbäume eines Neurons für das Auftreten der Richtungsselektivität des Neurons verantwortlich sein könnten. In ihrem Modell trifft sie die Annahme, dass die Ausrichtung der Asymmetrie in der tangentialen Ver-


Das letzte Kapitel fasst die Resultate zusammen.
Statement

This work uses 3D reconstructions of neurons that were obtained from experiments on anaesthetised cats. The experiments were done by Prof. Kevan A. C. Martin and colleges. The 3D reconstructions were made by John C. Anderson.

I performed the analysis of the 3D reconstructions in chapters 3, 4 and 5 and wrote all the MATLAB code that was necessary for the analysis.

Many ideas of how the reconstructions could be analysed in a sensible way came from John, Kevan and Prof. R. J. Douglas or were born in discussions and lab meetings.

Parts of chapter 3 were published in a paper (Anderson et al. 1999) of which I was a co-author. I defined a sensible measure of dendritic bias and performed the analysis of the correlation between dendritic bias and preferred stimulus direction.

Tom Binzegger
July, 2000
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Chapter 1

Introduction

1.1 The brain as a network of connected neurons

Our current view of the brain is that of a highly complex network of cells (neurons) which are specialised to process and exchange signals. This concept has its roots in the 1820’s when investigations of brain tissue with the light microscope (LM) revealed bits and pieces of cell bodies ('globule') and nerve fibers. Deiters distinguished in 1865 between two kind of nerve fibers originating from the cell body. Protoplasmic prolongation (the dendrites) often branched and becoming thinner when leaving the cell body and the axis cylinder (axon), a thin fibre that does not branch (Shepherd 1991, page 11-12). The view of the cell was still limited to a small region around the cell body. The first complete dendritic trees with apical dendrites (a single process which arises from the cell body, is large in diameter and has a long straight course toward the pial surface) and the local horizontal branching of the axon were discovered with better staining techniques developed by Camillo Golgi in 1873 (Shepherd 1991, page 41-42).

1.1.1 The neuron doctrine

It was then not clear what the function of the two processes was nor how cell bodies and their fibers interconnect. One plausible possibility considered was that the nerve fibers join by anastomosis, forming a continuous net as is known to be the case for the system of veins and arteries ('reticular' theory, Shepherd 1991, page 157-162). Evidence for this kind of connection was made by the observation that many nerve fibers end in fine brushes. However, observations of free endings could also be interpreted as evidence against the reticular theory and would favour the idea that nerve cells were individual, clearly separated units. This idea was motivated by the 'cell theory', introduced by Schwann in 1839, which states that all organs are formed by individual cells. It was finally Ramon y Cajal (Shepherd 1991, page 157-162) who brought up strong evidence that the cell theory also applied to the brain. He simplified the Golgi technique and improved upon it so that the results produced were more constant and more decisive. He revealed fibres whose endings were sharp, suggesting free endings without the fine brushes. A wave of
studies that followed culminated in Wilhelm Waldeyer's review in 1891 (Shepherd 1991, page 181-193), in which he summarised the new findings in a coherent theory ('neuron doctrine') which stated that the nerve cell, 'the neuron' is the anatomical and physiological unit of the nervous system.

Although the neuron doctrine became more and more accepted, it should be noted that the methods available at that time lacked the quality and resolution to solve this problem beyond any doubt. In the 1950's the electron microscope (EM) was first used to demonstrate the absence of a protoplasmic continuity between membranes (Palay 1956). At the same time limitations of the 'neuron doctrine' were also revealed by the discovery of 'gap junctions'. Gap junctions are points of contact between cells separated by a narrow gap, but across this gap there is direct (electrical) signal transmission between the two cells through pore-forming proteins that span the gap. However, the function of gap junction communication between neurons is still poorly understood, mainly because of a lack of tools that help to elicit their function (Perez Velaquez and Carlen 2000).

1.1.2 The Law of dynamic Polarisation

The functional role of the processes were still unknown. Golgi, for example, believed that the protoplasmic elongations ('dendrites') had only a nutritive function and the network was formed by the axon cylinders (Shepherd 1991, page 84-88). The view that essentially survived is that of Cajal, proposed by his 'Law of dynamic polarisation', which states that dendrites, soma and axon conduct nerve impulses. It further clarified the question of the direction of the propagating nerve impulses from the dendrites and soma to the axon. Cajal derived this law from several studies of pathways (e.g. olfactory pathway) where he observed, based on Golgi studies, that the neurons form dendrite-soma-axon-dendrite chains. The direction of the pathway was given by the flow of information which had to start from the peripheral neurons (Shepherd 1991, page 197-198).

The Law of Polarisation was an important contribution to the neuron doctrine. It emphasises the idea of a functional unit of the cell body with its axonal and dendritic processes. However, the existence of dendro-dendritic and axo-axonic synapses or the back-propagation of nerve impulses from the cell body to the dendrites show the limits of the 'Law'.

1.1.3 The synapse

Because the neuron doctrine proposed that the membranes between communicating cells are separated it was necessary to explain how the signals are actually transmitted from one neuron to the other. Such a concept was suggested by Sherrington in 1897. He proposed that the communication between an axon and a dendrite is made at sites of very close apposition with a 'surface of separation' (synapse) in between. The properties of the surface determines the transmission characteristics between cells (Shepherd 1991, page 227-228). EM investigations revealed membrane specialisations at sites of close apposition between an axon and a dendrite which
were interpreted as the anatomical substrate of the functionally defined synapse (e.g. Palay 1956). Further investigations revealed, based on anatomical features, two types of synapse. Type 1 synapses (or asymmetric synapses) have an asymmetric appearance because beneath the postsynaptic membrane the electron-dense staining is much more pronounced than that beneath the presynaptic membrane. In the close neighbourhood of the presynaptic membrane round vesicles can be observed. Type 2 synapse (or symmetric synapses) appear to be symmetric in the electron-dense staining of the two membranes and flattened vesicles can be observed (Gray 1959a; Gray 1959b; Colonnier 1968). The synapses with round vesicles (type 1) were associated with an excitatory effect of the postsynaptic membrane, while the synapses with flattened vesicles were associated with an inhibitory effect (type 2, Uchizono 1965).

1.1.4 Problems in the characterisation of the network

The three concepts formulated in the preceding section describe the brain as a network. The elements of the network, the neurons, are cells that form elongated processes that conduct signals to the cell body (dendrite) and away from it (axon). The transmission of the signal from one neuron to the other happens at close appositions of the processes where synapses are formed. Implicit in the assumption that the brain is a network of neurons is the notion that our capability to perceive the world and act on it is given by the way how the cells are connected and the way how each cell converts an input signal to an output signal. The exploration of this network is therefore a necessary step towards an understanding of the functioning of the brain. A basic characterisation of the network will finally involve for any given neuron the subset of neurons that exchange signals with it.

Such a characterisation is difficult to achieve for several reasons. The number of neurons that participate in the network is immense. This can already be appreciated when one focuses on a small region of the brain of the cat, the primary visual cortex (V1 or area 17). Cell counts of Nissl stained sections show that the number of cells in V1 must be very high, about 27 million (Beaulieu and Colonnier 1983). This number indicates that the strategy cannot be to simply list the subset of contacting neurons for each single neuron. Rather the principle must be found that allows the generalisation of connectivity patterns of all cells from the study of some few cells. There is some evidence (see section 1.2) that the cortex is uniform in the sense that small regions of the cortex contain the same basic set of cells which are connected in the same way and therefore process the information in a similar way ('microcircuit'). The network that is made by the cortical cells could therefore be viewed as the composition of a microcircuit that is repeated many times. If this is indeed true, the high number of cells in the cortex would not be a problem anymore. The study of the circuits of neurons in a local environment would allow for the characterisation of the whole cortical network.

A further problem is the large number of cells in the subset of a given cell. Estimates of the number of synapses in EM studies show that in cat V1 there are about $1.5 \cdot 10^{11}$ million synapses (Beaulieu and Colonnier 1985). If it is assumed
that each cell forms the same number of synapses it would follow that the number of neurons a cell contacts is about 5500. There is recent evidence that the number of synapses that connect two cells is in general rather small, so that each neuron receives convergent input from 100's of presynaptic neurons.

A final problem is the non-ambiguous characterisation of the neurons in the subset. Without a unique characterisation it is not possible to reveal sequences of connected cells. Such a characterisation system is not known to exist. However, it is possible to identify the neurons in the subset by their morphology, the approximate location and functional properties.

1.2 The network as a repetition of a basic circuit

1.2.1 The anatomical uniformity of the neocortex

The cortex is a highly folded sheet about 2mm thick. Starting with Meynert in 1867 it was soon recognised that different regions of the cortex have a different internal structure. Nissl stained sections, cut in a perpendicular direction to the cortical surface appeared to be horizontally layered, caused by differences in density, composition, shape and size of the cell bodies in the different layers. The appearance of the cell pattern in a layer could change for different regions on the cortex. Based on these differences the cortical surface was parcellated into different areas ("cytoarchitecture"). Other criteria like the degree of myelin of intrinsic or extrinsic nerve fibers were also used for the areal classification ("myeloarchitecture"). V1, for example, can be recognised by a distinct white line running through the middle of the section ("line of Gennari"), and the cell bodies are in general smaller compared to other regions. In cat V1 six layers are generally accepted (O’Leary 1941; Henry et al. 1979). In Nissl stained sections layer 1 is a zone of low cell density beneath the pial surface. The border between layer 2 and 3 is difficult to identify. Layer 2 consists mainly of smaller and less intensively stained pyramidal cell bodies and is seven to eight cell bodies thick. Layer 3 is composed of larger pyramidal cell bodies whose size increases towards the border to layer 4. The majority of neurons have a roundish cell body. Layer 4 is subdivided into two parts 4A and 4B on the basis of the size of the cell body (4A has larger cell bodies and is the upper part). Cells of layer 5 are relatively sparsely distributed. The layer is also divided into two parts. Some of the largest pyramidal cell bodies are found in its middle and lower portion (5B). Layer 5A contains smaller cell bodies. Layer 6 consists of cell bodies which tend to be arranged in columns in the direction of the radiating fibers. Layer 6 is also divided into two parts. Compared to 6A the density of cell bodies in layer 6B is lower.

The existence of different cytoarchitectonic areas does not seem to indicate a functional uniformity of the cortex, especially since it was shown that different areas subserve different functions. However, this doesn’t necessarily mean that the internal wiring of the different areas is different. It was already implicit in the work of Cajal and made explicit by his pupil Lorente de Nó that there is a basic architectural arrangement of cortical elements which is recognisable throughout the
1.2. THE NETWORK AS A REPETITION OF A BASIC CIRCUIT

cortical areas of mammalian species (Lorente de Nó 1938; Lorente de Nó 1949). Based on Golgi studies Lorente de Nó ignored the 'unimportant details' of cell number, cell form and size in the different areas. What remained constant was the arrangement of the plexus of dendritic and axonal branches, and therefore the arrangement of the connections between neurons. But what then must be explained is how the functional differences occur. A possible hint was given by the observation that the different areas correlate with differences in the target regions of the efferents of an area and with the differences in the brain regions that innervate the area (Sanides 1972; Creutzfeldt 1977; Mountcastle 1978; Powell 1981). The cytoarchitectural differences then do not reflect a fundamental different of internal wiring, rather they are a consequence of variable density of the input and output fibers or other factors such as developmental constraints (Creutzfeldt 1977). If indeed different regions are internally wired in a similar way one could expect also similarities between them. One such striking similarity is the number of cells in a small vertical column.

It is evident from the Nissl stained sections and implicitly contained in the concept of a parcellation of the cortical surface into homogenous regions (according to cytoarchitectural criteria) that the composition of cells within a layer of an area is similar everywhere. A corollary of this is that the number of cells contained in a vertical column ranging from the pial surface to the white matter is constant within an area (packing density). Indeed this was found to be true in the experiments of Rockel et al. 1980 (for a review see Peters 1987). When measuring the number of cells in a vertical cylinder (30µm wide and 25µm thick) ranging from the pial surface to the white matter, the number of cells counted was roughly constant in the area and ranged between 100 to 110 neurons. Actually, it was found that the packing density of cell bodies is quite constant even across species (Rockel et al. 1980). This number was independent of the area (prefrontal, primary, motor, somatosensory, parietal and temporal cortex) and mammal (mouse, rat, cat, Old World monkey, human). While this is also true for the primary visual cortex of the non-primates, the packing density of primate V1 was roughly 2.5 times higher (260 to 270 neurons, Rockel et al. 1980).

The constancy of cell density between areas was challenged by Beaulieu and Colonnier (1989). They showed that in the cat the number of cells beneath an area of 1mm² is only constant within certain groups of areas, but the numbers between different groups can change by up to 25%. However, even if the variation is within 25%, it is still an amazing finding, especially when one considers the volume differences of the cortices between e.g. cat and human (the human brain is about 40 times bigger).

Another constancy between different areas is the ratio between pyramidal and non-pyramidal cells. The cell body of pyramidal cells at the EM level can be recognised by different features, among them the triangular shape with an apical dendrite directed towards the surface and the absence of asymmetric synapses. In contrast, cell bodies of non-pyramidal cells are roundish, and have asymmetric as well as symmetric synapses. The proportion of pyramidal cells to non-pyramidal cells was found to be roughly constant (64 to 85% pyramidal cells) in motor and
visual cortices of cat, rat and monkey in small sections ranging from the pial surface to the white matter (Winfield et al. 1980; Powell 1981).

The interpretation of the constancy of cell body counts in different areas and species and the constant ratio between pyramidal cells and non-pyramidal cells was interpreted as an expression of a basic anatomical uniformity (as opposed to the cytoarchitectural heterogeneity) of the cortex. It was argued that the constancy in number and proportion (beneath a small surface area) between different areas and species are genetically defined properties (Powell 1981).

1.2.2 Vertical columns in an area

The previous section focused on evidence that the internal wiring of the different areas are similar. If this is really the case it would be enough to understand the wiring of a single area and generalisations to the wiring of the cortical network as a whole could be made. In the following we will focus on the network in an area and discuss evidence that allows the view that this network is composed of a repetition of a basic circuit. Here we focus on V1, although many of the results apply also to other areas (and therefore can also be seen as more evidence for the similarity between areas).

Lorente de Nó’s circuit was confined in a narrow cortical column involving neurons of all layers with input and output connections to other regions. He proposed that this circuit could be revealed anywhere in the cortex. The idea of vertical columns that are repeated over the surface of an area, possibly overlapping and each of them containing the same basic circuit, was supported by physiological findings.

Neurons responding to the same region in the visual space

The discharge of action potentials of cells of V1 can be influenced in a restricted area of the retina by shining light on the area. This area is referred to as the receptive field of the cortical unit, applying the concept introduced by (Hartline 1938) for retinal ganglion cells.

As a recording electrode moves parallel to the surface of the striate cortex, the position in the visual field from which the responses are evoked changes in a predictable manner. The receptive field location of many cortical cells were used to map the visual field onto the cortical surface of the monkey (Talbot and Marshall 1941; Daniel and Whitteridge 1961; Dow et al. 1981; Van Essen et al. 1984) and cat (Bilge et al. 1967; Tusa et al. 1978). This map was called the ‘retinotopic map’. A direct demonstration of the map was made possible by showing the anaesthetised animal a visual stimulus in the form of a polar coordinate system after intravenous injection of radioactively labelled 2-deoxyglucose. The resulting pattern of activity in the visual cortex showed a somewhat distorted Cartesian coordinate system whose lines represented the distribution of activity of the cells (Tootell et al. 1988).

Daniel and Whitteridge (1961) were the first to explore and quantify the map of the whole striate area in the monkey. They described the map by using the
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magnification factor which compares the distances between the visual field and the cortical surface in a given direction (i.e. a distance of \(d\) deg in the visual field corresponds to a distance of \(M \cdot d\) mm on the cortical surface, where \(M\) is the magnification factor). They found that the magnification factor at a given location in the visual field is only dependent on the distance ('eccentricity') of the location to the fovea and that the inverse of the magnification is proportional to this distance. As they showed, this data defines a simple three dimensional ('3D') surface (by stretching the polar coordinate system with the corresponding \(M\)) which, when appropriately folded, resembles the visual cortex. By determining the area of the simple surface they were able to determine the area of the visual cortex. For the cat the magnification factor is also largely dependent on the eccentricity in the visual field. It is about 2.5 at 1 deg eccentricity, about 1 at 5 deg and roughly 0.5 at 10 deg (Albus 1975a).

Based on the same data, Schwartz (1977) derived a mathematical model of the retinotopic map. The model essentially has the form of an analytic function \(w = \log(z)\) which maps a point in the visual field \(z\) onto a point in the (flattened) cortical surface \(w\). The magnification factor is then approximately given by the absolute number of the derivative of this function. This function maps concentric exponentially spaced circles in the visual space (centred at the fovea) into equally spaced vertical lines and radial lines onto horizontal lines. Two such lines intersect at right angles. Limits of this model are given by measured anisotropics in the magnification factor. Measurements in the monkey show that the magnification factor in the vertical direction (in visual space) can be bigger (less than a factor of two) than in the horizontal direction (Tootell et al. 1988).

The retinotopic map is not accurate on a microscopic level. Hubel and Wiesel (1962,1974b) showed that in the monkey the receptive field locations of cells recorded with an electrode penetrating perpendicular to the pial surface do not exactly overlap but are scattered. This indicates that the map is not perfectly two dimensional ('2D'). Both for the monkey (Hubel and Wiesel 1974b) and cat (Albus 1975a) it was demonstrated that the map breaks down in the horizontal direction for small distances. In the case of the cat it was shown that for two cells, horizontally displaced by less than 200\(\mu m\) from each other, no prediction about the direction of the relative displacement of one receptive field centre relative to the other could be made. The magnitude of the displacement ('receptive field scatter') increased with increasing eccentricity (on average about 1 deg at 1 deg eccentricity, about 2.5 deg at 5 deg eccentricity and about 3.5 deg at 10 deg eccentricity). Only when the cells were displaced more than 600\(\mu m\) from each other, the direction and magnitude of the displacement was reflected systematically by a displacement of the receptive field centres.

The average receptive field area also increased with increasing eccentricity (Hubel and Wiesel 1974b; Albus 1975a). The receptive field diameter of the cat was 0.7 deg at 1 deg eccentricity, 2.5 deg at 5 deg eccentricity and 2.7 deg at 10 deg eccentricity (Albus 1975a). The receptive fields of two cells separated horizontally by less than 200\(\mu m\) were contained in a disc in the visual field whose diameter increased with increasing eccentricity (1.2 deg at 1 deg eccentricity, 3.6 deg at 5 deg eccentricity...
and 4.8 deg at 10 deg eccentricity). As already mentioned, the magnification factor decreased with increasing eccentricity, so that the diameter of the discs corresponds to discs of about 3mm on the cortical cortex, independent of the eccentricity and layer (Albus 1975a). It follows that the cells sensitive to a given small region in the visual field are contained in a vertical cortical column of diameter 3mm.

Ocular dominance columns

Many cells in cat V1 get input from the two eyes (Hubel and Wiesel 1959). For some cells the influence of one eye is stronger than that of the other eye. In a vertical penetration of a recording electrode the dominance of one eye does not change for the different cells encountered during the descent of the electrode. For an electrode penetrating horizontally there is a gradual change of eye dominance from the dominance of one eye to an equal dominance of the other eye. V1 is the first site at which the information from the two eyes are mixed. It is therefore plausible to assume that the mechanism that generates binocularity comes from the convergence of thalamic afferents from different eyes onto the same cortical cell. The unequal sampling of the cortical cell from the thalamic afferents of the left or right eye would then determine the ocular dominance (Hubel and Wiesel 1977). Radioactive label injected into one eye of the cat indeed revealed a patchy distribution of the labeled axons in layer 4 (Shatz et al. 1977) which can explain the ocular dominance columns. The patches were roughly 0.5mm in diameter and had a centre-to-centre distance of about 1mm. A cortical cell located close to a patch would be predominantly contacted by the thalamic afferents of this eye and therefore would show a preference for this eye. A cell located between two radioactive labeled patches, formed by two thalamic afferents of the two eyes, would sample from both eyes equally and be binocular. For both the cat (LeVay et al. 1978) and monkey (Hubel and Wiesel 1972; LeVay et al. 1975), it was shown that the cross-sectional patches form a stripe like, irregular pattern in horizontal direction.

Orientation columns

The response of a cell to a bar that is swept over the receptive field is optimal if the bar has a specific orientation ('preferred stimulus orientation'). Hubel and Wiesel showed that cells of similar preferred stimulus orientation are arranged in vertical columns for cat (Hubel and Wiesel 1962; Hubel and Wiesel 1963) and then for monkey (Hubel and Wiesel 1968). The cross section of the orientation columns is round or slab-like and for the cat the diameter is about 100\(\mu\)m (measured along the smaller diameter in the case of slabs). Cells separated by 200\(\mu\)m had orientation differences < 30 deg. The horizontal arrangements of the orientation columns is quite orderly. If a recording electrode was penetrated horizontally, a gradual change in orientation could be observed with a change of 10 deg every 50\(\mu\)m or so which means a periodicity of about 1mm (Albus 1975b). The direction of rotation can change unpredictably in clockwise or counter-clockwise stimulus directions (Hubel and Wiesel 1974a). Occasionally, discontinuous jumps of up to 90 degrees in preferred stimulus orientation are seen (Hubel and Wiesel 1962; Hubel
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and Wiesel 1963; Hubel and Wiesel 1974a). More elaborate techniques allowed the study of the tangential arrangement of the columns. Anaesthetised animals, such as the cat, were presented with a large array of moving stripes of a given orientation after injection of the radioactive label 2-deoxyglucose. The radioactive labeling revealed a tangential pattern of slabs. This was also shown to be true for the cat (Löwel et al. 1987).

The drawback of this technique is that the animal must be killed to visualise the result and therefore does not allow us to show how the same piece of tissue might respond to several different stimuli. A clearer picture of the orientation columns evolved with the development of the technique of optical recording, in which neural activity is detected with a sensitive camera, either after first applying a voltage sensitive dye to the surface of the cortex, or by recording small changes in the reflectivity of neural tissue which occur in response to neural activity. In this way the activity evoked by stimuli of different orientations at different positions across the surface of the visual cortex was recorded (Blasdel and Salama 1986; Bonhoeffer and Grinvald 1991). The discrete jumps of the electrode recordings could be explained by the existence of singularities, where a single set of iso-orientation domains meets at a point or fracture, elongated regions characterised by a high orientation gradient, extending from the singularities (Swindale 1998). The existence of singularities is rare, for the monkey only about 8 per mm² (Swindale 1992).

In contrast to the ocular dominance columns, there was no obvious explanation for the generation of the orientation columns. A first clue came from the study of the axons of cortical neurons by other methods than that of Golgi. In fact, the Golgi method had a serious drawback. Only local axonal collaterals were impregnated using the Golgi technique, so that the horizontal extent of the axonal trees were not recognised for a long time. Evidence that there is more horizontal interaction within an area relied on degeneration techniques. From small lesions within the cortex it was possible to trace degenerating fibers over surprisingly long distances (up to 6mm in certain layers, Fisken et al. 1975). However, the degeneration studies could not differentiate between fibers arising from cells at distant sites, i.e. running within the cortex before giving off terminals, and fibers from intrinsic cortical connections.

Studies of tracing techniques using anterograde and retrograde transport which do not suffer from these limitations supported the view of long range horizontal intrinsic connections. The injection of HRP into a small patch of visual cortex in the tree shrew labelled the axons in a region of about 1mm² in diameter (Rockland and Lund 1982). The horizontal distribution of the axons appeared to be patchy, resembling very much the orientation stripes revealed by radioactive labeling with 2-deoxyglucose (Hubel and Wiesel 1977). Intracellular injection of HRP into individual cortical neurons of the cat indeed revealed long ranging horizontal axonal arborisations which had a patchy appearance (Gilbert and Wiesel 1979; Gilbert and Wiesel 1983; Martin and Whitteridge 1984a).

The patchy appearance of the axonal branches means that the cells prefer to connect to certain groups of cells and avoid connecting with the remaining cells. But what cells were in those groups? Experiments which revealed the relation-
ship between the axonal patches and the orientation columns were controversial. Matsubara et al. (1985) used physiological recordings to get a rough map of the orientation distribution on the surface of cat visual cortex, area 18. Bulk injections of HRP into physiologically identified locations were made and what they found was a preference of the cells to connect to orientations of orthogonally preferred stimulus orientations (Matsubara et al. 1985). An experiment involving radioactive label in the cat area 17/18 showed that bulk injections preferred to label axons located in columns of similar preferred stimulus orientation, i.e. cells tended to connect to cells of similar preferred stimulus orientation (Gilbert and Wiesel 1989). Reasons for these contradictory results are many. Technical difficulties as for example the correct superposition of the orientation columns and the patchy distribution of the labeled axons will influence the result considerably. As pointed out by Kisvarday et al. (1996), the size of the injection site or the location of the injection site can also influence the result. For example cells at a singularity prefer to connect to cells of all orientations equally (Kisvarday et al. 1996).

A refined analysis in cat areas 17 and 18 (Kisvarday et al. 1997) showed that about half (56%) of the connections were made with cells of similar orientation preference (orientation difference < 30 deg). 30% of the connections were made with cells of obliquely orientation preference (orientation difference between 30 deg and 60 deg) and 14% were made with cross orientated cells (difference > 60 deg). For the inhibitory cells slightly different values were revealed (similar orientation: 47, oblique orientation: 34, cross orientation: 19).

Correlation between retinotopic map, orientation and ocular dominance columns

The three column system (response of cells to the same small region in the visual field, response to the same orientation and to the same ocularity) are not independent of each other. For example, as an electrode crosses from one side to the other of a single ocular dominance column in layer 4 of the monkey, there is a clear progression through the visual field in a direction predicted by the topographic map of the visual field. As the border is crossed, the position of the receptive field jumps back in the visual field about a distance half of the width of the column (Hubel and Wiesel 1977). This 'two step forward and one step back' fashion assures that cells of both eyes have access to the same point in the visual space. For both the cat (Hübener et al. 1997) and the monkey (Blasdel and Fitzpatrick 1984; Blasdel et al. 1995) there is also a tendency for the borders of orientation columns to cross the ocular dominance borders at right angles. And recently, a direct correlation between the change of receptive field position and the change in preferred stimulus orientation of cells was reported (Das and Gilbert 1997). The larger the change of preferred stimulus orientation per 100 μm of cortex, the larger is the change in receptive field position per 100 μm or cortex.
1.2.3 Plausibility of repeated circuits

The existence of functional columnar systems makes the hypothesis of a repetition of a circuit, or several circuits, plausible. It is an argument of simplicity to assume that columns in the cortical space that essentially perform the same function do this in the same way, i.e. with the same circuit. This statement was enforced by Mountcastle (1978). He argues that the enlargement of the neocortex that had to be very quick for mammals, especially for primates, happened primarily in horizontal direction while the thickness remained rather constant. Such an enlargement could have been achieved best by the replication of a basic vertical neural module with the same basic circuit in it.

As noted above, Lorente de Nó (1949) emphasised that the plexus of the dendritic and axonal branches is quite characteristic for certain neurons. Indeed, the stereotypical form of the Golgi stained axons and dendrites allowed the distinction of morphologies into different classes. The different classes are normally restricted to specific layers and because it can be assumed that within a layer in an area the cells distribute themselves regularly, one can see that the area can be regarded as the composition of some stereotypically shaped neurons that are repeated over and over in the horizontal direction. This could well be the anatomical reflection of a network that is composed of a basic circuit that is repeated horizontally.

Two broad classes can be distinguished, pyramidal cells and non-pyramidal cells (Peters and Jones 1984). The characteristic feature of pyramidal cells is the apical dendrite. This is a prominent dendritic tree oriented radially and growing vertically from the cell body to the pial surface. The apical dendrite terminates often in an apical tuft where the dendrites ramify more or less extensively. At the base of the apical dendrite, close to the soma, oblique branched dendrites extend as collaterals from the shaft of the apical dendrites. Finally the system of basal dendrites extends outward from the lower portion of the pyramidal cell body. The pyramidal cells can often be recognised by the triangular form of the cell body. However, other shapes of pyramidal cell bodies were also observed (e.g. Feldman 1984). In Golgi studies only local axonal ramifications could be observed because myelinated axons are not stained (e. g. Somogyi 1978). With the introduction of better staining methods such as HRP it became clear that the axons of pyramidal cells can travel several millimetres and distribute their axonal branches in a characteristic, often patch-like way (Gilbert and Wiesel 1983; Martin and Whitteridge 1984a). The pyramidal cells form asymmetric synapses (table 1.1).

The non-pyramidal cells lack of a prominent apical dendrite. Of the non-pyramidal cells two major classes of cells that form spines on their dendrites are the spiny stellate and the star pyramidal cells. These cells occur exclusively within the middle part of the cortex (layer 4) (O'Leary 1941; Lorente de Nó 1949; Lund et al. 1979; Martin and Whitteridge 1984a). Spiny stellate cells can easily be distinguished from the pyramidal cells by the lack of an apical dendrite. The basal dendrites are emitted at multiple points from the cell body and have no orientation preference. Star pyramidal cells are a type in between these two cell classes. They have basal dendrites like spiny stellate cells, but have only a weakly developed api-
cal dendrite. Spiny stellate cells (table 1.1) and presumably also the star pyramidal cells (Lund 1984) form asymmetric synapses. It was proposed that the spiny cells form a continuum in shape ranging from the pyramidal cells to the spiny stellate cells (see Lund 1984). However, the three types differ in many respects from each other. Intracellular labelling of HRP showed that star pyramidal cells have a wide spread and diffuse axonal branching pattern, while pyramidal cells and spiny stellate cells prefer to innervate particular layers (Gilbert and Wiesel 1983; Martin and Whitteridge 1984a). In addition, spiny stellate cells have a much lower spine density than pyramidal cells and have many more excitatory synapses on their dendritic shaft (Anderson et al. 1994a).

Prominent types of neurons with spineless (i.e. smooth) dendrites are the basket cells (Szentagothai 1973), the chandelier (or axo-axonic) cells (Szentagothai and Arbib 1974; Szentagothai 1978a), the double bouquet cells (Szentagothai 1973) and the dendrite targeting cells (Tamas et al. 1997a: Tamas et al. 1998). All these cell types form symmetric synapses (table 1.1). One exception is the double bouquet cell for which the formation of asymmetric synapses were also reported (Somogyi and Cowey 1981). The dendrites of these cells have no apical dendrite, are usually shorter than some 100 μm and can grow radially in all directions from the soma. A characteristic feature of basket cells are the axons which form baskets around the soma of pyramidal cells which are formed by the convergence of 10 to 25 basket cell axons (Somogyi et al. 1983). The axon of the chandelier cells have a very characteristic shape which gave the cell its name. The axon boutons (small swellings on the axon) are arranged in vertically oriented cascades (about 330 in the cat, Freund et al. 1983) like the candles of a chandelier and contact the initial segment of the axon of pyramidal cells. The axon of the double bouquet cells are vertically oriented and confined to a narrow vertical column of several 100 μm, often spanning the whole depth (layer 1 to 6) of the cortex. While the basket cells contact primarily the cell body and the dendritic shafts of the target cells, the double bouquet cells the dendritic spine, dendrite targeting cells are smooth cells that mainly form synapses with the dendritic shafts. The axonal tree either arborises mainly in the close environment of the soma or is radially oriented, elongated and confined to a column. The soma of dendritic targeting cells were exclusively observed in layer 4.

1.3 Search for the basic circuit: Microcircuits

The concept of the synapse implies that a connection can only be made when an axon of one cell and the dendrite or soma of the other cell are in close apposition. This made it possible to reveal possible circuits based on Golgi studies by comparing the location of the sites of the input region (axon) with the sites of the output region (dendrites). The underlying assumption is that an input and an output region at the same location would interconnect. This resulted in a connection scheme of neurons in which the direction of flow of the signals was indicated.

One of the earliest circuits revealed in this way was presented by Lorente de Nó as a semi-diagrammatic drawing of a vertical chain of simplified but represen-
1.3. SEARCH FOR THE BASIC CIRCUIT: MICROCIRCUITS

tative neurons (fig 1.1). It explained the flow of an incoming signal via an afferent into an 'elementary unit' (Lorente de Nó 1949), i.e. a vertical cylinder from white matter to pial surface in which all cortical elements were represented. The connections between cortical elements were such that they could re-excite themselves. As pointed out by Sholl, many possible circuits could have been proposed from the same material and it would have been difficult to justify a specific one (Sholl 1956). What is needed are the formulation of exact rules according to which the circuits are formed, i.e. which subset of the neurons in the network exchange information with any given cell. As becomes apparent in the remainder of this chapter, these rules can roughly be classified into two classes. One class describes the possible subset of neurons in the network that could exchange information with a given cell. These studies involve the analysis of the shape of the dendritic and axonal patterns of stained cells with the LM. This analysis allows us to make predictions about close appositions of axons and dendrites. The subset of the candidate neurons typically are identified by their positions in the retinotopic map or modality maps like orientation columns. However, close apposition is only a necessary condition for a synaptic contact, but it is not sufficient. The other class of rules describes which close appositions actually form synapses between cells, i.e. it describes the neurons in the subset of neurons, defined by rules of the first class, that exchange information with the one cell. Typically, these cells are characterised by their morphology or by functional properties, e.g. if they act excitatory or inhibitory on other cells. These rules describe also the actual substructures of dendrites and axons that are involved in the formation of synapses, e.g. if a synapse is formed on a shaft or on a spine. Because synapses can only be observed with high magnification, the analysis of these rules involve the use of the EM.

1.3.1 Quantitative description of branching patterns

Random patterns

Sholl (1956) emphasised the importance of a quantification of the dendritic and axonal branching pattern so that objective rules could be used for a circuit's justification.

Axonal and dendritic trees appear to be of considerable complexity, so the question arises how they can be described quantitatively. Sholl solved this problem by taking the point of view that the branching pattern is drawn from a random distribution which is essentially of simple form. This is depicted in fig 1.2. Each circle describes the region in which the random pattern of basal dendrites are distributed. Because of a lack of experimental data for the axonal trees, the axon is represented only by local branches. An overlap of a circle and such an axonal branch cannot be interpreted as the existence of a connection. It is only possible to say how probable such a connection is and this probability depends on the probability that a dendritic branch in the circle is actually in close apposition to the axonal branch. One should think of the piece of cortex that is describe by diagram 1.2 as consisting of many thousands of neurons of which each neuron is drawn from one of the random
distributions. So many chains of neurons are actually described by the diagram. But some of the chains are more likely to exist than others.

Sholl characterised the 3D distribution of the dendritic trees of Golgi stained stellate and pyramidal cells by counting the number of intersections $n$ of dendritic branches with concentric spheres of varying radii $r$, measured from the soma along dendritic paths (Sholl 1953; Sholl 1956). He found that the basal dendrites followed all the same general law which can be described by the general form

$$n(r) = S_r \cdot e^{-\frac{r}{\beta_b}}.$$  

(1.1)

where $S_r = 4\pi r^2$ is the surface area of the sphere of radius $r$. According to this law, the number of intersections increases with increasing $r$ until it reaches its maximum at $r_{\text{max}} = 2/\beta_b$, with a maximum value of $S_{2/\beta_b} \cdot e^2$ intersections and then falls off towards zero. This law was shown to be true for the motor and visual areas of the cat (Sholl 1953; Anderson et al. 1994a) and for the visual cortices of the mouse (Haddara 1955) and rat (Larkman 1991). For example the spiny stellate cell of the cat reaches the maximum at about 100$\mu$m from soma with a maximum number of intersections of 40 and falls off to zero after 200$\mu$m (Anderson et al. 1994a). This results in parameters $\alpha_b = 0.0024$ and $\beta_b = 0.020$. Implicit in this description is the assumption that the dendritic trees are spherically symmetric. While this is
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Figure 1.2: Proposed microcircuit of Sholl (1956). Circles indicate the basal dendrites of pyramidal cells. Apical dendrites are indicated by the vertical lines leaving the centre of the circles towards the pial surface (top). Axons are indicated by vertical lines leaving the centre of the circles in downward direction.

to some extent true for the basal dendrites, it is certainly not true for the apical dendrites (relative to the soma). Although Sholl characterised these dendrites too and derived a similar law as for the basal dendrites, the information contained in a description solely based on the radius is rather limited in this case. The same is true for the axonal branching pattern which appears for many cells as highly asymmetric. Technical reasons prevented Sholl from characterising the local part of the axon. The Golgi technique stains the axonal field very incompletely, revealing often only some single fibers distributed locally around the soma. The idea of a statistical description of the cell morphology, as it was introduced by Sholl, triggered many theoretical studies about the probability of connections between cells.

A first approach was made by Uttley (1954a). The basic assumption was that two cells form a synapse at close appositions between the axon of one cell and the dendrite of the other cell. Uttley then calculated the number of close appositions based on Sholl's description of the basal dendrites and different axonal systems. Liley and Wright made a more careful approach following the ideas of Uttley (Liley
and Wright 1994). They treated the dendritic and axonal fibre systems as composed of small straight segments. It is assumed that the location of the segment, its length and orientation are drawn from random distributions which are independent of each other. It is further assumed that the orientation is uniformly distributed on the unit sphere, i.e. that each orientation is equally probable. A close apposition between a dendritic and axonal segment is formed if the axonal segment is close to the dendritic segment (i.e. closer than a critical distance \( s \)). \( s \) was chosen to be two times the summed diameter of the axonal and dendritic segments. Liley and Wright showed that under these assumption the total number of close appositions between an axonal tree located at \( 0 \) and a dendritic tree located at \( r_0 \) is given by

\[
N(r_0) = \frac{\pi s}{4} \int A(r)D(r - r_0) dV(r),
\]

where \( r = (x, y, z) \), \( r_0 = (x_0, y_0, z_0) \), \( A(r) \) is the axonal fibre density (i.e. the total axonal length per unit volume) and \( D(r) \) is the dendritic fibre density. \( A \) and \( D \) can be computed as follows. Let \( \rho_k(r) dV(r) \) denote the probability of an axonal (\( k = A \)) or dendritic (\( k = D \)) segment to be in the small volume \( dV(r) \) centred at \( r \). Denote by \( N_k \) the total number of segments and assume a binomial distribution with parameters \( N_k \) and \( \rho_k(r) dV(r) \) to find a given number of segments in the volume. Further define \( f_k(l) dl \) to be the probability to find segments of length \( (l, l + dl) \). It follows that

\[
A(r) = \bar{l}_A N_A \rho_A(r) \quad \text{and} \quad (1.3)
\]

\[
D(r) = \bar{l}_D N_D \rho_D(r), \quad \text{where} \quad (1.4)
\]

\[
\bar{l}_k = \int l f_k(l) dl
\]

is the average segment length (\( k = A \) or \( k = D \)). As one can see, the average number of close appositions is linearly related to \( s \), the critical distance. Actually it is not at all clear what a good value for \( s \) is. The existence of spines on dendrites and bouton terminaux (i.e. boutons that are displaced some \( \mu m \) from the axonal shaft and connected to it by a small neck) on axons makes the application of a constant \( s \) less appropriate. In addition, recent electron EM investigations show that for most close appositions no synapse is formed (Tamas et al. 1997a; Tamas et al. 1998).

For basal dendrites Sholl's law determines the fibre density \( D(r) \). In this case, the total length of dendritic fibre within two concentric spheres of radius \( r \) and \( r + dr \) (\( dr \) small) is roughly \( n(r) dr \) (assuming that the segment intersect the spheres at roughly right angles). Because the volume between the two concentric spheres is about \( dr S_r \), it follows for the dendritic fibre density

\[
D(r) = \frac{n(r)}{S_r}, \quad r = \|r\|. \quad (1.6)
\]

A recent approach used a similar concept of a contact zone (Hellwig 2000). But instead of working with an estimated distribution of axonal and dendritic arborisations the actual detailed morphologies were used (i.e. instantiations of a hypothetical distribution). In an in vitro study the axonal and dendritic morphology of eight
rat cortical superficial pyramidal cells stained with biocytin were used to calculate the connectivity for pairs of cells. This was done by the reconstruction of the cell morphology in 3D. A pair of cells was chosen and horizontally displaced from each other. For each position the number of close appositions (s about 1\(\mu m\)), which was again taken as an indication of the formation of a synapse, was calculated. For small cell separation the probability to connect (i.e. to have at least one close apposition) is high (0.4 to 0.9), as is the average number of close appositions (1 to 3.5). With increasing distance the probability falls monotonically and is small for cell separations larger than 500\(\mu m\). The total number of contacts that a cell receives on the dendritic tree from other superficial pyramidal cells is primarily made by cells with cell separations between 100\(\mu m\) and 300\(\mu m\) from the target cell. This is because only a small number of cells are in a very close neighbourhood to the target cell and for cells with separations larger than 300\(\mu m\) the probability to connect to the target cell is small (Hellwig 2000).

Other models involved the distribution of pre- and postsynaptic synapses instead of the distribution of axonal and dendritic processes, thus avoiding the introduction of a critical distance \(s\) (Braitenberg and Lauria 1960; Krone et al. 1986; Abeles 1991; Douglas et al. 1995). Loosely following Abeles, the general idea is outlined below. Let us focus on two types of cell, \(i\) and \(j\) for which the presynaptic parts of the axonal tree and the postsynaptic parts of the dendritic tree are characterised by probability distributions. We want to determine the average number of synapses \(N_{ij}(\mathbf{r}_0)\) that the cell of type \(i\) with cell body at \(0\) forms with the cell of type \(j\) with cell body at \(\mathbf{r}_0\). Let \(\mu_{ij}(\mathbf{r})dV(\mathbf{r})\) be the probability of finding in \(dV(x)\) a presynaptic part of the cell at \(0\) whose postsynaptic part is of type \(j\). Further define \(\nu_{ij}(\mathbf{r} - \mathbf{r}_0)dV(\mathbf{r})\) to be the probability of finding a postsynaptic part in \(dV(\mathbf{r})\) of the cell at \(\mathbf{r}_0\) whose presynaptic part is of type \(i\). Let \(\phi_{ij}(\mathbf{r})dV(\mathbf{r})\) be the probability to find a synapse in \(dV(\mathbf{r})\) that is formed between an axon of type \(i\) and a dendrite of type \(j\). Let us choose a synapse in \(dV(\mathbf{r})\). If \(L\) is the total number of synapses the cell of type \(i\) forms with the cells of type \(j\), the average number of such synapses in \(dV(\mathbf{r})\) is, assuming binomial distribution,

\[
l = L \cdot \phi_{ij}(\mathbf{r})dV(\mathbf{r}).
\]

Similarly, the average number of synapses in \(dV(\mathbf{r})\) whose postsynaptic cells are of type \(j\) and the presynaptic part of the cell at \(0\) is given by

\[
m = M \cdot \mu_{ij}(\mathbf{r})dV(\mathbf{r}),
\]

\(M\) the total number of such synapses, and the average number of synapses in \(dV(\mathbf{r})\) with the postsynaptic part from the cell at \(\mathbf{r}_0\) and the presynaptic part of cells of type \(i\) is

\[
n = N \cdot \nu_{ij}(\mathbf{r})dV(\mathbf{r}),
\]

\(N\) the total number of such synapses. The probability to find a synapse with the presynaptic part from the cell at \(0\) and the postsynaptic part from the cell at \(\mathbf{r}_0\) is given by \(nm/l^2\). The average number of such synapses in \(dV(\mathbf{r})\) is given by \(nm/l\). It is assumed that the existence of one synapse in \(dV(\mathbf{r})\) does not influence
the probability of finding another synapse (binomial distribution). The average number of synapses is then given by

\[ N_{ij}(r_0) = \frac{NM}{L} \int \frac{\mu_{ij}(r)}{\phi_{ij}(r)} \nu_{ij}(r - r_0) dV(r). \]  

(1.10)

The examples above make clear how important the spatial distribution of the axonal and dendritic processes are. It is therefore important to understand the principles that govern the shape of the axonal and dendritic branching patterns.

**Correlation of dendritic shape and receptive fields**

In retina there is a good correlation between morphology and physiology. The hope was that similar correlations would be found in the cortex. Such a comparison was made possible by recording intracellularly, characterising the physiology of the cell (e.g. the receptive fields) and subsequently injecting the cell with a dye such as fluorescent markers or the enzyme horseradish peroxidase (HRP) (Kelly and van Essen 1974; Muller and McMahan 1976; Gilbert and Wiesel 1979; Lin et al. 1979; Friedlander et al. 1979). The HRP diffuses throughout the cell into the finest processes and permits a detailed view of the morphology of the cell. This method had several advantages over the Golgi technique. It stained the axonal arborisation completely and the morphology of the cell could be directly compared with the physiological properties of the cell.

Studies of correlations between receptive field properties and morphology revealed no simple laws. For example based on intra-cellular recordings and injection of a fluorescent marker into cortical cells in the cat, it was suggested that pyramidal cells tend to have complex and stellate cells simple receptive fields (Kelly and van Essen 1974). However, further studies did not support these findings (Gilbert and Wiesel 1979; Martin and Whitteridge 1984a). In general, all morphological cell types in all layers can have simple or complex receptive fields. For the cat it was found that all the layers contain cells of simple and complex type (Bullier and Henry 1979; Martin and Whitteridge 1984). With the exception of layer 5 most layers were dominated by simple cells. However, there seems to exist some tendencies. For example the spiny stellate cells in layer 4 generally had simple receptive fields (one exception), as did most of the pyramidal cells of layer 2 and 3. The pyramidal cells in layer 5 had complex receptive fields. Basket cells also have receptive fields of simple or complex type (e.g. Martin et al. 1983; Somogyi et al. 1983).

For the dendrite as well as for the axons various types of correlations with receptive field properties were suggested. Colonnier suggested a correlation between the elongation of dendritic fields in horizontal direction and the preferred stimulus orientation of cells (Colonnier 1968). Similarly, Livingstone hypothesised that the asymmetry of the horizontal extent of dendrites does correlate with the preferred stimulus direction of cells (Livingstone 1998). Gilbert and Wiesel argued that one possible determinant (not necessarily the only one) of receptive field size is the size of the horizontal spread of the dendritic field (Gilbert and Wiesel 1979). Common to all these models is the idea that the receptive field of cortical neurons is determined
by the sampling of the dendrites from the retinotopic map, i.e. from the axons of the relay cells of the thalamus that project to the visual cortex. Although this idea is very attractive because of its simplicity, there is only little evidence for such a sampling mechanism. For the cat, indirect methods suggested a correlation between the elongation of the cortical dendritic field and the preferred stimulus orientation (Tieman and Hirsch 1982; Coleman et al. 1981). However, intracellular recordings with subsequent injection of HRP showed that none of the proposed correlations hold (Martin and Whitteridge 1984b; Anderson et al. 1999). This suggests that the sampling from the retinotopic map is not the only determinant of receptive field properties. For a more detailed discussion see chapter 3.

Correlation of axonal pattern and receptive fields

A positive correlation between receptive field properties and axonal fields were found in the tree shrew. The horizontal extent of the axonal tree of biocytin filled cortical cells were elongated and the axis of elongation was parallel to the preferred stimulus orientation of the cell (Bosking et al. 1997). For the cat similar elongated axonal fields were described (Gilbert and Wiesel 1983). Although only qualitatively, they described the axis of strongest elongation was parallel or perpendicular to the preferred stimulus orientation of the cell. As pointed out by Gilbert and Wiesel, caution must be taken when interpreting the elongations of the axonal fields because of possible anisotropics of magnification factors. For example it could well be that the area innervated by an elongated axon corresponds to a circular area in the visual field. So asymmetries in the visual field are not necessarily reflected on the cortical surface. Leaving that aside, elongated patchy axons are exactly what one would expect for the creation of large receptive fields and end-inhibition (the axis of strongest elongation parallel to the preferred stimulus orientation) or inhibitory side bands (the axis of strongest elongation perpendicular to the preferred stimulus orientation). In both cases the axon would need to connect to cells of similar orientations and skip cells of different orientations, i.e. a patchy behaviour would emerge.

Patchy connections

Thalamic afferents and horizontal connections between cortical cells often appear patchy in their distribution of axonal branches. The existence of patches indicates that some locations are more preferred for innervation than others. Both patch systems could be related to functional maps. The general principles that emerge are that cells tend to prefer to interconnect with target cells of similar properties. For the thalamic afferents the properties are the preference for one eye, for the horizontal connections it is the preferred stimulus orientation.

1.3.2 Identification of pre- or postsynaptic neurons

The existence of a synapse between two cells can only be demonstrated with the EM. Light microscopy can only resolve securely objects bigger than 0.3μm. So EM
studies are needed to recover the cortical chains that are actually present in the cortex. The larger magnification that is involved makes it difficult to relate the observations made on the EM level to the well known morphological features of cells that are seen in light microscopy studies. The soma can be recognised by its size and shape and the appearance of a nucleus. The main dendritic shaft and axon initial segments can be distinguished as they emerge from the soma. The axon, for example, can often be recognised by the myelin that surrounds it. The axonal bouton which emerges from the axon, can be recognised by the vesicles and mitochondria it contains. Dendrites can be identified by parallel running micro tubes. The dendritic spine, spinous projections from the dendritic shaft, can be recognised by the spine apparatus (Gray 1959a; Gray 1959b), lack of mitochondria and by the drum-stick like shape. By studying apposed membranes of axonal boutons and dendritic shafts, somata and spines in the cortex, Gray observed the existence of a region of the membrane that was thickened and associated with an accumulation of vesicles. He concluded that these specialised membrane regions must be the synapse. The cleft between the presynaptic and post-synaptic membrane was about 20 to 30 nm wide.

A harder problem to solve is the identification of a cell type under the EM. The strategy that was taken to solve this problem was to compare significant details of morphological structures as seen in Golgi stained cells and try to find these details again under the EM. For example the somata of pyramidal cells often have a triangular shape with a thick apical dendritic shaft leaving the soma in the direction of the pia. A similar shape can also be recognised under the LM. So it was concluded that the triangular shaped contours seen under the EM belong to pyramidal cells. In a similar way it can be concluded that a synapse formed with a spine must belong to a morphological type whose dendrites have spines. This lead naturally to the question of what type of cells have spiny dendrites. The only way to find an answer to such a question was to study the Golgi pictures in great detail and the appreciation of the possible importance of the detailed structure of the various axonal and dendritic patterns, something that was lost in the simplifications made by Lorente de Nó or Sholl (Lorente de Nó 1949; Sholl 1956). For example it had been customary to classify all non-pyramidal cells as 'stellate cells' (Sholl 1956) (e.g. cells with spines and without were in the same group). A result of a new wave of Golgi studies was the definition of a new classification scheme for cortical cells in terms of the axonal and dendritic arborisations for non-pyramidal cells (Lund 1973; Jones 1981).

Szentagothai was one of the first who studied systematically the microcircuit contained in a cortical column with the EM (Szentagothai 1978a). He determined close appositions between Golgi stained cells and tried to find similar constellations at the EM level. While this strategy of finding the 'best spatial fit' between LM and EM level was successful in the cerebellar cortex and hippocampus, it was more difficult to apply to the cortex. The great diversity of axonal patterns offered too many possible cell types as candidates for a good match.
EM studies of cortical connections

Progress was made by the use of more elaborate techniques, which allowed a direct correlation of observations on the LM and on the EM level. Single cells were injected intracellularly with HRP. The labeled cells were then reconstructed with a LM in order to reveal the morphological type. Subsequently the same tissue was prepared for EM analysis (Somogyi et al. 1979). A result of this procedure is a catalogue of fine structural criteria for the different cell classes that helps to distinguish between structures from pyramidal cells ('P') or smooth cells ('S') in EM preparations. S type dendrites contain numerous mitochondria and often show several synaptic contacts in a single section. In contrast, P type dendrites have a more palely stained cytoplasm and tend to have fewer synaptic contacts on the shaft and contain fewer mitochondria. Sometimes spines can be detected emerging from such dendrites. The percentage of area occupied by the mitochondria is about 20% from the total cross-sectional area of the dendritic structure in the case of an S type. For P type cells it is about 5% (Somogyi et al. 1983; Kisvarday et al. 1985). P type cell bodies often have a pyramidal form with a large nucleus. A major apical dendrite is directed towards the pial surface. The soma is mostly contacted by symmetric synapses. S type somata are packed with mitochondria and are densely covered with symmetric and asymmetric synapses. It was shown that the S type cells contain the neurotransmitter \( \gamma \)-aminobutyric acid (GABA) (Freund et al. 1983; Kisvarday et al. 1985) and are positive to the enzyme precursor glutamate decarboxylase (GAD) (Freund et al. 1983).

This set of criteria was applied to determine the post-synaptic targets of synapses of intracellularly labeled cells. The results of these investigations are summarised in table 1.1. Each row describes the post-synaptic targets of a labeled cell. The first entry ('no') is the reference number. The second entry ('cell') shows the number of synapses for which post-synaptic targets were investigated. The third entry ('type') shows the type of synapse that the labeled cell formed. Asymmetric synapses are denoted by 'as', symmetric by 'sy'. The next four entries show the percentage of investigated synapses on the axon that contacted a substructure such as a spine, a dendritic shaft, a soma or an axonal initial segment. The numbers in the parenthesis are the percentage of parent cells of the substructures that were classified as P-type or S-type. The last three entries show the percentage of analysed synapses that contacted a spiny cell (P-type), a sparsely spiny cell or a smooth cell (S-type). The class of sparsely spiny cells was used by McGuire et al. (1984). It is not clear if this type of cell is inhibitory of excitatory.

The analysis of the post-synaptic target distribution of an identified cell can be summarised as follows. The major targets of the thalamic afferents and the spiny cells (excluding the layer 6 pyramidal cells) are the spines (85%), followed by dendritic shafts (12%). An exception are the layer 4 projecting pyramidal cells in layer 6 that prefer to contact the dendritic shafts (70%) and followed by the dendritic spines (29%). Basket cells prefer to contact dendritic shafts (44%) and cell bodies (36%). Dendrite targeting cells form most of their synapses with dendritic shafts (85%) and sometimes dendritic spines (13%). The sample of double bouquet cells is
not homogenous. While two studies show that they prefer to contact dendritic spines (67%, shafts 33%), two other studies show that they prefer to contact dendritic shafts (80%, spines 13%). With exception of the basket cells, the cell bodies are hardly contacted (< 9%). Where verified, the contacted spines have spiny cells as parent cells. For the contacted shafts, on average 70% of the parent cells were spiny cells. For the layer 4 projecting layer 6 pyramidal cells this means that most synapses are formed on dendritic shafts of spiny cells in layer 4. In fact, this is in agreement with the observation that spiny stellate cells in layer 4 have a significant number of excitatory synapses on their dendritic shafts (Anderson et al. 1994a). The cell bodies that were contacted by thalamic afferents, a layer 4 pyramidal cell and one double bouquet cell, were all smooth cells. In contrast, another double bouquet cell formed all their cell body contacting synapses with spiny cells. A similar preference holds also for the the basket cells (95% of cell body contacting synapses were spiny cells).

These studies reveal a high specificity of connectivity on a small level (of the order of a μm) in the sense that different cell types prefer to contact different substructures. With the exception of the basket cells all cell types avoid to contact the cell bodies. Contacts with spines are preferred by the thalamic afferents, the pyramidal cells in layer 5 and 3, the spiny stellate cells in layer 4 and some of the double bouquet cells. Dendritic shafts are preferred by dendrite targeting cells, the layer 4 projecting pyramidal cells in layer 6 and some double bouquet cells. Only for the basket cells the preference for a particular substructure (excluding the axonal initial segment) is not pronounced. An extreme example is the axo-axonic cell that exclusively contacts the axon initial segment of pyramidal cells (Freund et al. 1983).

Despite this observed specificity, the synapse formation seems to be unspecific with regard to spiny or smooth cells. For the cells in table 1.1, the percent of contacted spiny cells is between 58 and 100% (mean 87%). This range is expected under the assumption that a cell forms synapses with the same probability onto spiny cells and smooth cells. In fact, the population of spiny cells in cat V1 is about 80% (Powell 1981).

Specificity for the synapse location on the dendrite can also be observed. For example basket cells tend to prefer the proximal part of the dendrite and the cell body, while the double bouquet cells and the dendrite targeting cells contact in general the more distal part. This is true for the smooth cells as targets (Tamas et al. 1998) as well as for the spiny cells (Tamas et al. 1997b). A xo-axonic cells contact the output domain (axon initial segment) (Freund et al. 1983).

There are two studies which attempted to identify the cells that projected to a labeled dendrite. The dendrites of two types of cells were investigated, the basket cell and the spiny stellate cell, both in layer 4. The relative proportions of the asymmetric and symmetric synapses on soma, proximal dendrite and distal dendrites for HRP labelled cells were determined under EM for both cell types. The results are shown in table 1.2 (Ahmed et al. 1997; Anderson et al. 1994a). Each row shows the analysis for different parts of the dendrite. The second column shows the number of synapses investigated. The third column shows the density of the synapses on the dendrite. The fourth and 5th columns indicate the percentage of
the synapses on spines and shaft on the proximal or distal region of the dendrite. The number in parentheses show the percentage of asymmetric (as) and symmetric (sy) synapses from the synapses on the spines or shafts, respectively. The last two entries show the percentage of asymmetric (as) and symmetric (sy) synapses. For both cell types the most common type of synapse on the dendrite is asymmetric. The relative distribution of asymmetric to symmetric synapses is different for the distal and proximal region (including soma). In both cases hardly any symmetric synapses can be found on the distal region. Again, this reveals a high specificity in contacts and is consistent with the view that smooth cells prefer to contact the proximal part of the dendrite.

The analysis of the map of synapses on these dendrites was pushed forward one more step by attempting to identify the cell types that project to the dendrite. This was made possible by the observation that different cell types form axonal boutons of different size. Measurements of the cross-sectional surface at the EM level of boutons from cells projecting to layer 4 revealed different typical values for different cell classes (Ahmed et al. 1994; Ahmed et al. 1997). For layer 6 pyramidal cells the mean value was $0.18 \pm 0.27 \mu m^2$, for the spiny stellate cells $0.32 \pm 0.34 \mu m^2$ and for the thalamic afferents $0.76 \pm 0.08 \mu m^2$. Boutons of basket cells forming synapses on somata were different in size ($0.66 \pm 0.7 \mu m^2$) than boutons forming synapses with shafts ($0.47 \pm 0.27 \mu m^2$). With this criteria it was possible to estimate that about 43% of the asymmetric synapses on dendrites and soma of the basket cell came from layer 6 pyramidal cells, 44% from spiny stellate cells and the remaining 13% were from thalamic afferents. For the spiny stellate dendrite, the estimates were that the layer 6 pyramidal cells made up about 45% of the asymmetric synapses, spiny stellate cells about 28% and thalamic afferents about 6%. Of the symmetric synapses about 84% were estimated to be from basket cells.

Additional intracellular recordings showed that basically all cell types in all layers can receive mono-synaptic inputs from the thalamus. The preference for mono-synaptic input is layer 4 and 6, the main termination zones of the thalamic afferents. Indirect inputs were mainly observed in the remaining layers 2, 3 and 5 (Bullier and Henry 1979; Martin and Whitteridge 1984a). As a result of these studies a microcircuit free from guesswork about the connections between cells was proposed (Douglas and Martin 1998).
Figure 1.3: Proposed microcircuit of Douglas and Martin (1998). Smooth cells and their connections are indicated in gray. Spiny neurons and their connections are indicated in black. The numbers to the left indicate the cortical layers.
Table 1.1: Post-synaptic targets of identified cells of the cat. Abbreviation of cell types (first column): The first character indicates the type, 'p' for pyramidal cell, 'sp' for star pyramidal cell , 'ss' for spiny stellate, 'b' for 'basket cell', 'db' for double bouquet cell, 'Ign' for thalamic afferents, 'aa' for axo-axonic cells (chandelier cells) and 'dtc' for dendrite targeting cells. It follows a number which indicates the layer of the soma. 2/3 means cells in layer 2 or layer 3. 'IgnX' is a thalamic afferent of type X, 'IgnY' of type Y. The number in parentheses indicates the number of cells in the sample. * marks cells from in vitro experiments. The remaining cells are from in vivo experiments. 1: Boutons in layer 2, 3 and 5 and boutons distal or proximal to soma did not show any difference in the target distribution. 2: Small basket cell. 4: One of the basket cells was a layer 4 clutch cell. 11: Large basket cell at the border of layer 3 and 4. Boutons in layer 3 were investigated. 12: Clutch cells (small basket cells in layer 4). 15: Pyramidal cells projecting to layers 5 and 6. 16: Large basket cell at the border of layer 5 and 6. 17, 18: Layer 4 projecting pyramidal cells. For further explanation see text.

<table>
<thead>
<tr>
<th>no</th>
<th>cell type</th>
<th>num</th>
<th>spine(P,S)</th>
<th>shaft(P,S)</th>
<th>soma(P,S)</th>
<th>ais(P,S)</th>
<th>P</th>
<th>sparse</th>
<th>S</th>
<th>reference</th>
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<td>1</td>
<td>p3(2)</td>
<td>191</td>
<td>as 87(100,0)</td>
<td>13(54,34)</td>
<td>0.5(0,100)</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Kisvarday et al. 1986</td>
</tr>
<tr>
<td>2</td>
<td>b2/3(1)</td>
<td>64</td>
<td>sy 9(100,0)</td>
<td>53(47,17)</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>3</td>
<td>b2/3(6)*</td>
<td>109</td>
<td>sy 5±5</td>
<td>45±11</td>
<td>50±14</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>4</td>
<td>b2/3(5)*</td>
<td>32</td>
<td>sy 1±10</td>
<td>43±12</td>
<td>52±13</td>
<td>4±3</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>5</td>
<td>db2/3(1)</td>
<td>117</td>
<td>sy 20(100,0)</td>
<td>73(46,8)</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>6</td>
<td>db2/3(3)</td>
<td>66</td>
<td>sy 5(100,0)</td>
<td>86(100,0)</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>7</td>
<td>db2/3(4)</td>
<td>117</td>
<td>sy 69±4</td>
<td>31±4</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>8</td>
<td>db2/3(2)</td>
<td>55</td>
<td>sy 65</td>
<td>35</td>
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<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>9</td>
<td>aa2/3</td>
<td>66</td>
<td>sy 0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>Freund et al. 1986</td>
</tr>
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<td>10</td>
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<td>43</td>
<td>as 74(100,0)</td>
<td>26(73,27)</td>
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<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
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</tr>
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<td>11</td>
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<td>44(84,16)</td>
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<td>0</td>
<td>90</td>
<td>0</td>
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<td>12</td>
<td>b4(2)</td>
<td>321</td>
<td>sy 27(100,0)</td>
<td>53(70,17)</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>13</td>
<td>dtc4(5)*</td>
<td>118</td>
<td>sy 11±7</td>
<td>64±9</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>14</td>
<td>dtc1(1)*</td>
<td>13</td>
<td>sy 15</td>
<td>85</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>15</td>
<td>p5(2)</td>
<td>313</td>
<td>as 80(100,0)</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>16</td>
<td>b5(1)</td>
<td>199</td>
<td>sy 41</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
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</tr>
<tr>
<td>17</td>
<td>p6(2)</td>
<td>151</td>
<td>as 28</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
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<td>Freund et al. 1986</td>
</tr>
<tr>
<td>18</td>
<td>p6(1)</td>
<td>151</td>
<td>as 30(100,0)</td>
<td>70(80,20)</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
</tr>
<tr>
<td>19</td>
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<td>27</td>
<td>as 93</td>
<td>7</td>
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<tr>
<td>20</td>
<td>Ign*</td>
<td>330</td>
<td>as 83</td>
<td>14</td>
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<td>0</td>
<td>90</td>
<td>0</td>
<td>5</td>
<td>Freund et al. 1986</td>
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<td>122</td>
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<td>15</td>
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<td>0</td>
<td>90</td>
<td>0</td>
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Table 1.2: Map of synapses formed on basket cell (b4) and spiny stellate cell (ss4) soma and dendrites. See text for explanation.
Chapter 2

Experimental methods

The focus of the work lies on the analysis of the 3D reconstructions of neurons in cat V1. This chapter presents in detail the methods of the experiments that were carried out to obtain these 3D reconstructions. It basically collects information from several papers (Martin and Whitteridge 1984a; Martin and Whitteridge 1984b; Freund et al. 1985; Douglas et al. 1988; Douglas et al. 1991; Anderson et al. 1994b; Anderson et al. 1999).

Preparations and maintenance of animals

A total of 31 cats of average weight $2.5 \pm 0.6\text{kg}$ (range between 1.75 and 4.0kg, for three cats the weight is unknown) were used for the experiments. All experiments were carried out by K. A. C. Martin and colleagues under the authorisation of animal research licenses granted by the Home Office of the U.K. and the Cantonal Veterinary Authority of Zürich.

In cats, anaesthesia was induced with a gas mixture of 1 – 3% halothane (May and Baker) in oxygen/nitrous oxide (30/70%). The femoral artery and vein were cannulated and the tracheotomy performed with the animals under gas anaesthesia supplemented with Saffan (alphaxolone/alphadolone, Glaxovet). After surgery the cats were paralysed with an intravenous infusion of the muscle relaxants gallamine triethiodide and tubocurarine and were artificially respired with a mixture of oxygen/nitrous oxide (30/70%) and adjusted to maintain the end-tidal CO$_2$ at 4.5%. Anaesthesia was maintained by intravenous Saffan or sodium pentobarbital (Sagatal; May Baker; $2 - 3mg^{-1}kg^{-1}hour^{-1}$). Benzocaine cream was applied to all pressure points.

Electroencephalogram (EEG), blood pressure, heart rate, end-tidal CO$_2$, and rectal temperature (maintained at $37^\circ C$) were continously monitored. The anaesthetic regime kept the EEG in the pattern of slow wave sleep.

Neutral contact lenses were placed over the cornea and appropriate correction lenses were placed in front of the eyes to focus them on the tangent screen or oscilloscope, 1.14m away.
Electrical stimulation

Pairs of varnished tungsten stimulating electrodes were inserted stereotaxically in the optic chiasm (position OC), the optic radiations immediately above the lateral geniculate nucleus (LGN, position OR1), the optic radiations 4 mm below the recording site (position OR2) and in the equivalent OR2 position in the opposite hemisphere (to identify cells receiving input from the opposite hemisphere and the callosal projection neurons). Bare tips of 1 mm were used for the OC and OR1 electrodes and 4 mm for the OR2 and callosal electrodes. The precise placement of the electrodes was determined by recording evoked potentials through the electrode and adjusting the position until a strong visually evoked potential was obtained from the correct position in the visual field. The positions of the OC and the OR1 electrodes were checked by gross dissection in the fixed brain and the positions of the remaining two pairs were seen in histological sections of both hemispheres.

Square wave stimuli of 1 – 5 V for 100 – 200 μs were used for electrical stimulation. The stimulation pulses were triggered after a delay of 3 – 5 ms by a naturally occurring action potential. This resulted in a more consistent activation of the cells being recorded (Henry et al. 1979). Some spontaneously active cells were completely inhibited for a period of 10 ms after the electrical stimulation and could not be activated electrically.

Recording electrode and ionophoresis of HRP

Glass pipettes were filled with 4% horseradish peroxidase (HRP; Boehringer Grade 1) in 0.2 M KCl and 0.05 M Tris (Sigma, St. Louis, MO), pH 7.9. The tip of the recording electrode was bevelled to about 80 – 100 MΩ (at 300 Hz).

A plastic cylinder was placed around the craniotomy (over the post-lateral gyrus between Horsley-Clarke co-ordinates P3 and P6), which was filled with agar and topped with paraffin wax after the recording pipette had been placed in position above the cortex. A stepper motor advanced the pipette in 2 μm steps until an extracellular response was recorded.

In all but a few cells the receptive field characteristics and responses of the cell to electrical stimulation were examined extracellularly by hand-held stimuli or by stimuli displayed on an oscilloscope. This was done because it took at least 15 – 20 min to obtain a plot of the receptive fields, examine the binocular response and to obtain the latency values for electrical stimulation. A stable intracellular recording for this period could not expect to obtain consistently. After studying the cell, an attempt was made to impale it by advancing the electrode in 2 μm steps and passing positive pulses of 1 – 2 nA, while the cell was being visually stimulated. If the cell was successfully impaled, as seen by a drop in the DC potential (40 – 70 mV) and the appearance of large-amplitude action potentials and slow-wave activity, the receptive field was again examined (in order to confirm that it was the same cell) before ionophoresis HRP into the cell with positive pulses of 2 – 4 nA in a 200 ms on / 50 ms off duty cycle for a total duration of about 1 min. Although we could have continued recording intracellularly in many instances, we generally withdrew the electrode as soon as we were satisfied that the receptive field properties were
correct and that enough HRP had been ionophoresed into the cell. In some cases the ionophoretic current inactivated the spike-generating mechanism, although non-spiking potentials continued to be recorded at stable base-line DC levels.

For the thalamic afférents the electrode was advanced toward the white matter until axons were encountered. Based on extracellular recordings the receptive field properties of the axons were examined with hand-held stimuli or stimuli displayed on an oscilloscope. If the recording had been maintained throughout the tests, an attempt was made to advance the electrode into the axon. If this was successful, as seen by a drop in the DC potential of 40mV or more and the appearance of spikes > 20mV, the receptive fields were re-examined. HRP was then ionophoresed into the axon using currents of 2—8nA for up to 5 minutes.

Intracellular recordings were made with Neurolog NL102 preamplifier (Digitimer) and in some cases and Axoclamp 2A preamplifier (Axon instruments, Burlingame, CA, USA). The microelectrode voltage and current were logged by a CED1401 (Cambridge Electronic Design) intelligent interface. The CED1401 also controlled intracellular current injection, and extracellular stimulation. The microelectrode voltage signal was filtered (Kemo VBF/3 anti-alias filter, 24 or 48dB · octave^-1 Butterworth or Kemo VBF/33 elliptic filter, 135dB · octave^-1, frequency is between 0.5 and 0.7kHz) before being digitised (12bit) at 2kHz.

The preamplifier signal was also passed through a threshold crossing spike detector (NL200 Digitimer) whose logic output was led to the CED1401 for on-line construction of peri-stimulus time histograms.

Whenever a recording was stable enough, the receptive field properties were determined by an automatic procedure. Visual stimuli, whose shape and trajectories were controlled on-line by microcomputer (380Z, Research Machines, in-house program written by Prof. Rodney J. Douglas), were generated by a Picasso CRT Image Generator (Innisfree Ltd, USA) for display on a Hewlett Packard P1304A or Tektronix 604 oscilloscope. Their displays were synchronised with the CED1401 and the 380Z. Various experimental protocols and their parameters could be selected by a menu-driven executive program that ran on the 380Z. In particular, any combination of stimulus size, direction, speed and contrast could be selected. The 380Z also maintained a comprehensive database, and provided near real-time data analysis and graphical displays that were used to monitor the progress of the experiments.

All extracellular and intracellular recordings consisted of multiple trials. Extracellular trials were averaged to provide a peri-stimulus time histogram, but each intracellular trial was stored. Each trial consisted of a control period during which a neutral contrast field was displayed, followed by a test period of nearly equal duration during which the stimulus was displayed. For extracellular recordings the actual duration depended on stimulus parameters such as the velocity and the length of the trajectory. The intracellular trials were all 4s duration. The intertrial interval was at least the duration of two trials. The presentation of stimuli was randomised. For analysis of subthreshold events, action potentials were digitally stripped from the intracellular records by truncating them at their bases and then averaging the several trials.
Receptive field mapping

Receptive fields were classified as simple (s type) or complex (c type) using the criteria proposed by Henry (1977) and Henry et al. (1979). The main criteria being that simple cell receptive fields consist of subfields for which either a light stimulus (ON field) or a dark stimulus (OFF field) discharges the cell. Here a light stimulus is a moving light edge or a stationary flashed light bar while a dark stimulus is a moving dark edge or a stationary flashed dark bar. For complex cells light stimuli and dark stimuli discharge the cell everywhere in the field. Simple cells with one subfield (ON or OFF) were denoted by s1, cells with two subfields (ON and OFF) were indicated by s2, and so on. Concentric receptive fields of cortical cells or thalamic afferents were distinguished as ON (on type) if a light stimulus activated the centre of the receptive field and as OFF (off type) if a dark stimulus activated the receptive field centre.

A standard protocol was used to determine orientation and direction preference by using hand-held bars and edges. The range of orientations to which the cell would respond was plotted on a tangent screen and the optimum orientation was taken to be the line bisecting the orientation range. The preferred stimulus direction was taken as the direction of the optimally orientated stimulus for which the larger response was elicited. Based on the strength of directionality, the neurons were classified into three groups, 'non-directional' (when the responses to forward and backward motion were similar), 'direction preference' (when responses were clearly biased for one direction of motion) or 'direction selective' (where the cell responded almost exclusively to one direction of motion). This measure was compared with a quantitative direction index

\[\text{DI} = \frac{A_p - A_{np}}{A_p + A_{np}}\]

which was determined from the post-stimulus time histograms that were made for 23 cells recorded in these experiments. \(A_p\) and \(A_{np}\) are the total number of spikes elicited by the cell when the stimulus moved in the preferred and non-preferred direction over the receptive field. Virtually all the cells that were classified by hand as being in the second and third class had direction indices in the range 0.5 to 1.0. This range corresponds to Livingstone’s (1998) criterion of direction selectivity, i.e. the response to one direction of stimulus motion is at least 3 times larger than the response to the opposite direction.

The width and length of the receptive fields were determined with hand-held bars and edges using the minimal response field protocol (Barlow et al. 1967). The receptive field position was defined as the centre of the rectangle that is defined by the length and width of the receptive fields. All receptive field positions lay within 14 deg of the fovea.

It was further tested to see if a cell shows the properties of end-zone inhibition (formerly called 'hypercomplex' by Hubel and Wiesel, 1965). Such cells diminish the response of an optimal stimulus bar when lengthening the bar. If the response of the cell was completely suppressed, the end-zone inhibition was classified as H. For partial suppression of the response the end-zone inhibition was classified as PH.
Further tests for length summation were also carried out. Cells that respond as well to optimally orientated short bars as to optimally oriented bars had their receptive fields classified as SP ('special complex', Gilbert 1977). Cells that responded best to an optimally orientated long bar were classified as ST ('standard complex', Gilbert 1977).

The binocularity or ocular dominance of cells was assessed using the standard dominance scale of Hubel and Wiesel (1962), ranging from 1 (cell is activated by contralateral eye only) to 7 (cell is activated by ipsilateral eye only). Binocular cells have a value of 4.

In order to classify thalamic afferents as X or Y-type, a battery of tests was applied (Friedlander and Stanford 1984; Martin and Whitteridge 1984a). 1) Responsiveness of the cell to large (20 deg), rapidly moving (> 200 deg/sec) visual stimuli of contrast sign opposite to that of the receptive field centre, 2) linearity of spatial summation within the cell's receptive field, determined using a sinusoidally counter-phased grating pattern that was at the spatial frequency slightly below the cell's spatial frequency cut off, 3) the latency difference of a response at OC and OR1.

Thalamic afferents were classified as X type if they showed linear spatial summation to a counter-phased high spatial frequency grating in their receptive field (i.e. had a demonstrable 'null' response), did not show a consistent excitatory response to the large and fast moving target and had OC - OR1 latency differences of 1.9ms or more. Axons were classified as Y type if they showed non-linear spatial summation (no 'null' response could be found for the counter-phased gratings), showed a consistent excitatory response to the large fast moving target, and had OC-OR1 latency differences of 1.7ms or less.

Histological procedures

Twelve to 18 hours after the first neuron had been recorded, the cat was given a lethal dose of anaesthetic and then perfused through the heart with physiological saline followed by a solution of 2.5% glutaraldehyde-1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The block of tissue containing the intracellularly filled neurons was serially sectioned in the coronal plane at a thickness of 80μm using an Oxford Vibratome.

Shrinkage of the tissue caused by the perfusion was estimated to be 11%. Further shrinkage artefacts were kept negligible in sections containing HRP-filled processes by osmicating (1% osmium tetroxide in 0.1 M phosphate buffer, 45 to 60min), dehydrating with alcohols and propylene oxide and mounting the sections on slides in Durcupan ACM resin (Fluka) under a cover-slip (cured for 2 days at 56°C). This processing allowed the material to be examined at both the light and the electron microscope level.

The estimates of the tissue shrinkage was performed as follows. During an in vivo experiment two vertically oriented tungsten electrodes were placed in V1 with a known distance (1.2mm) between them. After perfusion the brain was removed and sectioned at 80μm thickness (verified with the LM, ×100 oil objective) in the coronal
The section containing the two marks made by the electrodes was processed as described above and the distance between the marks was measured after each stage in the processing. We observed a shrinkage of 11% after perfusion but no more shrinkage after any of the further stages. We also checked for changes in the thickness of the section but found none. This was done by measuring the thickness of the section with the LM (×100 oil objective) after processing and comparing it with the thickness measured from sections taken from the Vibratome. It is important to emphasize that the shrinkage in material prepared by this protocol is very much less than the conventional methods where shrinkage can be as much as 80% (Martin and Whitteridge 1984b).

**Laminar boundaries**

Laminar boundaries where determined using the criteria of Henry et al. (1979). Layer 1 can be recognised in the sections with the LM by its low cell density. The border between layer 1 and 2 is made at the onset of an increased cell density. Layer 2 consists of pyramidal like cell bodies which are relatively small in diameter. The border between layer 2 and 3 was not determined. Towards the border to layer 3 and 4 the proportion of cells with larger cell body diameters increases. Pyramidal cells in layer 4 occur only rarely. The majority of cell bodies are round. Layer 4 can be subdivided into layers 4A and 4B on the basis of cell body size. The larger cell bodies occur in layer 4A (upper part). Cells in layer 5 are sparsely distributed. Layer 5 can also be divided on the basis of cell body size. Some of the largest pyramidal like cell bodies in V1 are found in the lower part of layer 5. The top of these cell bodies form the border between 5A and 5B. Layer 5A contains small pyramidal like cell bodies. The distribution of the cell bodies is irregular in all the layers described so far. In contrast, the cell bodies of layer 6 are arranged in (to the pial surface) vertical columns.

For those cells in which the axon extended over large (> 1mm) antero-posterior distances, it is not possible to show in the coronal projection the correct relationship between the axonal arbor and the laminae boundaries obtained from a single section. Since the relationship of the axonal and dendritic arbours to the cortical layers was of utmost importance we have ensured that this relationship remains as accurate as possible, even if it has meant distorting the thickness of the laminae as shown in the two-dimensional drawings.

**Cell reconstruction**

Neurons were reconstructed by J. C. Anderson in three dimensions with the aid of a LM (Leitz Dialux 22) with drawing tube attachment magnified to ×400 (×40 objective and ×10 eye pieces) attached to an in-house 3D reconstruction system (TRAKA).

TRAKA was written in PASCAL running on a personal computer by Prof. R. J. Douglas and D. Botha. The reconstructions were characterised by a list of data points and stored for further usage. Each data point consists of a code describing the digitised structure (axon or bouton) and its three spatial co-ordinates and thickness
The axonal arborisations are complex and often extend through many histological sections. The measurement error of the digitised structures was estimated by measuring four boutons ten times. The standard deviation was smaller than 0.6µm for all 3 coordinates x, y and z. The data was not rescaled to account for tissue shrinkage.

In order to bring all reconstructed cells into the same coordinate system (x, y, z in fig 2.1 A), the reconstructions had to be rotated around x in order to correct for the inclination of the layers. The rotation angles were determined by eye as follows. If an apical dendrite was present, it was rotated about x (medial - lateral axis) until the dendrite was parallel to the vertical plane (xy-plane). If no apical dendrite was present, the efferent part of the axon was used instead. If no efferent axon was present, but the neuron had axonal ramifications, the cell was rotated around x until the horizontal extent of the axon was parallel to the xz-plane. The rotation angles ranged between 0 deg and 28 deg. There were two exceptions to that, one cell had to be rotated about −33 deg, another about −23 deg. A rotation about z (anterior - posterior axis) was needed for correction of how the tissue section was mounted onto the glass slide in order to reconstruct the cell. The angles ranged between −110 deg to 100 deg. In order to determine the angle, the reconstructed neuron was rotated until either the apical dendrite or the efferent axon was parallel to the yz-plane, or the horizontal axonal extent of the ramification pattern was parallel to the xz-plane.

Retinotopic map

Based on data from 8 cats it was previously estimated that the representation of the vertical meridian in the cortex for a region within 4mm anterior to the representation of the fovea has an angle of about 13.6 ± 2.9 deg to the mid line of the skull (Martin and Whitteridge (1984b), see also fig. 2.1 B and D). The receptive fields of cells in these regions can therefore be mapped onto the cortex by first flipping the mapped receptive field about the horizon (H), then rotating it about 14 deg about the fovea and finally applying a magnification factor that is appropriate for this region (about 1 deg per mm). This is illustrated in fig. 2.1 C and D. If the cell is on the left hemisphere, the rotation is about 14 deg in clockwise direction, if it is on the right hemisphere it is rotated counter-clockwise about the same amount.
Figure 2.1: Mapping the receptive field onto the cortical surface. A: \((x, y, z)\) is a reference coordinate system of the world. A cat looks with the left eye in a direction parallel to the \(z\)-axis onto a plane ('visual field') which is parallel to the \(xy\)-plane and displaced about \(d\) units along the \(z\)-axis. The fovea of the eye looks at the point of the plane which is denoted by \(F\). Also indicated is the vertical meridian ('VM', vertical line through \(F\)) and the horizon ('H', horizontal line through \(F\)). A point \(p\) in the visual field is described by the azimuth ('az') and elevation ('el'). The distance of \(p\) from \(F\) is given as the eccentricity ('ec'). B: Top view of the cortex of the cat. The shaded region shows a part of the left visual field (the shaded region indicated in A) when projected by the retinotopic map onto area 17. Also indicated are the projected F, VM and H. IAP: inter oreal plane. C: Outline of a receptive field of a cell which is located close to \(F\). The small arrow indicates the preferred stimulus direction. D: The receptive field and the small arrow are projected by the retinotopic map onto the cortical surface. This involves a flip of the receptive field around the \(H\)-axis and a subsequent clockwise rotation of 14 deg about \(F\). The projected preferred stimulus direction is then compared with the dendritic field of the cell (indicated).
Chapter 3

Asymmetry of dendritic morphology

Models of receptive field properties often assume that a cell samples input from very specific locations in the retina. One possible way to justify how such specific wiring can occur is based on the assumption that the dendrites connect unselectively to the array of thalamic afferents and the shape of the dendrites determines from which locations in the visual field information is sampled. For example an extensive horizontal dendritic tree can sample inputs from distant visual fields, generating in this way a large receptive field. Similarly, elongated dendritic fields would result in elongated receptive fields and asymmetric dendritic fields would result in asymmetric receptive fields. In this chapter it is investigated to what extent the above mechanism plays a role in the formation of receptive field properties.

3.1 Correlation studies

3.1.1 Receptive field size

For the cortex, Gilbert and Wiesel suggested that large dendritic trees have large receptive fields because they are able to collect input over a wide area of the visual field (Gilbert and Wiesel 1979). Such a correlation was indeed observed for the cat retina (Peichl and Wässle 1979). The bigger the receptive field diameter of retinal ganglion cells, the bigger the dendritic field diameter.

Gilbert and Wiesel's suggestion was based on purely qualitative data. In order to correlate the two measurements one has to quantify receptive field size and dendritic field size. The determination of the receptive field size is problematic because the measurements of the receptive field borders can depend on the stimulus that is used to map the receptive field. For example if a cell responds to length summation, the receptive field length (measured along the axis of preferred stimulus orientation) is dependent on the length of the stimulus. The length of the receptive field and therefore the natural measure of the area of the receptive field are therefore unreliable measurements. It was proposed that the width of the receptive field (measured along the axis perpendicular to the preferred stimulus orientation) is a
more reliable measure of receptive field size (Orban 1984).

The correlation between receptive field size (as measured by the width) and the size of the dendrites was carried out for cells in cat V1 (Martin and Whitteridge 1984b). Intra-cellular records from cells in the cat visual cortex were made and the receptive field size was determined. Subsequently HRP was injected to reveal the dendritic morphology of the cell. The size of the dendritic field was measured by the width of the horizontal extent of the basal dendrites when measured in direction perpendicular to the preferred stimulus orientation of the cell. Twenty four cells were recovered, including cell types from all layers and all receptive field types. The receptive field centres were displaced less than 5 deg in vertical and less than 10 deg in horizontal direction from the fovea. No correlation was found between the two measurements for the 24 cells (fig 3.1 A). It could still be true that a correlation exists for subpopulations of cells, as for example the simple cells in layer 4 which are the major targets of the thalamic afferents. However, it is more reasonable that other factors determine the receptive field size, such as the type of the receptive field or the eccentricity. For example the receptive fields of complex cells tend to be bigger than simple cells (Hubel and Wiesel 1962). Although their measure of receptive field size was the area, later studies supported these findings (Orban 1984). The receptive field size increases with increasing eccentricity (Hubel and Wiesel 1974b; Albus 1975a) and different layers have a preference for different receptive field sizes. The deeper layers (6 and 5) have in general larger fields and the upper layers (4, 3 and 2) smaller receptive fields (Gilbert 1977). In this study the receptive field was measured by the length, but Gilbert reports that the same relation is also true for the area and thus also for the receptive field width.

3.1.2 Orientation selectivity

Relations between dendritic shape and receptive fields were found again in the retina of the cat. Retinal ganglion cells have a weak preference (about 9 times smaller than that of cortical cells) for a specific orientation of a sinusoidal grating (Levick and Thibos 1982). The preferred stimulus orientation of a cell is dependent on its position. There is a tendency of the cells to prefer gratings which were radially aligned, i.e. parallel to the line joining the cell and the area centralis (Levick and Thibos 1982). Because most retinal ganglion cells also have a weakly radially elongated dendritic field, it was concluded that the orientation of the dendritic field elongation correlates well with the preferred stimulus orientation (Leventhal and Schall 1983).

The model of Hubel and Wiesel (1962) assumes that the subfield of simple cells is constructed by the convergence of a set of thalamic afferents whose receptive fields are overlapping and arranged in a row. Such a wiring involves a high degree of specificity. Recent evidence for a specific fine structural wiring of geniculate cells onto a simple cell comes from Reid and Alonso (1995). Their study suggests that geniculate receptive field centres which have strong overlap with the centre of a simple receptive field have a high probability of connection to the cortical cell (67%), as long as the two centres were of the same sign (both off or both on).
3.1. CORRELATION STUDIES

Figure 3.1: Correlation study between receptive field and dendritic field shape of cortical cells. The cells were physiologically characterised by intracellular recordings in vivo, injected with HRP and subsequently reconstructed in 3D with a computerised LM. A. Shown is the scatterplot of dendritic field width and receptive field width. B. For the same cells a scatterplot of preferred stimulus orientation and axis of largest elongation of the dendritic field is shown. From Martin and Whitteridge (1984b).

The smaller the overlap, the smaller the probability of the contact (strong overlap 86%, just overlapping 19%). The probability that two overlapping receptive fields of different sign would connect is very small (6%).

Colonnier (1964) suggested a possibility how such a wiring could be achieved. The receptive field of a cell whose dendritic field samples unselectively from the array of thalamic afferent would show an elongated receptive field if the horizontal extent of the dendrites were elongated. The orientation of the elongation would determine the orientation of the elongated subfield and therefore the preferred stimulus orientation. Colonnier showed in a Golgi study of V1 (of cat, rat and monkeys) that such elongated dendritic fields in fact exist. Most non-pyramidal cells (i.e. 62 from 81, Colonnier 1964) had a length to width ratio of more than 1.5, where the length was the diameter of the dendritic field when measured along the axis of maximum elongation. The width was measured perpendicular to this axis. Essentially all orientations were observed and therefore a purely anisotropic magnification factor effect could be excluded (Colonnier 1964).

However, one has to show that a positive correlation exists between the orientation of the elongated dendritic field and the preferred stimulus orientation of a cell. Two studies aimed to test the presence of such a correlation indirectly by rearing experiments. It was observed that many cells preferred horizontal stimulus orientations when the cat was reared while being exposed to horizontal lines and vertical stimulus orientations when the cat was reared while being exposed to vertical lines. But subsequent Golgi impregnation revealed that the dendrites of layer 3 pyramidal cells of the cats exposed to vertical lines had a preference to be horizontally elon-
gated, and the dendrites of the cats exposed to horizontal lines had a preference to be vertically elongated (Tieman and Hirsch 1982). So although they observed a correlation, it was not the one proposed by Colonnier (1964). In addition, they also observed that the elongation of the dendritic fields of layer 4 stellate cells is weak and is not influenced by the different rearing. This is interesting, because layer 4 is one of the main recipients of thalamic afferents.

The other study, also with cats that were exposed to horizontal or vertical lines, focused on the stellate cells in layer 4 (Coleman et al. 1981) and found an elongation of the dendritic fields in accordance with Colonnier’s model. However, the results of both groups remain difficult to interpret. The essential limitation of this method, as pointed out by Tieman and Hirsch (1982), is that it is not possible to relate directly the preferred stimulus orientation of the cell with the orientation of the dendritic field elongation, because they recorded extracellularly. It is not clear if the Golgi stained cells that had an elongated dendritic field in the horizontal direction had a preferred stimulus orientation perpendicular to this direction.

In their study of a correlation between receptive field size and dendritic field size, Martin and Whitteridge (1984b) tested also the hypothesis of a correlation between preferred stimulus orientation and the orientation of the dendritic field elongation. Because they recorded intra-cellularly with subsequent injection of HRP, they could address this question directly. For each cell they determined the preferred stimulus orientation and also the orientation of the dendritic field elongation of the reconstructed dendritic field. No correlation was revealed for the whole population of cells, sampled from all layers and cell types (fig 3.1 B). As Tieman and Hirsch (1982) noted, for the subpopulation of the layer 3 pyramidal cells of this sample, 5 of 6 cells had a difference between preferred stimulus orientation and orientation of dendritic field elongation that was smaller than 45 deg. Tieman and Hirsch interpreted this as a confirmation of the hypothesis that the preferred stimulus orientation of superficial cells and their orientations of the dendritic field elongation are parallel.

3.2 Direction selectivity

3.2.1 Livingstone's model

Barlow’s model

Many models that explain direction selectivity are based on a model that was proposed by Barlow and Levick (1965) for the rabbit retina. In this model the preference for a direction is generated by a sequence of excitation and (delayed) inhibition of the cell that is triggered by the moving stimuli. If the stimuli moves in the non-preferred direction, first inhibition and then excitation is evoked. Because the inhibition is delayed (or long lasting), it prevents the cell from firing if excitation comes in. If the stimulus moves in the preferred direction, the cell is first excited and makes the cell fire before the inhibition comes into action. For the cortex, several such models were proposed (Heggelund 1981; Koch and Poggio 1985; Berman et al.
3.2. **DIRECTION SELECTIVITY**

1992; Suarez et al. 1995). The direction sensitive cell is excited by some thalamic afferent, the inhibition is mediated by inter-neurons converging onto the cell. The inter-neurons themselves are excited by some thalamic afferent whose receptive fields are displaced in relation to the receptive field of the other thalamic afferent. A candidate for the inhibitory neurons are the basket cells because they have a tangential spread of up to 1mm which is enough to guarantee a spatial displacement in the visual space (Martin 1988).

**Rall’s model**

Single dendrites are also able to compute direction selectivity (Rall 1964; Segev and Rall 1998). For the simple case of a passive dendritic branch with excitatory synapses that are distributed along it, the depolarisation in the soma caused by the activation of the synapses is dependent on the spatiotemporal activation sequence of the synapses. If the synapses are activated one after the other from the distal tip of the dendrites towards the soma, the depolarisation is larger and more delayed than when the activation pattern is in the opposite direction. This difference in depolarisation could be used by the cell to compute the preferred stimulus direction. The dendrites act in this model as a delay line, so that for the preferred stimulus direction the depolarisation of the first activated synapse (the most distal one) needs longer to arrive at the soma than the depolarisation of the most proximal synapse. This delay makes it possible that both depolarisations arrive at the soma at the same time where they are summed, causing a strong depolarisation. An activation sequence in the opposite direction has the effect that the single depolarisations arrive at different times at the soma and the depolarisation of the soma will be less.

**Livingstone’s model**

It is not difficult to see how the models of Rall and Barlow actually could work in concert for a cortical neuron. The synapses that transmit the visual information to the cortical cell must be arranged on the dendritic tree such that a bar, moving in the preferred stimulus direction over the receptive field, will trigger the synapses from distal to proximal (Rall’s model). The proximal synapses are inhibitory, while the more distant synapses are excitatory (Barlow’s model). While there is anatomical evidence for the second assumption of this model, it is less clear how far the highly specific wiring, that is needed in the first assumption, is true.

Recently Livingstone (1998) proposed that this combination of models could be applied to cells in the cortex. She explained, similar to Colonnier (1964) for the preferred stimulus orientation, the highly specific arrangement of synapses on the dendritic tree by an asymmetric arrangement of the dendritic branches in respect to the soma. The idea is that an asymmetric dendrite that samples from the array of the thalamic afferents would automatically result in the desired synaptic arrangement (fig 3.2).

Strong asymmetries in the tangential distribution of basal dendrites are known to exist in the monkey V1 for a special type of pyramidal cells at the border between layer 5 and 6, the Meynert cells (Winfield et al. 1983). These cells can be recognised
by their large cell bodies (average diameter is 30\( \mu m \)) and by their basal dendrites that can extend horizontally up to 0.6\( mm \) (Winfield et al. 1983). However, they are rare, only about 25 (of a total 150000) cells under a square millimetre of cortex (Winfield et al. 1981). But about 30\% of all cells in primate visual cortex respond three times stronger in the preferred stimulus direction than in the null direction (Orban 1984), and direction selective cells are found in all layers (Orban 1984). Livingstone suggested therefore that her model works not only for the most plausible candidate, the Meynert cells, but also for other cortical cells which show a (weaker) dendritic asymmetry of the basal dendrites (Livingstone 1998).

Actually it is not clear if such sampling would indeed produce this arrangement of synapses because the innervation zone of the thalamic afferents in layer 4 is much bigger than the extent of the basal dendrites. However, given that it works, the model predicts the following two properties that are testable. First, the tangential projection of a cell’s dendritic tree is asymmetric, and second, the angle between the direction of largest asymmetry (‘dendritic bias’) and the direction preference of the cell (‘preferred stimulus direction’), mapped to the cortical surface by the retinotopic map, has to be about 180\(^\circ\) deg. We tested how close these two predictions are in agreement with experimental data.

### 3.2.2 Experimental procedure

Intracellular recordings from neurons \( (n = 32) \) in cat V1 with subsequent injection of HRP were made. For each cell the preferred stimulus direction \( (\phi_{\text{pref}}) \) was determined. The labeled dendrites of the neurons were reconstructed in 3D by means of a computer-assisted microscope (see chapter 2). In addition 3 Meynert cells with unknown physiology were reconstructed, one in a kitten, 2 in monkeys. Under anaesthesia, the monkey Meynert cells were retrogradely filled from tiny extracellular ionophoretic injections of biocytin into individual layers of V1. This method fills some neurons in a Golgi-like way. After appropriate survival times, the brains were fixed and processed (by V. Meskenaite) to reveal the biocytin and osmicated and embedded in resin to eliminate differential shrinkage. The cells were then reconstructed in 3D.

The strength of direction preference was qualitatively classified into three classes. Class 1 cells responded equally to a stimulus moved both forwards and backwards (‘non-directional’ cells). Class 2 contained cells which showed a clear bias in the response to one direction of motion but also responded to the opposite direction (‘direction preferential’ cells). Cells of class 3 responded almost exclusively to one direction of motion (‘direction selective’ cells).

In order to correlate the dendritic bias and the preferred stimulus direction, the preferred stimulus direction was mapped onto the cortex by the retinotopic map (see chapter 2).
3.2. DIRECTION SELECTIVITY

Figure 3.2: Livingstone’s model of direction selectivity. It is assumed that the dendritic field is highly asymmetric (indicated by a single branch) that samples the visual information from the thalamic afferents (vertical lines). It is further assumed that the synapses on the dendrites (triangles) are formed in a neighbourhood preserving way so that close locations in the visual space (indicated by the circles in the visual space, the receptive fields of the thalamic afferents) correspond to neighbouring synapses on the dendrite. Consistent with the known anatomy it is further assumed that the synapses onto the soma are inhibitory (open triangles), mediated by inhibitory cortical cells that relay the visual information from the thalamic afferents. A bar moving in the preferred stimulus direction will activate on the dendrite a spatiotemporal sequence of synapses from distal to proximal locations that sum up in the soma to a large depolarisation. A bar in the opposite direction will inhibit the cell and activate the synapses from proximal to distal so that the soma is depolarised only slightly.
3.2.3 Definition of dendritic bias

In order to determine the dendritic bias of the dendritic field, the projection of the line segments onto the tangential plane was used (surface view). For the pyramidal cells only the basal dendrites were considered and the apical dendrite was chopped off 200 μm above the soma. The dendrites of the cells were represented by dots which were equidistantly distributed over the branches. The dendritic bias was defined as follows (see fig 3.3). Let \( L \) be the the radius of the smallest circle, centred at the soma, that circumscribes the dendritic tree of a cell. The 'outer dendritic field' of the dendrite is the set of all dendritic dots \( q \) that lie outside the disc of radius \( \frac{2L}{3} \) (centred at the soma). The dendritic length distribution \( D(\phi) \) of the outer dendritic field was then determined as a function of the direction. Let \( C(\phi) \) be a cone of angular width 30 deg, centred at the soma and with orientation \( \phi \) (i.e. the mid-line of the cone and the positive x-axis in the tangential plane form an angle of size \( \phi \)). The dendritic length distribution \( D(\phi) \) in the direction \( \phi \) is then given by the number of dendritic segments \( q \) in the cone,

\[
D(\phi) = |\{q|q \in C(\phi), |q| > \frac{2L}{3}\}|. \tag{3.1}
\]

The dendritic bias \( \phi_{dend} \) is defined as the maximum \( \phi_{dend} \) of \( D \), i.e.

\[
D(\phi_{dend}) = max_{\phi} D(\phi). \tag{3.2}
\]

As a measure of the strength \( B(\phi) \) of the asymmetry of the outer dendritic field in the direction \( \phi \) we took the difference

\[
B(\phi) = D(\phi) - D(\phi + 180) \tag{3.3}
\]

between the density at \( \phi \) and the density at the opposing direction \( \phi + 180 \). \( B(\phi) = 0 \) means that the dendritic tree is symmetric at \( \phi \) and the larger \( B(\phi) \), the larger is the asymmetry in the direction \( \phi \).

3.2.4 Relation between dendritic bias and preferred stimulus direction

Livingstone’s model implies that the dendritic bias \( \phi_{dend} \) and the preferred stimulus direction \( \phi_{pref} \) should have an angular difference of about 180 deg (see 3.2). We checked this by correlating the dendritic bias and the preferred stimulus direction of the 32 cells. Fig 3.4 shows the tangential projections of the cells, with dendritic bias and preferred stimulus direction indicated. Also shown are an additional 3 Meynert cells for which no physiological data was available and therefore were not used for the analysis. The smallest angular difference between the two directions \( \phi_{dend} \) and \( \phi_{pref} \) shows no significant deviation from uniform randomly distributed angles between 0 and 90 deg (\( p = 0.9 \), Kolmogorov–Smirnov test, fig. 3.5 A, B). This is true for the whole population of the 32 cells and visual inspection shows also that cells of a single type show no tendency for preferred differences. Increases or
3.2. DIRECTION SELECTIVITY

Figure 3.3: Computing the dendritic bias. The basal dendrites of a layer 6 pyramidal cell are drawn in a top view. The soma is the blob at the centre of the field. The dendritic segments were approximated and replaced by equally spaced dots q. A: The dendritic bias was calculated as follows. The length, $L$, is the Euclidean distance in the tangential projection from the soma to the most distal dendritic tip. $\phi$ is the angle between the midline (dashed line) of the cone $C(\phi)$ and the x axis. For a given $\phi$, the summed length ($D(\phi)$) of all dendrites lying within the outer third (shaded area) of a 30 deg cone $C(\phi)$ was calculated. This threshold excluded possible bias from many short dendrites. B: The distribution $D(\phi)$ (shaded for visibility), calculated at 1 deg intervals for the full 360 deg range, is rescaled by $\frac{L}{3D(\phi_{dend})}$ and plotted on a circle of radius $\frac{2}{3L}$. $D(\phi_{dend})$ is the maximal value obtained for $D$. The dendritic bias is defined as the angle $\phi_{dend}$ (deg). The preferred stimulus direction $\phi_{pref}$ of the neuron is indicated by the large filled arrow. The coordinate system is indicated by the x and z axis. Length of x and z axes is 50 $\mu m$.

decreases in the cone width did not alter this conclusion. The result did not change when $D(\phi)$ was replaced by the middle part of the dendritic field

$$D(\phi) = \{|q| q \in C(\phi), \frac{L}{3} < |q| < \frac{2L}{3} \}$$  \hspace{1cm} (3.4)

or the inner part of the dendritic field

$$D(\phi) = \{|q| q \in C(\phi), |q| < \frac{L}{3} \}$$  \hspace{1cm} (3.5)

or the whole dendritic field

$$D(\phi) = \{|q| q \in C(\phi), |q| > 0 \}.$$  \hspace{1cm} (3.6)

It also did not change if the dendritic bias $\phi_{dend}$ was taken as the direction of the longest dendritic branch.

The dendritic asymmetry $B(\phi_{dend})$ in the direction of the dendritic bias $\phi_{dend}$ (closed dots in fig 3.5 C), the dendritic asymmetry $B(\phi_{pref})$ in the direction of the preferred stimulus $\phi_{pref}$ (open squares) and the largest asymmetry $B_{max} =$
max \\phi B(\phi) \text{ (height of vertical lines)} \text{ are compared for the individual cells (fig 3.5 C). It shows that the dendritic asymmetry } B(\phi_{dend}) \text{ is in general very close to the largest asymmetry } B_{max} \text{ while the asymmetry } B(\phi_{pref}) \text{ is normally small or even negative. This indicates that there was no dendritic bias along the axis of preferred stimulus direction. Two exceptions show a large negative value, indicating that the longest dendrites are oriented 180 deg from the preferred stimulus direction, as Livingstone's hypothesis requires.}

The relationship between the dendritic asymmetry } B(\phi_{dend}) \text{ (radius in fig 3.5 D) and the smallest angular distance between } \phi_{dend} \text{ and } \phi_{pref} \text{ (polar angle in 3.5 D) shows again that most dendritic fields do not have a strong asymmetry and where the asymmetries are large (compared to the values in the whole sample) the dendritic bias is not necessarily opposed to the preferred stimulus direction (fig 3.5 D). If Livingstone's model were correct, large dendritic biases and angular differences close to 180 deg would be expected (i.e. all dots would be on the left side close to the horizontal in fig 3.5 D).}

3.2.5 Discussion of results

Intracellular recordings of cortical cells in vivo with subsequent injection of HRP made it possible to correlate the asymmetry of the horizontal extent of dendrites with the cell’s preferred stimulus direction (fig 3.4). No correlation could be detected between these two variables (fig 3.5 A and B). This result was independent of several different quantifications of dendritic field asymmetry (dendritic bias). This lack of correlation could be observed for the whole population of cells but also for cells within a cell class. Of special interest are the spiny stellate cells in layer 4 which are the most prominent target of thalamic afferents. For this class of cells no correlation could be detected (fig 3.5 s4). This is also true when only cells with large dendritic bias were considered (fig 3.5 D).

There are several possibilities that can distort this result. The preferred stimulus direction of the cell had to be projected onto the cortical surface of the retinotopic map in order to compare it with the dendritic bias. For the locations of the receptive fields of the analysed cells the mapping involves a rotation of the preferred stimulus direction by 14 deg. Local distortions of the visual map which would increase or decrease the rotation angle can finally not be excluded.

A further argument for not seeing a correlation is the use of a wrong definition for the asymmetry of the dendritic field. It is actually true that there are many different ways how such an asymmetry could be quantified and each of them will give different directions of dendritic bias. For example, the dendritic bias defined by the direction of the longest dendritic path will be different from the dendritic bias that is defined by taking into account the distribution of the dendritic length per unit area. We therefore tested different definitions of dendritic bias which all resulted in a lack of correlation. However, we can not be certain that there is no sensible definition of dendritic bias that also reveals a correlation.
3.2. DIRECTION SELECTIVITY

Livingstone’s model

The model of Livingstone (1998) predicted a dendritic bias that is about 180 deg opposed to the preferred stimulus direction of the cell. The lack of a correlation between dendritic bias and preferred stimulus direction therefore doesn’t support the model. As fig 3.4 (A and B) shows, the angles are distributed rather uniformly in the possible range between 0 and 180 deg. In the context of Livingstone’s model it appeared to us as appropriate to detect asymmetries of long delay lines. We therefore defined dendritic bias by the direction of largest dendritic length of the outer dendritic field that is contained in a cone that rotates around the soma. The strength of the dendritic bias was measured by the difference in dendritic length contained in the outer third part of the cone that is oriented in the direction of the dendritic bias and the dendritic length that is contained in the outer third part of the opposing cone.

The largest difference in total dendritic length between two opposing cones was 250 μm. If this difference were caused by a single dendrite, it would have been 250 μm longer than the dendrite on the opposing side. In general the difference was usually caused by the summation of several dendritic fragments, so that individual dendritic lengths in the two opposing cones were rather similar. This means that the asymmetry is in general not caused by the existence of dendritic paths that are much longer than all others. It is also intriguing to note that the difference of the lengths in the two opposing cones was often close to 0 when the axis of the cones were orientated parallel to the preferred stimulus direction (fig 3.5 C).

Simulation studies

Further evidence not in agreement with Livingstone’s prediction comes from simulation studies (Anderson et al. 1999). A detailed reconstruction of a Meynert cell (fig 3.4, third cell from the left in bottom row) from the monkey V1 was used to create a detailed compartmental model of the soma and dendrites. A very asymmetric case was used by assuming that the dendrites with the longest dendritic path (770 μm) was the only dendrite that sampled from the retinotopic map (2000 excitatory synapses on the dendrites, 100 inhibitory synapses on the soma). As expected it was possible to compute the preferred stimulus direction from the voltage difference seen in the soma for a bar moving in the optimal or non-optimal direction (peak voltage difference about 12 mV for a bar velocity in the range 30 – 40 deg/sec (assuming that the cell is located at 10 deg – 15 deg eccentricity). The cell responded best for stimuli velocity of 77 deg/sec. However, cortical cells located in the same region respond best to slower velocities, about 10 deg/sec (Livingstone 1998). This suggests that the mechanism proposed by Livingstone needs too high stimulus velocities to compute the preferred stimulus direction optimally.

It is concluded that there is little evidence that the asymmetry in the tangential organisation of the dendrites can contribute much to the computation of the preferred stimulus direction. It is more likely that direction selectivity involves a collective computation of a network of cells.
3.3 Conclusions

It is concluded that there is little evidence that the asymmetry in the tangential organisation of the dendrites can contribute much to the computation of the preferred stimulus direction. It is more likely that direction selectivity involves a collective computation of a network of cells.
3.3. CONCLUSIONS

Figure 3.4: Dendritic bias of the 35 cells. The basal dendrites of 35 reconstructed cortical neurons as seen from the cortical surface are shown. The shaded area indicates the dendritic length distribution of the outer part of the dendritic tree as it was defined in fig 3.3. The dendritic path from soma to the most distal tip is drawn in bold. Filled dots indicate the dendritic bias ($\phi_{dend}$). Arrows and arrowheads indicate the preferred stimulus direction $\phi_{pref}$: a filled arrow (with neck) indicates a cell whose strength of direction preference was in class 3; a filled arrowhead indicates a cell in class 2 and an open arrowheads indicates a cell of class 1 (non-directional). In addition, three Meynert cells were shown. The two monkey Meynert cells (mm) and the cat Meynert cell (mk) were not recorded physiologically. For abbreviations see table 1.1. Scale bar = 100µm.
Figure 3.5: Relationship between dendritic bias and preferred stimulus direction. A: Filled dots show the smallest angular difference (positive) between the dendritic bias $\phi_{dend}$ and the preferred stimulus direction $\phi_{pref}$. For non-directional cells (class 1) the differences for both directions are shown (open dots). B: Histogram of the smallest angular differences between $\phi_{dend}$ and $\phi_{pref}$ (bin size 30 deg) as shown in A. Values larger than 10 or smaller than 2 (dashed horizontal lines) are highly unlikely when a uniform random distribution is assumed ($p = 0.045$). C: The asymmetry $B(\phi)$ of the dendritic field in the direction of the dendritic bias ($\phi = \phi_{dend}$, dots) and preferred stimulus direction ($\phi = \phi_{pref}$, squares) are shown. The maximal value of asymmetry $B_{\text{max}} = \max_\phi B(\phi)$ is indicated by the height of the vertical lines. Class 2 and 3 cells are indicated by filled dots, class 1 cells (non-directional) by open dots. Abbreviations of cell types as in fig 3.4, me: Meynert cells). D: Polar plot of the asymmetry along the dendritic bias (radius of length $B(\phi_{dend})$) and the smallest angular difference between $\phi_{dend}$ and $\phi_{pref}$ (polar angle).
Chapter 4

Spatial distribution of boutons

While the axon can have an extent of several millimetres and is best observed at the LM level, the synapses are very small and can only be recognised with the EM. However, the presynaptic components of cortical synapses are located on swellings of the axons ('boutons'). These boutons are either directly attached to the side of a continuing axon ('boutons en passant'), or arise at the termination of a small axonal branch ('boutons terminaux'). Boutons are roughly 0.5 to 3μm in diameter (Kisvarday et al. 1985; Gabbott et al. 1987) and each gives rise to between 0 and more than 3 synapses (most values are between 1 and 2), dependent upon the kind of neuron from which the axon arises or on the target the contact is made (Gabbott et al. 1987; Anderson et al. 1994b; Freund et al. 1985).

Because of their relatively large size, boutons are visible in suitably prepared histological material using a conventional LM. Thus, the spatial distributions of the boutons provide a convenient estimate of the global spatial distribution of the synapses made by an axon. This chapter characterises this spatial distribution. Emphasis is on a quantitative approach. The boutons were reconstructed and digitised in 3D. A cluster algorithm is applied to the data and a quantitative model is derived for the bouton clouds of individual cells.

The neurons (n = 30) examined in this study were obtained from V1 of anaesthetised adult cats. The receptive field properties of the neurons were obtained by intracellular recordings. Subsequently HRP was injected intracellularly in order to label the cells (see chapter 2).

4.1 Qualitative description of the axonal tree

The axonal trees and the boutons that were formed on the axonal trees were studied. The sample contained the main types of cells from layer 2 to 6. Fourteen pyramidal cells, 3 spiny stellate cells, 8 basket cells, a star pyramidal and a double bouquet cell. In addition, the afferents of 3 relay cells of the dLGN entering V1 were reconstructed.
Receptive field properties

The receptive field properties of the reconstructed cells are listed in table 4.1. The first column ('label') shows the label that is referred to in this work in order to identify the cell. The small characters indicate the type of cell. 'p': pyramidal cell, 'sp': star pyramidal cell, 'ss': spiny stellate cell, 'lgn': thalamic afferent, 'b': basket cell and 'db': double bouquet cell. The subsequent number indicates the layer of the cell body. In general the layers 2 and 3 are viewed as one layer, indicated by '2/3'. The capital characters indicate the order within a class of cells. The second column ('cell name') shows the cell name as it is used in the laboratory. The third column ('hemis') indicates the hemisphere in which the reconstructed cell was located. The remaining columns describe the receptive field properties of the cells. The fourth column ('type') describes the receptive field types, following the classification scheme of Henry et al. (1979). 's' followed by a number n: simple cells with n subfields, 'c': complex cells, 'off': off centre - on surround receptive fields, 'on': on centre - off surround receptive fields. The next column ('oc') indicates the ocular dominance of the cell by a number between 1 (cell dominated by contra lateral eye) and 7 (cell dominated by ipsi lateral eye). A value of 4 indicates a cell that is driven by both eyes equally well (Hubel and Wiesel 1962). The next column ('orient') describes the preferred stimulus orientation of the cell in degrees, measured from the horizon in the visual field in counter clockwise direction. The column 'direct' lists the preferred stimulus direction of the cell. The number describes the angle of a vector. The cell responded best to a bar which was moved into the direction in which the vector points. The angle is measured in degrees, in counter clockwise from the horizon. The number in parentheses indicate the directionality ('1': non-directional, i.e. the response to forward and backward motion were similar, '2': direction preferential, i.e. the responses were clearly biased for one direction of motion, '3': direction selective, i.e. when the cell responded almost exclusively to one direction of motion). The column 'sum' indicates length summation ('yes' for ST type, 'no' for SP type). The last column ('end inh') indicates end-zone inhibition ('yes' for H or PH types, 'no' if no end-zone inhibition was observed). See chapter 2 for a detailed explanation of the different receptive field properties.

Thalamic afferents

The visual information from the retina is relayed in the dLGN and transmitted to V1 by the thalamic afferents. Their main innervation zone is layer 4. Layer 6 and layer 3 are also innervated, but weaker. Physiological evidence suggest that cells in all layers can receive direct thalamic input (Martin and Whitteridge 1984a). Within layer 4 the axonal terminal pattern can be constrained to the lower part (layer 4B), the upper part (layer 4A) or throughout layer 4 (Freund et al. 1985; Humphrey et al. 1985). Two main classes of thalamic afferents (X and Y types) can be distinguished using physiological criteria. A distinguishing feature are latency differences. The Y types have a difference smaller than 1.8ms in latency for responses elicited at the optic chiasm and in the optic radiation just above the LGN. X cells have a latency larger than 1.9ms (Bullier and Henry 1979; Freund et al. 1985). The two types
4.1. QUALITATIVE DESCRIPTION OF THE AXONAL TREE

have also different morphological characteristics. The arborisation of the Y type is 3 to 4 times larger than that of the X types and the main branch at the point of entry is about twice as thick (3 to 4μm).

The innervation preference of the three reconstructed thalamic afferents in our sample supported the view that layer 4 is the main innervation zone. When seen in the coronal plane (fig 4.1), the axonal branches where mainly confined to layer 4 and sometimes a second minor innervation zone in layer 6. The distribution of branches was patchy for all three cells. One was an X type (Ign:A), the remainder being Y types. The X-type had an OFF-centre receptive field. Both Y-afferents had an ON-centre receptive field. The X-afferent was the only afferent which did not innervate layer 6.

Layer 4 spiny cells

The main recipient of the thalamic afferents are the spiny cells in layer 4, although smooth cells are also contacted. In our sample, all but one of the 5 spiny cells in layer 4 were simple cells, the remaining one a complex cell. This reflects the fact that most cells in layer 4 are simple. Based on morphology, the spiny cells in layer 4 can be roughly divided in three types (Martin and Whitteridge 1984a). The pyramidal cell in layer 4 (n = 1, fig 4.2 p4) has an axon that innervates layer 2 and 3. Some branches descend and reach layer 6. The receptive field was complex. The second group are the star pyramidal cells (n = 1, fig 4.2 sp4). The axonal ramification is widely branching and very diffuse. The receptive field was simple, but complex receptive fields were also found ((Martin and Whitteridge 1984a)).
The last group are the spiny stellate cells \((n = 3\), fig 4.2 ss4:A-C). The axon of the spiny stellate in our sample ramify locally in layer 3 and layer 4 and have long horizontal patchy ramifications, also innervating layer 3 and layer 4. This pattern was also observed previously (Gilbert and Wiesel 1979; Martin and Whitteridge 1984a; Gilbert and Wiesel 1983). The spiny stellate cells had a simple receptive field although one with a complex receptive field was also found (Anderson et al. 1994b). The spiny stellate cells in our sample had a descending axon to layer 6 which did not enter the white matter.

**Figure 4.2:** Coronal view of reconstructed spiny cells in layer 4.

### Pyramidal cells in layer 2 and 3

The reconstructed axons of the pyramidal cells in layers 2 and 3 \((n = 5\), fig 4.3) innervated the local environment of the soma, extended horizontally in layer 2 and 3 and also projected to layer 5 and sometimes layer 4. The axons formed distinct clusters. Similar patchy axonal distributions have already been described (Martin and Whitteridge 1984a; Gilbert and Wiesel 1979; Gilbert and Wiesel 1983). In Gilbert and Wiesel (1983) a pyramidal cell was found whose axon was entirely...
4.1. QUALITATIVE DESCRIPTION OF THE AXONAL TREE

restricted to layer 3, although this may be exceptional. Of the 5 reconstructed cells in our sample two had a simple and two a complex receptive field. For one cell (p2/3:C) the physiology is not known.

Layer 5 pyramidal cells

Three basic patterns of innervation of the layer 5 pyramidal cells have been described (Martin and Whitteridge 1984a). The axon can innervate predominantly the superficial layers (layers 2, 3 and sometimes layer 1), predominantly the deep layers (layers 5 and 6) or both. The pyramidal cell reconstructed here (fig 4.4 p5) belongs to the last group. It ramified extensively in the local environment of the soma in layer 5 but also projected extensively to layers 2 and 3 where it made distinct clusters. Layer 5 has the highest proportion of complex cells (Hubel and Wiesel 1962; Henry et al. 1979; Martin and Whitteridge 1984a). The reconstructed pyramidal cell was simple. It lacked an efferent axon.
Layer 6 pyramidal cells

Six of the reconstructed pyramidal cells with soma in layer 6 \((n = 7)\) had an axon that mainly ramified in layer 4, avoiding the deep layers and the local region around the soma \((4.4 \text{ p6:A-F})\). Three dimensional rotations show that the horizontal extent of the axons in layer 4 were either restricted to a local region of the apical tuft \((\text{p6:B, p6:C, p6:E and p6:G})\) or showed some considerable horizontal spread within layer 4 \((\text{p6:A and p6:D})\). With one exception \((\text{p6:A})\), these cells had an efferent axon leaving the white matter and probably innervating the LGN \((\text{Katz 1987})\).

One layer 6 pyramidal cell did not project to layer 4. Its axon was restricted to the deep layers, mainly the local region around the soma \((\text{p6:F})\) and constitutes another subclass of pyramidal cells in layer 6. The part of the axon leaving the white matter is assumed to project to the claustrum \((\text{Katz 1987})\). A figure of this cell was already published in Martin \((1988)\).

The innervation of layer 4 by pyramidal cells in layer 6 has previously been noted \((\text{Gilbert and Wiesel 1979; Gilbert and Wiesel 1983; Martin and Whitteridge 1984a})\). However, recently, layer 6 pyramidal cells were revealed with axons innervating layers 2 and 3 \((\text{Hirsch et al. 1998})\). It was suggested that complex pyramidal cells in layer 6 prefer to contact cells in layer 2 and 3, while simple layer 6 pyramidal cells prefer to innervate layer 4 \((\text{Hirsch et al. 1998})\). From our sample of layer 6 pyramidal cells with ascending axons, two innervated layer 3 in addition to layer 4 and had complex receptive fields \((\text{fig 4.4 p6:C and E})\). Three of the remaining 4 cells \((\text{fig 4.4 p6:A, B, D and G})\), whose axon were restricted to layer 4, had simple receptive fields. For the remaining cell no physiology was obtained \((\text{table 4.1})\). However, Hirsch et al. \((1998)\) pointed out themselves, that this is not a strict rule. There are complex layer 6 pyramidal cells that innervate mainly layer 4 \((\text{McGuire et al. 1984; Martin and Whitteridge 1984a})\).

Smooth cells

Of the 9 smooth cells in our sample 8 of them were basket cells and one was a rare example of a double bouquet cell of layer 2 or 3 \((\text{fig 4.5 db2/3})\). The double bouquet cell had a simple type receptive field. The axon was exclusively vertically oriented, traversing all layers from 6 to 2 and confined in a vertical column, which gives it the defining shape \((\text{Somogyi and Cowey 1981})\). The figure of this cell was already published in Martin \((1988)\).

It was estimated that about 20% of the GABAergic neurons are basket cells \((\text{Kisvarday 1992})\). However, considering the fact that most filled smooth cells were basket cells, this estimate seems to be rather conservative. Based on the size of the soma, the basket cells can be divided into three groups \((\text{Kisvarday 1992})\). The large basket cells have a cell body of about \(30\mu m\) and are observed in deep and superficial layers. The cells of the second type have a soma diameter of about \(20\mu m\) and appears exclusively in layer 4 \((\text{clutch cells})\). The cells of the third type have soma diameter of about \(10\mu m\) and are observed in the superficial layers. Typically basket cells strongly innervate the local environment around the soma. However, large basket cell axons typically form long arms of up to \(1mm\), running horizontally
4.1. **QUALITATIVE DESCRIPTION OF THE AXONAL TREE**

in the layer of the soma. In addition, many basket cells have vertically projecting collaterals, innervating other layers.

The layer 5 basket cell (fig 4.5 b5) forms a long arm in layer 5. It could therefore be a large basket cell. It had a simple type receptive field. The axon was restricted to layer 5 and sent some collaterals to layer 6. Layer 5 basket cells projecting to the superficial layers were revealed in earlier studies (Kisvarday et al. 1987).

Two of the 4 basket cells had a complex receptive field (b4:A and b4:D), one was simple (b4:C) and one had a thalamic type off-centre on-surround receptive field (b4:B). The main axon was restricted to layer 4 and only occasional collaterals
entered the superficial or deep layers. The axonal field lacks of large horizontal arms which suggests that they are clutch cells.

Only one of the basket cells in layer 2 or 3 provided physiological data (b2/3:B). It had a simple receptive field. Again the local environment is strongly innervated. In addition, one of the basket cells in layer 2 or 3 (fig 4.5 b2/3:B) ramified strongly in layer 5. It is reasonable to assume that b2/3:B is a large basket cell and b2/3:A and B are small basket cells.

Figure 4.5: Coronal view of reconstructed smooth cells.
### 4.1. Qualitative Description of the Axonal Tree

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<tr>
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<td></td>
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<td>right</td>
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<td>s1</td>
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<td>c</td>
<td></td>
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<td>51(2)</td>
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<td>c</td>
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<td>1.3×1.3</td>
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</table>

Table 4.1: Receptive field properties of the different cells. For explanation see text.
CHAPTER 4. SPATIAL DISTRIBUTION OF BOUTONS

4.2 The determination of clusters

The spatial distribution of the axonal branches are rather non-uniformly distributed in space. Often the axons are arranged in tight clusters (e.g. the thalamic afferents, or the pyramidal cells in layers 2, 3 and 5). In the coronal view the clusters were often hidden and only the top view could reveal the highly structured distribution of boutons (e.g. 4.4 p6:A). The axonal clusters indicate quite precisely the bouton clouds. This was shown in previous studies were the axonal branches and the boutons were treated separately (Gabbott et al. 1987). This was also true for this study, some exceptions being where the axon formed a cluster but no boutons were seen (e.g. 4.3 p2/3:A, the cluster in the lower right corner), probably due to incomplete filling of distant collaterals. Because the bouton clusters are such a prominent feature of many cells, a description of the bouton distribution will involve some characterisation of these clusters.

The appearance of clusters were described in many studies but an objective measure was not applied which determined which bouton belongs to a cluster and which not. For tight and well separated clusters this is not a problem. But as soon as the clusters are close together or more diffuse, the decision about the borders of a cluster and the number of clusters in the distribution is a matter of opinion unless some strict rules are applied. Therefore we used a cluster algorithm in order to determine the subset of boutons which form a cluster.

4.2.1 The cluster algorithm

Principle component analysis

Before the cluster algorithm is discussed we need to introduce the 80%-ellipsoid of a set of points in the 3D space. The principle component analysis, applied to this set, determines an ellipsoid whose axis is the principle axis of the data points, the diameters are about twice the standard deviations of the data in the principle axis system and the origin is the centre of gravity of the data. The 80%-ellipsoid is a scaled version of this ellipsoid that contains about 80% of the data points. If the bouton cloud is normally distributed, the (theoretical) scaling factor for the ellipsoid is 2.2.

The mean shift algorithm

The cluster algorithm used to decompose the cloud of boutons into clusters is a simple iterative procedure that shifts each dot (the location of the bouton in the 3D space) to the centre of gravity of the dots in its neighbourhood (a sphere of radius \( h \)), so that the dots become concentrated into a number of tight clumps. This type of cluster algorithm is called 'mean shift algorithm' (Cheng 1995). All dots that belong to such a clump constitutes a cluster in the original data set. The outcome of this procedure is a partition of the data set into clusters and is dependent on the radius \( h \). If \( h \) is large, the resulting cluster will be the whole bouton cloud and if \( h \) is very small, each bouton constitutes a cluster. The question is which \( h \) one has
4.2. THE DETERMINATION OF CLUSTERS

to choose for an appropriate decomposition of the bouton cloud into clusters. The following procedure was used to find an appropriate $h$.

For each $h$ a 3D distribution $M(h)$ was determined. $M(h)$ is specified in detail below. It is essentially a mixture $M(h)$ of 3D normal distributions where each normal distribution is an estimate of the bouton distribution in the clusters that were revealed by the cluster algorithm. As a measure of the discrepancy $MSE(h)$ between the estimated distribution $M(h)$ and the distribution of boutons the mean square error $MSE(h)$ between the corresponding radial distributions was taken. This was done by calculating the mean square error $MSE(h)$ of the radial distribution of the bouton cloud (i.e. the distribution of the Euclidean distances origin - bouton location) and the radial distribution of the model (average of 5 Monte Carlo simulations of $M(h)$). The $h$ for which $MSE(h)$ is minimal was selected as the appropriate $h$.

Mixture of 3D normal distributions

For each $h$ a mixture of 3D normal distributions $M(h)$ is defined as follows. Let $C_i$ ($i = 1, 2, ..., n, n+1, ..., m$) be the clusters recognised by the mean shift algorithm for a particular $h$. $C_j$ ($j = n+1, ..., m$) are clusters for which it is true that: 1. the number of boutons in the cluster is less than 5% of the total number of boutons, 2. the ratio of the shortest and longest diameter of the 80% ellipsoid of the cluster is larger than 0.2. These clusters will be ignored in the model. The first point makes sure that only clusters with enough boutons are considered. The second point ignores clusters which are very elongated, normally consisting of boutons which are aligned on a small piece of an axonal branch. Other characterisations of clusters which are to ignore or retain are possible and can influence the result.

Define for each cluster $C_i$ ($i = 1, 2, ..., n$) the centre of gravity $x_i$, the covariance matrix $S_i$, and the relative number of boutons in $w_i$ (i.e. a normal distribution is fitted to each cluster). The $w_i$ are taken relative to the total number of boutons in all the $C_i$ ($i = 1, 2, ..., n$). Then set $M(h) = \sum_{i=1}^{n} w_i G(x_i, S_i)$.

Final comments on the cluster algorithm

If the clusters defined in points 1 and 2 above were not ignored, $MSE(h)$ would decrease with increasing $h$ as more and finer clusters approximate all the tiny non-uniformities in the bouton distributions (fig 4.6 A and B stippled line). The result is that a very accurate model of the bouton distribution can be achieved for the price of a complicated model ($n$ large). However, we would like to have the model simple ($n$ small) and as accurate as possible. We achieved this by ignoring the small and elongated clusters. This has the effect that for small $h$ many clusters will be ignored because the cluster algorithm produced many small clusters. This will result in an inaccurate model $M(h)$ (fig 4.6 A and B, solid lines). In order to get a more accurate model a larger $h$ has to be selected. Less clusters will be produced by the cluster algorithm and less of them will be ignored because the clusters will be in general bigger. The model will be more accurate. However, if $h$ is too large, the cluster algorithm will reveal only few but large subsets of boutons as clusters which
again will result in an inaccurate model. The \( h \) for which \( MSE(h) \) is minimal is between these two extreme cases. For this \( h \) small clusters were ignored to keep the model simple but not too many clusters were ignored, so that the model is still accurate.

For the actual determination of the minimum of the curve \( MSE(h) \) only the values \( h \in \{50, 100, 150, 200, 300, 400, 500, 600\} \) were considered.

Figure 4.6: Data clustering. A: The mean square error (MSE) between the fitted model and the bouton cloud of a layer 6 pyramidal cell (p6:A) as a function of the parameter \( h \). B: The number of clusters used for the construction of the model as a function of \( h \). Dashed curves: no clusters are ignored in the construction of the model. The most accurate model (MSE smallest) is for \( h = 50 \), but the model is also very complex (143 clusters). Solid curves: clusters that were too elongated and too small in bouton number were ignored. The most accurate model is for \( h = 200 \). The model is not as accurate as the model for \( h = 50 \) in the unrestricted case but only 6 clusters were used.

4.2.2 A model of the bouton cloud

A cluster algorithm was used to determine in an objective way the clusters in a bouton cloud. A compact description of the clusters was achieved by the representation of the clusters by a mixture of 3D normal distributions. Of course one can see it also the other way round. The distribution of bouton clouds was fitted by the 'best' mixture \( M(h) \) of 3D normal distributions and the components can be interpreted as a description of the clusters. 'Best' refers here to the most accurate model from the set of models \( M(h) \), \( h \in \{50, 100, 150, 200, 300, 400, 500, 600\} \).

In this section we present in numerical form the parameters of the mixture of 3D normal distributions. Much of the analysis of the clusters given in the next section relies on these numerical values. In order to be able to compare the constellation of clusters of different cells the cortex had to be flattened in order to correct for the curvature of the cortex. The parameters in the tables listed refer to the corrected values.
4.2. THE DETERMINATION OF CLUSTERS

Correction for cortical curvature

The flattening of the cortex was done as follows (fig 4.7). Each cluster had its location determined within the cortical lamina. This was done by eye using the location of the layers as seen in the microscope and rotations of the cell in the 3D space. For each cortical layer that contained clusters a reference point was determined (point R in 4.7). This point was always on the vertical axis and had, relative to the soma, the same height as a nearby centre of a cluster in the same layer (B in 4.7). If no such cluster centre was available, the mean of the heights of the cluster centres in the layer were taken. Although taking the mean does not work in general, visual inspection shows that for our cases it worked well. The boutons of each cluster in the layer (cluster with centre A in 4.7) were then rotated about the reference point in a plane that is determined by the vertical axis and the bouton that has to be rotated. The rotation angle (a) is given by the angle of the horizontal plane through the reference point and the line between the reference point (R) and the centre of the cluster that has to be rotated (A). Clusters of the cells p6:A, p6:B, p6:C, p6:D, p6:F, p5, ss4:A and ss4:B were rotated in this way.

The parameters

Tables 4.2 and 4.3 summarise the result of the clustering process. Each cluster is described by its location, extent and relative number of boutons. With the assumption that the boutons are normally distributed in the cluster, the bouton cloud is thus completely defined. For comparison, fig 4.8 shows the bouton cloud of an original cell (4.8 A, D, G), a Monte Carlo simulation of the unflattened model (4.8 B, E, H) and a Monte Carlo simulation of the flattened model (4.8 C, F, I)

\[
M = \sum_{i=1}^{n} w_i N(\mu_i, \Sigma_i). \tag{4.1}
\]

The parameters \(w_i, \mu_i\) and \(\Sigma_i\) are taken from the tables 4.2 and 4.3. \(N(\mu, \Sigma)\) is the 3D normal distribution with mean \(\mu\) and covariance matrix \(\Sigma\), that is

\[
N(\mu, \Sigma)|_x = \frac{1}{2\pi^{3/2}\det(\Sigma)^{1/2}} e^{-\frac{1}{2}(x-\mu)'\Sigma^{-1}(x-\mu)}. \tag{4.2}
\]

Referring to the tables, one has \(\mu = (\mu_1, \mu_2, \mu_3)'\), and \(\Sigma = (\Sigma_{ij})_{i=1..3,j=1..3}\). Because \(\Sigma\) is symmetrical by construction, the matrix is already defined by knowing the entries \(\Sigma_{11}, \Sigma_{21}, \Sigma_{22}, \Sigma_{31}, \Sigma_{32}, \Sigma_{33}\).
Figure 4.7: Flattening the cortex. The bouton clusters of a cell are schematised by ellipses and their centres (A and B). In order to flatten the cortex, the cluster with centre A is rotated around R to the new location A'. R is a point on the vertical axis with the same height as a nearby cluster (B) which is in the same layer as cluster A. The rotation angle is indicated by $\alpha$. 

Rotated patch

Soma
4.2. THE DETERMINATION OF CLUSTERS

Figure 4.8: Monte Carlo simulation of a bouton cloud. Left panel (A, D and G): raw data of the reconstructed cell p6:A. Only the boutons are shown (small dots). The star indicates the soma which is at the coordinates (0,0,0). Middle panel (B, E, and H): Monte Carlo simulation of the model of the bouton cloud (small dots). No correction was made for the curvature of the layers. Right panel (C, F and I): Monte Carlo simulation of the model of the bouton cloud. Correction for curvature was made. A, B, C: 3D view. D, E, F: Coronal view. G, H, I: Top view. Units are mm.
Accuracy of the model

The entries in the table refer to the flattened bouton cloud. In order to give a measure of how good the model fits to the real data, the unflattened model was used for comparison. For both sets of data the distance from the boutons to the soma (or at the point where the thalamic afferent leaves the white matter and enters layer 6) was determined and a cumulative probability distribution of the radii was computed. Then the greatest distance \( D \) between these two curves was determined. The bigger the distance the worse the model. In fact it is possible to calculate the probability \( p \) that a difference is bigger than the observed difference \( D \) (Kolmogorov-Smirnov test, see Press et al. 1997). Ten Monte Carlo simulations of the model were made, and \( D \) was calculated for each. On average both the \( D \) and the \( p \) values were around 0.05 (\( D = 0.0557 \pm 0.0285, p = 0.0513 \pm 0.1275 \)). On a 5%-significance value, only the model distributions of p6:B, ss4:C, p2/3:E, lgn:A, lgn:C and b5 are not significantly different from the distribution of the real data. This indicates that the models should be taken only as a first approximation to the real distribution of boutons. Visual inspection confirms that on a coarse level the model provides a good approximation to the data. Only on a smaller scale do the single clusters fail to follow a 3D normal distribution. This is demonstrated in fig 4.8. The bouton clouds in A (raw data) and B (simulated data) resemble each other on a larger scale, although \( p = 10^{-9} \). Fig 4.9 shows the radial distribution of the bouton clouds of each cell (thick line), a radial distribution of a simulated cell (superimposed thin line) and the \( D \) value for this simulation. Again, one can see that the thin line roughly follows the thick line.
Figure 4.9: Comparison of bouton distribution with model. The radial histogram of the bouton cloud (thick line) and the bouton cloud of a Monte Carlo simulation of the model (thin line) are shown for each cell in order to compare the goodness of fit of the model. Bin size of histogram is 20µm. y-axis: number of events. x-axis: distance from the soma to the boutons (radius). The number in the parentheses to the left of the cell label is the D value (largest vertical distance between the curves), which is a measure of how good the model curve fits the raw data. The higher the number, the worse the fit. Note that the left and right panels do not have the same scale in the horizontal direction.
Table 4.2: List of parameters of the model bouton clouds. The (flattened) bouton cloud of each cell (left row) was approximated by a mixture of normal distributions of the form \( \sum_{i=1}^{k} w_i N(\mu_i, \Sigma_i) \). Each term \( w_i N(\mu_i, \Sigma_i) \) describes a cluster whose parameters are given by the last ten rows of numbers in the table, \( w_i \) is the relative number of boutons in the cluster \( i. \) \( \mu \) is \( (\mu_1, \mu_2, \mu_3)' \) is the location of the cluster origin. \( \Sigma \) is the covariance matrix of the boutons in the cluster. \( \Sigma \) is symmetric and it is therefore well defined by the values \( \Sigma_{11}, \Sigma_{22}, \Sigma_{33}, \Sigma_{12}, \Sigma_{13}, \Sigma_{23}. \) The second row on the left shows the total number of boutons \( B \) in the bouton cloud. The third row from the left shows a goodness of fit \( D \) of the (unflattened) model to the real data. The higher the value \( D, \) the worse the fit. The next row indicates the relative number \( w_0 \) of boutons (relative to \( B \)) that were skipped in order to create the model. The number of clusters per cell \( n \) is shown in the next row and finally the row \( L \) shows the layer of the (cluster origin).

<table>
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<th>( n )</th>
<th>( L )</th>
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### 4.2. THE DETERMINATION OF CLUSTERS

#### Table 4.3: Second part of the list of model parameters of the bouton clouds.

| Label | B   | H   | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D10 | D11 | D12 | D13 | D14 | D15 | D16 | D17 | D18 | D19 | D20 | D21 | D22 | D23 | D24 | D25 | D26 | D27 | D28 | D29 | D30 | D31 | D32 | D33 |
|-------|-----|-----|----|----|----|----|----|----|----|----|----|----|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 4450  | 2   | 3   | 0.50| -14| 61  | 5   | 22256| -8838| 14033| -1768| -1298| 19633|
| 2901  | 2   | 5   | 0.88| 14  | -46  | 1   | 18717| -759| 16329| -6375| 1731| 21227|
| 5780  | 6   | 4   | 0.33| 7   | 695  | -36| 23116| -2096| 19034| -10377| -857| 16215|
| 1920  | 3   | 3   | 0.69| 80  | 705  | 50 | 20282| 10584| 17315| 1538| 2158| 16253|
| 4105  | 2   | 4   | 0.86| 1   | 590  | -16| 31569| -8995| 25309| 25309| 18973| 1281| 51846|
| 5754  | 3   | 4   | 0.63| -28 | 573  | -39| 57070| 21872| 28836| -4840| -8499| 55104|
| 4372  | 5   | 2   | 0.80| -25 | 586  | 50 | 16532| 3614| 20075| 3668| 14883| 14223|
| 2702  | 3   | 6   | 0.70| -71 | 73   | -25| 51723| 13505| 37415| 3471| 160| 38851|
| 8907  | 1   | 4   | 0.99| -48 | 498  | 13 | 21144| 2827| 15973| 7852| 9242| 28174|
| 2400  | 4   | 4   | 0.39| 322 | 638  | -137| 19588| -6689| 4963| -4455| 10099| 29061|
| 5738  | 4   | 4   | 0.28| -265| 641  | -312| 10524| 3585| 5601| 1732| 8376|
| 2878  | 7   | 4   | 0.29| -119| 954  | 1320| 22166| -105| 7311| 2848| 772| 15196|
| 3553  | 5   | 4   | 0.32| -1311| 1101| -646| 5790| -6890| 3462| -1965| -1676| 7471|
| 3200  | 5   | 4   | 0.32| -1355| 1101| -411| 8665| -703| 3287| 350| 1775| 4166|
| 3553  | 5   | 4   | 0.32| -1311| 1101| -646| 5790| -6890| 3462| -1965| -1676| 7471|
| 3200  | 5   | 4   | 0.32| -1355| 1101| -411| 8665| -703| 3287| 350| 1775| 4166|

Table 4.3: Second part of the list of model parameters of the bouton clouds.
CHAPTER 4. SPATIAL DISTRIBUTION OF BOUTONS

4.3 Analysis of bouton clouds

In the last section the bouton cloud was decomposed into clusters and, when necessary, subsequently flattened in order to account for the curvature of the layers. The result of the cluster analysis is summarised in tables 4.2 and 4.3. Figures 4.11 and 4.20 show the borders of the clusters for each individual cell in a coronal and top view.

4.3.1 Global description of bouton clouds

Total bouton number

For each cell the number of boutons on the axonal tree were counted. As already mentioned, the number of boutons gives an estimate of the number of contacts that a cell makes. Fig 4.10 A (closed dots) shows a plot of the bouton counts for each cell. The range of the bouton counts is large, from 1026 to 8967 boutons per axon. On average the number of boutons is 3963 ± 1810.

Spiny cells, thalamic afferents and smooth cells had about the same number of boutons (spiny cells and thalamic afferents 4014 ± 1856 (range [1026, 8967]), smooth cells 3843 ± 1803 (range [1713, 6446])). This is interesting because the spiny cells distribute their boutons within a much wider volume than the smooth cells.

Global volume and bouton density

In fact, the volume occupied by the spiny and thalamic afferents was, measured in units of 300\(\mu\)m cubes (i.e. a cube with side length 300\(\mu\)m), 33 ± 30 (range [4, 121]), while that of the smooth cells 3 ± 3 (range [0, 8]). In particular the spiny stellate cells (ss4) and the pyramidal cells in layers 2 and 3 (p2/3) occupy large volumes (fig 4.10 B). The volume was measured by calculating the volume of the ellipsoid that encloses 80% of the boutons of a cell and whose centre is the centre of gravity of the boutons. The volume occupied by spiny cells and thalamic afferents is roughly 11 times higher than that occupied by the smooth cells. Because the average number of boutons in the 80%-ellipsoid is roughly the same for the two groups, it follows that the density of boutons is much lower (about 11 times) for the spiny cells and thalamic afferents (222 ± 376 boutons per 300\(\mu\)m-cube, range [16, 1774]) than for the smooth cells (2406 ± 2296 boutons, range [205, 7769]).

Because of the clustered distribution of the boutons, the usage of the 80%-ellipsoid is in general an overestimate of the volume that is occupied. Therefore, the densities must be regarded as lower estimates. These values represent the overall bouton density of the boutons. Also note that the determination of the 80%-ellipsoid can be regarded as the roughest model of a bouton cloud in the class of mixture of 3D normal distributions (one normal distribution is used, \(n = 1\)).
4.3. ANALYSIS OF BOUTON CLOUDS

4.3.2 Description of clusters

The ignored clusters

Because the clusters which were too elongated and contained only few boutons were ignored, the union of the boutons in the clusters is in general only a subset of the bouton cloud. On average the number of clusters ignored ranged between 0 and 25 for individual cells. For 70% of the cells the number of clusters ignored was smaller than 6. The total number of boutons in the ignored clusters was smaller than 20% (on average about 7%) of the total number of boutons in the cloud (fig 4.10 A, open dots). It follows that there is only little loss of information in the spatial distribution of the boutons when the smaller clusters are ignored.

Classification of clusters

For further analysis it seems reasonable to classify the clusters depending on their location. In order to unbend the cortex, the clusters had to be assigned a layer. This is the layer of the cluster centres. We will use this information to determine the layer that a cell prefers to innervate. This was done by adding the number of boutons in the clusters of each layer. The layer with the greatest number of boutons we will call the primary layer of innervation. The layer with the second largest number of boutons is called secondary layer of innervation and so on. The clusters are divided further into proximal or distal clusters. The clusters are called
proximal if their horizontal displacement from the vertical axis running through the soma is small, i.e. when the 80%-ellipsoid of the cluster intersected the vertical axis running through the soma (e.g. cluster B in 4.7). This can easily be decided with the help of figures 4.20 and 4.11 which show the coronal view and the top view of the clusters.

The relation between cluster and layers

As can be seen in fig 4.11, many clusters are elongated. The axis of the biggest elongation is often more or less parallel to the layer borders (e.g. the pyramidal cells in layer 2 and 3 or the thalamic afferents). This suggests that many clusters have the tendency to avoid crossing laminar borders. However, there are clear exceptions from this rule. For example a single cluster can be highly elongated in vertical direction so that it innervates extensively more than one layer (e.g. fig 4.11 sp4). We quantified this for each cluster by calculating the percentage of boutons that are contained in the layers that the cluster occupies (see fig 4.11). With the exception of the star pyramidal cell (sp4) the clusters of each cell preferred to innervate the layer of their cluster centre (i.e. more than 50% of the boutons of the cluster were in this layer). With the exception of one or two clusters of the cells p6:C, p6:E, p4, sp4, ss4:A, ss4:C, b4:B and db2/3 the clusters of all cells had more than 80% of the boutons in one layer (a single cluster of sp4 innervates layer 4 and layers 2 and 3 equally strong with about 40%). This suggests that for most cells the clusters tend to innervate a single layer.

The distribution of the boutons in the layers

The number of boutons in the different layers is shown for each cell (fig 4.12). Because some clusters innervate extensively more than one layer this distribution reflects with more accuracy the distribution of the boutons in the different layers (as does simple summing of the boutons of clusters of the associated layers). With the exception of sp4 and db2/3 all cells have more than 50% of the boutons in the primary layer of innervation. In fact most cells (77% of all cells) have more than 70% of the boutons in this layer.

Proximal and distal clusters

Three kinds of proximal clusters can be identified (fig 4.11). The first type innervates the region around the apical dendrite. This is typically the case for deep pyramidal cells that have a projection to the superficial layers (e.g. fig 4.11 p6, p5 and p4). The second type innervates a local region around the soma. All cell types of our sample but for the thalamic afferents, which have no dendrite in the cortex, and some of the layer 6 pyramidal cells, form this type of proximal cluster. The third kind of proximal cluster innervates a region which is displaced downwards from the soma. Typically the pyramidal cells of layers 2 and 3 have this kind of proximal cluster (exception is p2/3:C). Only the cells p2/3:D and lgn:C have distal clusters in the non-preferred layers of innervation.
Figure 4.11: The coronal view of the 80% ellipse of the cluster is drawn for each cell. The clusters were first rotated so that their centre of gravity is located in the vertical plane $E = \{(x, y, z)|z = 0\}$. The rotation was about the vertical axis that runs through the soma, $\{(x, y, z)|x = 0, z = 0\}$. The bold ellipses are the 80% ellipses of the cluster after projecting the boutons of the cluster onto the plane $E$. The white dot indicates the centre of gravity of the clusters. The thin horizontal lines indicate the borders of the 6 layers. For visibility layer 4 was shaded in gray. Also shown are for each cell the dendritic trees (thin lines). If no dendritic tree is shown it was not digitised. Scale bar 500$\mu$m. For the coordinate system $(x, y, z)$ see fig 2.1.
### Figure 4.12: The relative number of boutons in each layer is shown for individual cells. Not shown are layers for which the values were smaller than 5% of the total number of boutons. The shaded area indicates the primary layer of innervation. The number to the right of each bar indicates the layer.

<table>
<thead>
<tr>
<th>Layer</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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</thead>
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<tr>
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<td>15</td>
<td>2/3</td>
<td>12/3</td>
<td>15</td>
<td>2/3</td>
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<td>15</td>
<td>2/3</td>
<td>12/3</td>
<td>15</td>
<td>2/3</td>
<td>12/3</td>
</tr>
<tr>
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<td>15</td>
<td>12/3</td>
<td>4</td>
<td></td>
<td></td>
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<td>16</td>
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<td>12/3</td>
<td>15</td>
<td>2/3</td>
<td>12/3</td>
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<td>2/3</td>
<td>12/3</td>
<td>15</td>
<td>2/3</td>
<td>12/3</td>
</tr>
<tr>
<td>sp4</td>
<td>15</td>
<td>12/3</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b4</td>
<td>15</td>
<td>12/3</td>
<td>4</td>
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<td></td>
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<td>15</td>
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<td>12/3</td>
<td>15</td>
<td>2/3</td>
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</tr>
<tr>
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<td>2/3</td>
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</tr>
<tr>
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<td>12/3</td>
<td>15</td>
<td>2/3</td>
<td>12/3</td>
</tr>
</tbody>
</table>

#### 4.3.3 Number, size and bouton density of clusters

In the following we will characterise the clusters. First, for each cell the number of clusters is determined. Then for each cluster the number of boutons it contains and its size is calculated. From this the density of boutons in single clusters is determined. The results are presented for six different groups of clusters (fig 4.13).
4.3. ANALYSIS OF BOUTON CLOUDS

The different groups are formed according to the following criteria which are applied to each cluster. 1) the cluster is proximal or distal, 2) the cluster is located in the primary layer of innervation or not and 3) the type of cell is spiny or smooth. Thalamic afferents are not considered in this overview, but are discussed in the detailed descriptions which follows next.

Number of clusters

For the 30 cells 106 clusters (91 in the primary layer of innervation, 15 in the other layers) were determined. This results in an average of 4 ± 2 clusters per cell. The maximum number of clusters that occurred was 9 (fig 4.15 p2/3:C), the minimum was 1 (fig 4.15 p6:G, sp4, b4:C, b2/3:C). The superficial pyramidal cells, the spiny stellate cells and the thalamic afferents all showed a tendency to produce many clusters (5 ± 2, range 3 to 9) when compared to the remaining cell types (3 ± 1, range 1 to 6).

One might expect some correlation between the total number of clusters and the number of boutons, e.g. the more clusters the more boutons. However, this is not the case. A scatter-plot between the total number of boutons and the number of clusters a cell forms shows no correlation (fig 4.14), i.e. \( r = -0.11, p = 0.56, a = 4.04, b = -0.00 \). Here and for the remainder of this work, \( r \) denotes the correlation coefficient, \( a \) and \( b \) describe the regression line through the pairs of data (i.e. \( x \to a + b \cdot x \)) and \( p \) is the probability that one observes an absolute correlation coefficient larger or equal to \( |r| \) when the variables were uncorrelated. Thus a small value of \( p \) indicates that the variables are correlated (see Bevington and Robinson 1994, page 198-200).

The number of clusters and the number of boutons are also uncorrelated for cells within the group of thalamic and spiny cells (\( r = -0.19, p = 0.42, a = 4.94, b = -0.00 \)) as well as within the group of the smooth cells (\( r = 0.10, p = 0.79, a = 1.95, b = 0.00 \)). It also remains true for a single class of cells as for example the layer 6 pyramidal cells (\( r = -0.24, p = 0.61, a = 3.59, b = -0.00 \), not shown).

**Primary layer of innervation:** All cells formed a proximal cluster in the primary layer of innervation. The majority of the distal clusters in this layer are formed by spiny cells. Smooth cells form on average about one distal cluster in the primary layer (fig 4.13 A). This cluster innervates a region close to the proximal cluster (fig 4.13 F). Each spiny cell forms on average about two distal clusters, many of them innervating regions far away from the soma, on average 800µm. This is about twice the distance of a distal cluster belonging to a smooth cell and reflects the more local character of the smooth cells. For both groups the existence of distal clusters in other layers than the primary layer of innervation is rare (fig 4.13 A).

**Remaining layers:** Most clusters in the remaining layers are proximal. From the 12 cells with clusters in the remaining layers only the thalamic afferent lgn:C and the pyramidal cell p2/3:D formed more than one cluster in the remaining layers (2 and 3 clusters). Eleven of the 12 cells (exception is p2/3:C) formed a proximal
cluster. The formation of clusters in these remaining layers seems to be dependent on the cell type. While the layer 6 pyramidal cells and the spiny stellate cells did not make a proximal cluster in the remaining layers (one exception is p6:B), most pyramidal cells in layers 2 and 3 did so (also one exception, p2/3:C which has only a distal cluster in the secondary layer of innervation). Typically the primary and secondary layer of innervation were not neighbouring layers for spiny cells but had a layer in between (fig 4.11). For the smooth cells this was only the case for a superficial basket cell (fig 4.11 b2/3:B). The other smooth cells made the proximal clusters in neighbouring layers (fig 4.11 b4:B and db2/3).
4.3. ANALYSIS OF BOUTON CLOUDS

Figure 4.13: Characterisation of clusters. The clusters were classified into 8 groups. The first distinction was made between clusters from spiny cells (the bars with white background) and smooth cells (the bars with the shaded background). For each class the clusters were further classified as proximal (black bars) or distal (dark gray bars). The proximal and distal clusters were additionally classified as being located in the primary layer of innervation (P) or not (N). The last class has no member. A: For each class the number of clusters per cell is shown. The mean and standard deviation (thin line) in each class were calculated for the following quantities. B: boutons per cluster, C: relative number of boutons per cluster, D: cluster volume (volume of 80%-ellipsoid), E: Average bouton density in a cluster and F: horizontal displacement a cluster from the soma. Thalamic afferents were excluded.
Bouton number and cluster number are independent

Figure 4.14: Independence of bouton and cluster number. Scatter plot of the number of boutons of a cell and the number of clusters the cell forms, showing that the two variables are independent of each other. Closed dots: spiny cells and thalamic afferents, open dots: smooth cells.
Figure 4.15: The number of clusters in the the primary layer of innervation (shaded bar) and in the secondary layer (empty bar) are shown for each individual cell. The number in each bar indicates the layer.
The number of boutons per cluster

73% of the total number of clusters contained less than 1000 boutons (449 ± 238, range [52, 969], fig 4.16 A and B). The total range of boutons in the clusters is between 52 and 8920 (mean 1046 ± 1348) which indicates a huge variation. The cluster that contained 8920 boutons was formed by the layer 6 pyramidal cell p6-G. This cell made exactly one compact cluster consisting of nearly all the boutons of the cloud. The proximal clusters in the primary layer of innervation seem to play a special role. As one can see in fig 4.13 B, these clusters contain on average many more boutons than the remaining clusters, independent of being spiny or smooth (on average about 5 times more, mean 2500 to 3000 boutons). Again, a high variance is observed for the number of boutons in these clusters, for the smooth cells as well as for the spiny cells. A similar result can be observed when the relative number of boutons in the clusters are considered (fig 4.16 C and D, these are the weights \( w_i \) in the model of the mixture of normal distributions). On average the proximal clusters in the primary layer of innervation contain more than 60% percent of the total number of boutons (60 ± 27 for the spiny cells and 83 ± 16 for the smooth cells).

A model for the weights: The question arises over the large variance observed in the number of boutons of the proximal clusters in the primary layer of innervation. Can it be explained by the different number of clusters a cell forms? The problem was approached by classifying the cells into groups of similar cluster numbers. For each group the weights \( w_i \) (in descending order) are shown in fig 4.17 A-G. There is a systematic decrease in the first weight \( w_1 \) for increasing cluster number. However, the remaining weights do not seem to change much. For example the second weight is always around 0.2, the third around 0.15 and so on. This impression is confirmed when the mean and standard deviation of the weights \( w_i \) of a given order \( i \) are computed (fig 4.16 H). The first weight has the highest standard deviation while for the remaining weights the standard deviation remains small.

A simple rule that could explain this behaviour is that the largest cluster is always divided according to the same ratio \( u : 1 - u \) (\( u > 0.5 \)), i.e. the boutons in the largest cluster are reduced to \( 100 \cdot u \% \) and the remaining \( 100 \cdot (1 - u) \% \) percent of the boutons are used for the formation of an additional cluster. For a cell with two clusters,

\[
w_1 = u \quad \text{and} \quad w_2 = 1 - u.
\]  

(4.3)

For a cell with three clusters,

\[
w_1 = u^2, \quad w_2 = 1 - u \quad \text{and} \quad w_3 = u \cdot (1 - u).
\]  

(4.4)

And for a cell with \( n > 3 \) clusters, one obtains

\[
w_1 = u^{n-1}, \quad w_2 = 1 - u, \quad w_3 = u \cdot (1 - u), \quad \ldots, \quad w_n = u^{n-2} \cdot (1 - u).
\]  

(4.5)

The comparison of this model for \( u = 0.82 \) is shown as a stippled line in fig 4.17. As one can see, the match is quite good, although the model is quite simple. The model is very sensitive to the parameter \( u \). A value of 0.9 or 0.7 results in a very poor fit.
Characterisation of the weights: The largest weight $w_1$ is for most cells (27 of 30) the proximal cluster in the primary layer of innervation (fig 4.20). However, there are some exceptions. The cells ss4:A, ss4:B and p2/3:D have distal clusters for which the weight is largest. In these cases the proximal cluster in the primary layer had the second or third largest weights (from at least 4 clusters). The characterisation of the remaining weights is less clear. We tested to see if the weights decrease with increasing horizontal displacement of the cluster from the vertical axis running through the soma. Only the distal clusters of the primary layer of innervation were considered. The thalamic afferents were ignored. No correlation was detected ($r = 0.06$, $p = 0.70$, $a = 0.13$, $b = 0.00$). This can also be observed in fig 4.20, e.g. p6:A.

Figure 4.16: The number of boutons in the individual clusters are shown for each cell. Clusters in the primary layer of innervation are indicated by filled dots. A: Number of boutons in proximal clusters. B: Number of boutons in distal clusters. C: Relative number of boutons in proximal clusters. D: Relative number of boutons in distal clusters.
CHAPTER 4. SPATIAL DISTRIBUTION OF BOUTONS

Figure 4.17: The weight distribution as a function of cluster number. A-G: For each cell the weights (relative number of boutons per cluster) were drawn in descending order (weight distribution). Cells of similar cluster numbers were grouped together and plotted in one of the figures. The stippled lines indicate the weight distributions derived from the model (see text). H: The mean and standard deviation of the first weight (rank 1) of each cell, second weight (rank 2) of each cell, etc. are shown. The stippled line indicates the mean of the weights derived from the model. x-axis: rank of the weights. y-axis: mean of weights.
4.3. ANALYSIS OF BOUTON CLOUDS

The volume and density of the clusters

**Volume:** The volume of the clusters were measured by determining the volume of its 80%-ellipsoid. We give the volume in units of 300\(\mu m\) cubes. Typically the basal dendrites of pyramidal cells or the dendrites of stellate cells occupy of volume of a 300\(\mu m\) cube. The cluster volume ranged from 0.1 to 22.3 (mean 2.0 ± 2.9), covering therefore a huge range. However, from the 30 cells only 5 cells have volumes larger than five 300\(\mu m\) cubes (p6:C, p6:D, p6:F, ss4:A and sp4). The average cluster volume without these cells is 1.4 ± 1.1 (fig 4.18 A and B, fig 4.13 D). The corresponding diameters (i.e. the diameters of spheres of similar volumes as these clusters) have a mean of 379.9 ± 115.2\(\mu m\) (range [160.2, 636.4]).

**Density:** A pronounced difference between basket cells and spiny cells is revealed when calculating the density of boutons in the clusters. The density was calculated by dividing the number of boutons contained in the 80%-ellipsoid by the volume of the ellipsoid (fig 4.13 E, fig 4.18 C and D). With the exception of b5, the bouton density is much higher in the proximal clusters of the primary layer of innervation (4323±1198 boutons per 300\(\mu m\) cube, range [3143, 6352]) than that of the spiny cells and thalamic afferents (550 ± 466, range [37.1, 1947]). For the spiny cells the average is 399±270, (range [29, 1257]), for the smooth cells (without b5) 1577±1141, (range [180, 4205]). Large variations can be observed in the density values, which cannot be explained easily by simple dependencies. For example no correlation could be found for the horizontal displacement of the distal clusters in the preferred layer and the density of boutons in the distal clusters (\(r = -0.34, p = 0.02, a = 1092.02, b = -0.72\)) or the volume (\(r = 0.20, p = 0.17, a = 145.67, b = 0.06\)). In addition, no convincing relationship between the independent parameters \(n\) (number of clusters of a cell) and \(b\) (total number of boutons of a cell) could be established with the density of clusters or the cluster volume. It is very likely that other parameters than \(n\) and \(b\) have an influence on the cluster volume or density, such as for example the thickness of the layer.

4.3.4 Horizontal arrangement of clusters

**Horizontal displacement**

The horizontal displacement of the proximal clusters (as measured by the distance of the vertical line through the soma and the centre of gravity of the boutons in the cluster) never exceeded 180\(\mu m\) (fig 4.19 A). The distal clusters had horizontal distances ranging from 84\(\mu m\) to 2103\(\mu m\) (without the thalamic afferents, fig 4.19 B). The smooth cells tended to form a distal cluster in the neighbourhood of the proximal cluster. The average displacement is therefore small for these cells (401 ± 221, range [84, 805]). In contrast, spiny cells (without thalamic afferents) formed clusters at average displacements of 794 ± 284, (range [197, 1408]). Here the large distance observed for a distal cluster in the non-preferred layer of the pyramidal cell p2/3:D was ignored. This distance is about 2\(\mathrm{mm}\).
Figure 4.18: Volume and density analysis of clusters. Volume of 80% ellipsoid of the
of proximal (A) and distal (B) clusters. Closed dots: primary layer of innervation, open
dots: remaining layers. C and D: Density of the boutons in the proximal (left panel)
and distal clusters (right panel). The density was taken as the number of boutons in the
80%-ellipsoid divided by its volume. Closed dots: primary layer of innervation, open dots:
remaining layers.

We also determined the distance (measured in the horizontal direction) between
nearest neighbours of clusters (fig 4.19 C). Only clusters in the preferred layer of
innervation were considered. Not surprisingly, the smooth cells have small near-
est cluster distances (338.3 ± 213.6, range [120.5, 791.0]). For the spiny cells and
thalamic afferents the distances are greater (574 ± 207, range [270, 1140]).
Figure 4.19: Horizontal displacement of clusters. A: Horizontal displacement of the centre of gravity of the proximal clusters from the soma. B: Horizontal displacement of the centre of gravity of the distal clusters from the soma. For the thalamic afferents the horizontal displacement from the point of entry into layer 6 was taken. Closed dots: clusters in primary layer of innervation. Open dots: clusters in the remaining layers. C: Nearest horizontal separation between clusters. Only the clusters in the preferred layer were considered.
Elongated axonal fields

The relation between the distribution of the bouton cloud in the primary layer of innervation and the receptive field properties of the cell is examined here. For a comparison the preferred stimulus orientation and the preferred stimulus direction of a cell was projected on the cortical surface by the retinotopic map (see chapter 3). The bouton cloud was distinguished into two parts, a proximal part which is formed by the boutons of the proximal cluster in the primary layer of innervation and a distal part which is formed by the boutons of the distal clusters in the primary layer of innervation. The boutons and cluster centers were projected onto a horizontal plane and were the proximal and distal part were then examined separately.

**Proximal part:** The axis of largest elongation ('cluster orientation') of the projected proximal cluster was determined and compared with the preferred stimulus orientation of the cell. In addition the direction of asymmetric bouton placement relative to the cluster centre ('cluster direction') was measured and compared with the preferred stimulus direction of the cell (fig 4.21 A and B).

The cluster orientation was defined as the longer axis of an ellipse that was fitted to the projected boutons of the proximal cluster (principle component analysis). For a measure of elongation of the proximal cluster ('strength of elongation') the ratio of the larger and smaller ellipse diameters was taken. The cluster direction was defined as the vector pointing from the soma to the centre of gravity of the projected boutons in the proximal cluster. The longer the vector, the stronger is the tendency of the boutons to be aligned in a certain direction relative to the soma. The length of the vector is therefore a measure of the strength of the asymmetry ('strength of asymmetry').

Fig 4.21 A shows the smallest angular difference between the preferred stimulus orientation of the cell and the cluster orientation (an angle between 0 and 90 deg). The smooth cells tend to have a cluster orientation which is oblique (between 30 and 60 deg) or perpendicular (> 60 deg) to the preferred stimulus orientation while the cluster orientation of the spiny cells were in general parallel (< 30 deg) or obliquely oriented. This relation is also maintained when only the strongly elongated clusters are considered (black dots, strength of elongation > 1.25) which did not show a strong asymmetry (strength of asymmetry < 50μm). Cells for which the preferred stimulus direction was determined, the smallest angular difference between the cluster direction and the preferred stimulus direction (an angle between 0 and 180 deg) is shown in fig 4.21 B. When clusters with a strong asymmetry were considered (strength of asymmetry > 50μm, closed dots in fig 4.21 B) the angular difference was in general between 60 and 120 deg.

**Distal part:** The second part of the axonal field is formed by the projected boutons of the distal clusters. Consider the clusters of, for example, p6:A in fig 4.20. Four clusters are fairly well aligned perpendicular to the axis of preferred stimulus orientation. The remaining cluster is arranged perpendicular to the preferred stimulus orientation. In this respect the location of the clusters of this cell seems to be highly correlated with the axis of preferred stimulus orientation.

Another kind of correlation can also be found. Very often a cell consist of a
4.3. ANALYSIS OF BOUTON CLOUDS

proximal cluster and an additional distal cluster in the primary layer of innervation. For example for the cell p6:E the distal cluster is arranged perpendicular to the preferred stimulus orientation of and directed away from the preferred stimulus direction. Both the correlation of the location of the clusters with the preferred stimulus orientation and with the preferred stimulus direction are investigated below.

For each distal cluster in the primary layer of innervation the smallest angular difference between the centre of gravity of its projected boutons and the preferred stimulus orientation of the cell were determined (relative to the soma, fig 4.21 C). This is an angle between 0 and 90 deg. The clusters of the layer 6 pyramidal cells were aligned either perpendicular (> 60 deg) or parallel (< 30 deg) to the preferred stimulus orientation but never oblique. This should be contrasted with the proximal cluster orientation for which the smallest angles never exceeded 60 deg. The distal clusters of the basket cells tended to be perpendicular aligned to the preferred stimulus orientation. This was also the case for their proximal cluster orientation. The remaining pyramidal cells (p5, p2/3) and the spiny stellate cells did not show any preference for certain angles. Also note that the pyramidal cell p2/3:E shows the opposite arrangement of the distal clusters to that observed for the layer 6 pyramidal cells.

From the 13 cells for which the preferred stimulus direction was determined, 10 had distal clusters perpendicular aligned to the preferred stimulus orientation (> 60 deg, fig 4.21 D). From these 4 had exactly one distal cluster in the same direction as the preferred stimulus direction of the cell and 3 had exactly one distal cluster in the opposite direction. The remaining 3 cells had distal clusters in both directions.

Covered Area

The area over which the axon distributes the boutons in horizontal direction was measured by the area of the convex hull of the border points of the 80% ellipses which are given by the clusters in the primary layer of innervation (fig 4.22 A). The units are given in 300\( \mu \)m-squares, i.e. by squares of side length 300\( \mu \)m. The convex hull is indicated for each cell in fig 4.20 as a stippled closed line. The total area ranged from 0.4 to 23 300\( \mu \)m-squares. The spiny cells and thalamic afferents occupied in general a higher area (11.3 ± 6.6, range [1.1, 23.0]) than the smooth cells (2.0 ± 1.9, range [0.4, 6.0]). However the rule is not strict. There are basket cells that occupy a larger area than spiny cells (e.g. b5 and p6:E). The percentage of area that is actually covered by the summed area of the ellipses is for the spiny stellate cells and the pyramidal cells in layer 2 and 3 often less than 50% of the area of the convex hull.
Figure 4.20: Horizontal arrangement of clusters. For each cell the top view of the clusters (ellipses) with their centre of gravity (open small dots) are indicated. Each ellipse was determined by applying a principle component analysis onto the boutons in a cluster, after having them projected to a plane parallel to the cortical surface. The ellipse contains 80% of the boutons in the cluster. Black ellipses are clusters in the primary layer of innervation (indicated by the label in parentheses), gray ellipses indicate clusters in the remaining layers. The number i in the ellipse indicates the rank of the relative number $w_i$ of boutons in the cluster (the cluster with largest relative number is 1). The stippled line indicates the convex hull of the border points of the ellipses in the primary layer. White squares indicate the location of the soma. White long bar: preferred stimulus orientation of the cell. Short white bar perpendicular to the long bar: preferred stimulus direction of the cell. A bar oriented parallel to the preferred stimulus orientation and swept in the direction of the short bar from soma to tip responds optimally. Length of large bar: 1mm. Length of short bar: 500μm.
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Figure 4.21: Correlation between receptive field properties and the bouton cloud. A and B: Correlation of distribution of boutons of the proximal cluster in the primary layer of innervation with the preferred stimulus orientation (A) or preferred stimulus direction (B) of the cell. A: For each cell the smallest angular difference between the preferred stimulus orientation and the cluster orientation was determined (open and closed dots). The closed dots indicate clusters for which the elongation is strong and the strength of asymmetry is small. B: For each cell the smallest angular difference between the preferred stimulus direction and the cluster direction was determined (open and closed dots). The closed dots indicate clusters for which the strength of asymmetry was large. C and D: Correlation of location of distal clusters with preferred stimulus orientation (C) and preferred stimulus direction (D). C: For each distal cluster the smallest angular difference between the preferred stimulus orientation and the vector soma to cluster centre were determined. Closed dots: distal clusters with horizontal displacement larger than 750\(\mu\)m. Open dots: distal clusters with horizontal displacement smaller that 750\(\mu\)m. D: Similar plot as in C. Only the cells for which the preferred stimulus direction is known were drawn (open and closed dots). Clusters which are displaced along the preferred stimulus direction are shown as closed dots. Clusters that are displaced into the opposite direction are indicated by open dots.
Figure 4.22: Occupied area of clusters. A: Estimate of the total area over which the axon spreads the boutons in the primary layer of innervation (stacked bar). Black bar: area of the proximal cluster in the primary layer. Gray bars: area of distal clusters in primary layer. B: Percentage of the total area that is occupied by the clusters in the primary layer.
4.4 Discussion

The bouton distribution of thalamic afferents and cortical cells in the cat V1 were studied systematically. The vertical as well as the horizontal component of the distributions were described quantitatively. This involved the decomposition of the bouton clouds into clusters. The clusters and their location relative to the soma where then described relative to the layers as well as within a layer.

4.4.1 The decomposition of the bouton cloud into clusters

The choice of a cluster algorithm

The patchy appearance of the bouton distribution strongly suggested the application of a cluster algorithm as a first step in the investigation of the bouton distribution. Several cluster algorithms are available and the result will generally depend on the algorithm used. We applied a mean shift algorithm in which the boutons are contracted into different clumps, each clump representing a cluster. This algorithm is appealing because it is easy to implement. In an iterative procedure each bouton has to be shifted towards the centre of gravity of the boutons in its neighbourhood (which is a sphere of radius \( h \) centred at the bouton). The main problem of this algorithm is to find an appropriate value \( h \) that determines the number and size of the clusters in the bouton cloud and is therefore crucial for the outcome. We approached this problem in the following way. For each decomposition of the bouton cloud into clusters, depending on \( h \), a bouton density was constructed as follows. First, the small and elongated clusters were ignored. Second, for the remaining clusters the boutons in each cluster were estimated by a tri-variate normal distribution. The resulting global density was compared with the true bouton density and the \( h \) with the best match was taken. This procedure could loosely be summarised as the search for a mixture of 3D normal distributions that explains the data as accurately as possible with as few clusters as possible.

Relation to the EM algorithm

A very popular clustering algorithm is the 'EM algorithm' (McLachlan and Krishnan 1997). This algorithm uses a parametrised model (e.g. a mixture of 3D normal distributions) and finds the parameters that minimise the maximum likelihood of the given data by searching the whole parameter space. The single components of the mixture can then be interpreted as clusters. The attraction of this algorithm is certainly the clear concept on which it is based. However, certain problems should be noted. First, the algorithm does not assign individual boutons to a cluster. Only the borders of the clusters are known. Second, the maximum likelihood function is unbounded in our application (the function approaches infinity for parameters for which the covariance matrix becomes singular). So the largest local maximum has to be found. The problem is even worsened when the data points tend to be locally aligned in a chain (McLachlan and Krishnan 1997, page 71-72). This is certainly the case for the boutons, because they are located on the axon (note that similar
chains were explicitly ignored in our cluster algorithm, although for other reasons). Third, the EM algorithm does not provide the number of mixtures that must be used for the fit. This problem is similar to the appropriate choice of $h$ in our case. Additional strategies as those used here (i.e. the ignoring of small elongated clusters and finding the best fit) would be necessary. However, despite these problems it would be instructive to compare the outcome of the algorithm with our results.

Performance of the cluster algorithm

In general the cluster algorithm worked well enough for our purpose. Rather than looking for a detailed model of the bouton cloud, we aimed to find a minimal model which captures the major clusters. The compromise we made was that some of the minor clusters were ignored, which makes the model lighter, but the fit to the bouton cloud is worsened. The goodness of the fit between the model and the bouton cloud (both 3D densities) were made by the comparison of the radial distributions of the bouton cloud and the model. For the bouton cloud the radial distribution is simply the histogram of the distances from the cell origin to the soma. For the model, Monte Carlo simulations were used to produce model boutons and from them the model radial distribution. The Monte Carlo simulation would not be necessary if the theoretical radial distribution of the model were known.

The strategy we used in order to find a minimal model was to compare the models for each $h$ with the bouton distribution and to choose the best match. One problem was that for some cells each model had about the same good or bad fit (i.e. $MSE(h)$ constant) although for each $h$ different number of clusters were obtained. So our method did not help to decide what a good model is. In such cases $h$ was selected so that the resulting clusters were close to a solution which was determined by eye. This point could probably be improved by using a more sensitive measure of goodness of fit, for example the total difference in volume of the 3D bouton and model distributions.

A comparison between the selected model and the real data is given in fig 4.9. The comparison reflects the compromises we made in favour of a simple model. The first one was mentioned above (ignoring some small clusters). The second assumption has been that the boutons are normally distributed within a cluster. This is certainly true to some extent, but clear counter examples can also be found. For example, many bouton clouds showed 'fingers'. These fingers were often distributed diffusely over the space so that the boutons were aligned along well-separated axonal paths. The concept of a cluster is difficult to apply in these cases.

4.4.2 Quantitative description of bouton cloud

Relation to other studies

The spatial distribution of boutons and the axonal ramification pattern in 3D in cat cortex has been the subject of few studies and only some of these gave quantitative numbers with which we could compare our data.
Number of boutons: Bouton counts for cells of cat visual cortex are available from other studies for thalamic afférents, basket cells and pyramidal cells of layer 3. With exception of the layer 3 pyramidal cells all cells were filled intra cellularly with the markers HRP or biocytin. The layer 3 pyramidal cells were revealed by bulk injection of biocytin. The total number of boutons counted for the thalamic afférents were different for the X and Y types (Humphrey et al. 1985). For the X types they found a range between 1000 and 4800 boutons (mean 2620). For the Y type more boutons were found, ranging between 2100 to 6700 (mean 4280). The thalamic afférents of our sample (n=3, one X type and two Y types) do not reflect this difference between X and Y types and the numbers (between 2400 and 2900) are rather at the lower end of the range observed by Humphrey et al. (1985).

Bouton counts of small basket cells in layer 4 ranged between 2876 and 3773 (Kisvarday et al. 1985; Kisvarday et al. 1987). This agrees well with our estimates of bouton numbers for layer 4 basket cells (2600 to 3800). In our sample the superficial basket cells formed considerably more boutons (5700 to 6200) than all other basket cells. This is not reflected in another study of a large layer 3 basket cell (Kisvarday et al. 1993). This cell had only 2755 boutons. As was pointed out in the original study this number is an underestimate because proximal boutons could not be counted. In addition it could also be due to some difference between area 17 (where our sample comes from) and area 18 (where Kisvarday et al.'s (1987) cell came from). In the same study a large layer basket cell at the border of layer 5 and 6 of area 18 was revealed, carrying 1599 boutons. Our layer 5 basket cell formed 2010 boutons and was clearly incomplete. Buhl et al. (1997) counted the number of boutons from smooth cells. The counts were performed on labelled cells of in vitro experiments, hence the numbers must be regarded as lower limits. The basket cells, all located in layers 2 and 3 had an average bouton number of 4442 ± 1212 (range 2580 to 5685). Three of the 6 cells had descending axonal processes to layers 5 and 6, forming about 3 to 20% of the boutons there. A smaller number of boutons were found for the dendritic targeting cells in layer 4 (n=5, average number 3439±543, range 2991 to 4308). Double bouquet cells, located in layers 2 and 3 had the highest number of boutons (n=1, average number 6171 ± 1964, range 5469 to 9048). In contrast, the double bouquet of our sample had less than 2000 boutons. With 76% of the boutons in layer 2 and 3, the double bouquet cells showed a clear preference of innervation of the superficial layers.

The number of boutons of superficial pyramidal cells was counted in an vivo study of Kisvarday et al. (1992). For the ten pyramidal cells the bouton number ranged between 69 and 1289 boutons (mean 417). The authors think that the cells were filled quite completely. If this is true this study differs considerably from our study. In our sample all pyramidal cells in layers 2 and 3 had at least 2800 boutons. 3 of the 5 cells had in fact more than 5400 boutons.

Number and volume of clusters: Patchy formations of boutons or axonal ramifications were reported by others for cells in all layers, covering both spiny cells and smooth cells. In general the description remained qualitative. However, some studies estimated the cluster diameter, the inter-cluster distance or the number of
Gilbert and Wiesel (1983) observed clusters of boutons of pyramidal cells and spiny stellate cells in the layers 6, 5, 4 and 2/3. They point out that the exact number of clusters was difficult to assess because they were not always clearly separated. A rough estimate of their published figures gives cluster numbers between 3 and 6 per cell. Also from their figures one can estimate the cluster diameter which is between 200 and 400\(\mu m\). The centre-to-centre distance of clusters from two pyramidal cells in layer 2 and 3 were quite different. They report that one had an average separation of about 800\(\mu m\), the other of about 1.3\(mm\). In their biocytin study of the layer 2 pyramidal cells Kisvarday et al. (1992) counted between 4 and 8 clusters, each containing an average of 80 to 125 boutons. The average separation was 1.1\(mm\). Another pyramidal cell in layer 2 or 3 had clusters between 300\(\mu m\) and 400\(\mu m\) in diameter and inter-cluster distances of 0.5 to 1\(mm\) (Kisvarday et al. 1986). The cell formed clusters in layer 2, 3 and in layer 5. The clusters in these two layers tended to be radially aligned, i.e. occupying the same column. This formation was also observed for a pyramidal cell revealed by Gilbert and Wiesel (1983) and Martin (1988). It could also be observed for some of the cells in our sample (e.g. fig 4.3 p2/3:D or fig 4.1 lgn:C). A patchy distribution of boutons was observed for a spiny stellate cell in layer 4 (Martin and Whitteridge 1984a). This cell had about 7 clusters and the inter-cluster distance was 1\(mm\). Gabbott et al. (1987) revealed two pyramidal cells in layer 5 that projected to layer 5 and 6. They reported elongated clusters of boutons separated by roughly 500\(\mu m\).

Clusters of boutons from basket cells were also reported. Kisvarday et al. (1985) revealed two bouton clusters of a basket cell at the border of layer 5 and 6. One cluster was formed locally around the soma, the other was formed in layer 2 or 3. Both clusters had diameters between 200 and 250\(\mu m\). Smooth cells in general tend to distribute their boutons differently to those of spiny cells. Basket cell axons can, similar to spiny cells, travel horizontally for several mm, but they tend to form finger like structures rather than clusters. Boutons of axo-axonic cells synapse with the initial segment of the pyramidal cells by making multiple boutons there. These boutons are organised in vertical rows and each such row can be seen a small cluster. A axo-axonic cell in layer 2 or 3 formed about 340 rows (Freund et al. 1983). Finally, the basket cell at the border of layer 5 and 6 revealed by Kisvarday et al. (1985) had also a subsystem of smaller clusters (diameter of about 100\(\mu m\)). Such subsystems were also observed for thalamic afferents (Gilbert and Wiesel 1983) and is therefore not a unique feature of basket cells. It was suggested that the smaller clusters reflect specific sampling from a segregation in alternating ON and OFF centres (Gilbert and Wiesel 1983). The fact that the small basket cell in layer 4 had a similar subsystem as the thalamic afferents was taken as an indication that the two cells have common targets in layer 4 (Kisvarday 1992).

The distribution of the boutons made by the thalamic afferents is in general also patchy and there is evidence that they form the anatomical substrate of the ocular dominance columns. The diameter of the clusters is between 300 and 400\(\mu m\) (Freund et al. 1985) and their number ranges from 1 to 4. The centre-to-centre distances of the clusters were reported to be between 300 and 600\(\mu m\) (Humphrey
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et al. 1985) up to 800 \( \mu m \) (Gilbert and Wiesel 1983).

To summarise, the number of clusters reported for the spiny cells is 3 – 8, with centre-to-centre spacings of 0.5 to 1.3 \( mm \) and cluster diameter between 200 and 400 \( \mu m \). For thalamic afferents the number of clusters is between 1 and 4 with centre-to-centre spacings of 300 to 800 \( \mu m \) and a diameter range of 300 to 400 \( \mu m \). For the basket cells only one cell with 2 clusters, in different layers, was observed. Both the centre-to-centre spacing and the diameter was between 200 and 250 \( \mu m \).

A comparison with our data confirms the observed data well. In our study, the number of clusters ranged from 1 to 9 (fig 1.15) which covers the range given above. While this is also true for the subset of our spiny cells (1 to 9 clusters, 4 \( \pm 2 \)) the number of clusters for the thalamic afferents is much bigger than the reported numbers, ranging from 4 to 7. With the exception of one of the basket cells (b4:A), the number of clusters of the basket cells is 1 or 2, again confirming the observed results. The cell b4:A had 5 clusters, all quite small (diameters smaller than 116 \( \mu m \)). Recalling that the basket cells can have substructures it could well be that this is such a cell and the cluster algorithm detected the sub-clusters. The average cluster diameter we measured for the smooth cells was 293 \( \mu m \) and for the spiny cells 390 \( \mu m \) (ignoring the few very large clusters). This confirms the result that the smooth cells have smaller clusters than the spiny cells. The thalamic afferents had a mean diameter of 320 \( \mu m \) which is also well within the previously observed range. The same is true for the centre-to-centre spacing. We observed a mean of about 593 \( \mu m \).

Proximal and distal clusters

The distinguishing of the clusters into proximal and distal regions of the axon is reasonable when one considers that cells in V1 are arranged in vertical columns of similar properties, such as iso-orientation columns. The proximal cluster will generally innervate the same iso-orientation column as that in which the soma lies while distal clusters innervate other iso-orientation columns. A comparison between the two cluster types will reflect differences of how the cell innervates its own column versus remote columns.

An additional classification was made between clusters in the primary layer of innervation (i.e. the layer which is innervated by the majority of the boutons of a cell) and the other layers. Every cell had a proximal cluster in the primary layer. This cluster is quite distinct from the distal clusters and also from the proximal clusters in other layers. The cluster contains more boutons (often more boutons than the total number of the remaining boutons) and its diameter and bouton density is often larger (fig 4.13 A-E). However, within the population of proximal clusters in the primary layer of innervation the variance of the properties can be large, especially the relative number of boutons. This variance can to some extent be explained by a dependence on the number of clusters that a cell forms.

In fact, as fig 4.17 shows, the relative number of boutons \( w_i \) in the clusters can be predicted quite well from the number of clusters \( n \) that the cell forms. The \( w_i \) can be derived by applying a simple algorithm to the clusters. If a cell has two
clusters, one of them containing 82% of the boutons, the other the remaining 18%. If the cell has three clusters, the relative number of boutons can be derived by first forming two clusters as described above. Then the cluster that contains 82% of the boutons is further divided into a cluster that contains 82% of the boutons and the other 18%. One has to proceed similarly for more than three clusters. In our model of sums of normal distributions, the relative number of boutons are the weights of the normal distributions. The dependence of the weights $w_i(n)$ on $n$ reduces the parameters which are necessary for the determination of the model. The decrease of the number of boutons in the proximal cluster with increasing cluster number could reflect some limitation of the number of boutons that a cell can produce.

We also tried to express other quantities such as the volume of a cluster and further its bouton density as a function of the order of the clusters, the number of clusters it forms and the total number of boutons. However, the case is not so clear and further analysis is required (fig 4.18 E and F). It is also probable that another dependency influences these quantities, such as cell class or location within the cortex (i.e. curvature of the layers). In this case a much larger sample of reconstructed boutons would be needed to do any significant analysis, because the number of samples per cell class is often quite small (i.e. 6 for the largest group of layer 6 pyramidal cells, but even in this group the introduction of subgroups would be justified, as for example cells projecting to layer 4 or to layer 6).

**Vertical projections**

The bouton cloud of a cell occupied extensively not more than one or two layers, thus expressing a high degree of layer specificity (fig 4.12). Two exceptions aside (sp4 and db2/3), each cell prefers one layer (primary layer of innervation) to all the other layers, i.e. the relative number of boutons in the primary layer is larger than 60%. Fig 4.23 A shows the average relative occupation of the layers by the boutons of a cell. The thickness of the border of a square represents the relative number of boutons.

If we assume that most boutons in a layer form synapses with dendrites of cells in this layer and that excitatory cells primarily form synapses with excitatory cells, one can recognise main sequences of layers that are connected by the major projections of excitatory cell types. So at least 80% of the thalamic afferent boutons are formed in layer 4. The excitation from the thalamic afferents is directed to layer 3 by the spiny stellate cells (more than > 80% of the boutons). The main innervation of the pyramidal cells in layer 2 and 3 are the layers 2 and 3 and only about 20% to 30% of the boutons are formed in the secondary layer of innervation, layer 5. The layer 5 pyramidal cells form, depending on the subtype, boutons in layer 2 and 3 (between 60% and 80%) or in layer 5 and 6 (percentage of boutons in different layers not known). Depending on the subtype, layer 6 pyramidal cells form more than 80% of the boutons either in layer 4 or in layers 5 and 6. Several other sequences certainly exist. For example branches of the apical dendrites of the layer 6 pyramidal cells in layer 4 and 5 and can sample excitatory input from the spiny cells that are contained in or project to these layers. Furthermore thalamic afferents form boutons also in
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layer 6 (ign:C about 30%). Finally it is true that all cell types form boutons in other than the primary layer of innervation, although, in general less than 20%.

We attempted to estimate the total number of boutons that all cells of a given type forms within any layer. This number is the product of the number of boutons that a cell of a given cell type forms in the layer (given by fig 4.23 A) and the number of cells of that cell type. For simplicity we assumed that the axon of each cell forms the same number of boutons, i.e. 5000 boutons. The number of cells within a cell type are listed in table 4.4. Only the excitatory cells were considered.

The number of cells of a given cell type was estimated as follows. The number of cortical cells in the different layers and the number of thalamic afferent are known (Beaulieu and Colonnier 1983; Peters and Payne 1993). The number of cells beneath a $mm^2$ of cortical surface are listed in the third column of table 4.4. 80% of the cells are excitatory, the remaining cells inhibitory. With the exception of layer 1, this ratio is independent of the lamina (Gabbott and Somogyi 1986). In the fourth column of table 4.4 the relative number of cells of different types in a layer are listed. Layers 2 and 3 is unproblematic because only one excitatory cell type of constant shape is known to exist, the pyramidal cells.

For the spiny cells in layer 4 the situation is more problematic. At least three types exist, the spiny stellate cells, the star pyramidal cells and the layer 4 pyramidal cells. About 40% of the spiny cells in layer 4 are pyramidal or star pyramidal cells (Martin and Whitteridge 1984a; Tarczy-Hornoch et al. 1999), the remaining are spiny stellate cells. In addition we need to distinguish the spiny stellate cells into two subtypes. One forms boutons mainly to layer 2 and 3 (i.e. ss4:C), the other in layer 4 (i.e. ss4:A and B, see also Martin and Whitteridge (1984)). The ratio between these two subtypes as well as the ratio between star pyramidal cells and pyramidal cells are not known. We therefore assumed in both cases a ratio of 1.

Of the excitatory cells in layer 5, the layer 5 pyramidal cells, it is again necessary to form two subtypes. One forms boutons predominantly in layer 2 and 3, the other in layer 5 and 6. We assumed a ratio of 4:1. This estimate is based on the observation that layer 2 and 3 projecting pyramidal cells have a small soma size, while layer 5 and 6 projecting pyramidal cells have a large soma size (unpublished observation). The ratio 4:1 of small to large somata of pyramidal cells in layer 5 was reported in a study of Peters and Yilmaz (1993). Our sample contains only a pyramidal cell that projects to layer 2 and 3. We therefore assumed that the layer 5 and 6 projecting cells forms an equal amount of boutons in both layers.

The excitatory cells in layer 6, the pyramidal cells, also appear into two subtypes. One of them projects primarily to layer 4, the other forms boutons in layer 6. The number of layer 4 projecting pyramidal cells was estimated to be 75% (Katz 1987; Ahmed et al. 1994). The total number of thalamic afferents projecting to area 17 was estimated to be 360000 (Peters and Payne 1993). The total surface area of V1 is about 399$mm^2$ (Anderson et al. 1988). It follows that the number of thalamic afferent per $mm^2$ is 902. The relative numbers for the different cell types per layer are summarised in column 5 of table 4.4. The last column shows that absolute number of cells of a cell type under a $mm^2$ of cortex.

It follows that the total number of boutons formed by excitatory cells in a
vertical column of $mm^2$ surface area is about 5000 · 62582, where 62582 is the total number of excitatory cells beneath a $mm^2$ of cortical surface, including the thalamic afférents. The percentage of boutons formed by the excitatory projections between the different layers is depicted in fig 4.23 B. For each cell type only the paths that involved the two highest percentages of boutons were shown. Most excitatory boutons were used for the projection of layer 6 to layer 4 (14%), from layer 4 to layer 2 and 3 (16%) and for the self-innervation of layer 4 (14%) and layer 2 and 3 (26%). These projection paths are shown as a bold line in the fig 4.23 B. We can also give estimates of the relative contribution of the excitatory boutons in a layer. For layer 4 about half of the excitatory boutons are from layer 6 pyramidal cells and about half from spiny cells in layer 4. The thalamic afferent boutons are represented only very weakly, about 3% of the excitatory boutons in layer 4. In layer 2 and 3 most excitatory boutons are formed by the pyramidal cells in this layer (56%) followed by the boutons of the spiny cells in layer 4 (34%). The excitatory boutons of layer 5 are dominated by the boutons of the pyramidal cells in layer 2 and 3, the excitatory boutons in layer 6 by the layer 6 pyramidal cells. Not shown is the projection of the layer 6 pyramidal cells back to the LGN. Katz (1987) estimated that about 60% (i.e. about 8400 cells under a $mm^2$ of cortex) of the layer 6 pyramidal cells are back projecting to the LGN. So the back projection outnumbers the thalamo-cortical projection by about a factor of 10.

<table>
<thead>
<tr>
<th>ref</th>
<th>layer</th>
<th>cells ($mm^2$)</th>
<th>cell types</th>
<th>rel cell num</th>
<th>cells/type ($mm^2$)</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
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<td>0.5 · 0.6 · 0.8 = 0.4</td>
<td>6576</td>
</tr>
<tr>
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<td>27400</td>
<td>ss4(L2/3)</td>
<td>0.5 · 0.6 · 0.8 = 0.4</td>
<td>6576</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>5900</td>
<td>sp4(L2/3)</td>
<td>0.5 · 0.4 · 0.8 = 0.16</td>
<td>4384</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5900</td>
<td>p4</td>
<td>0.5 · 0.4 · 0.8 = 0.16</td>
<td>4384</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>17300</td>
<td>p5(L2/3)</td>
<td>0.75 · 0.8 = 0.6</td>
<td>3540</td>
</tr>
<tr>
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<td>1180</td>
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<td>17300</td>
<td>p6(L4)</td>
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<tr>
<td>9</td>
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</table>

Table 4.4: Number of spiny cells per cell type and layer. 'ref': reference number. 'layer': layer of soma. 'cells': Number of cells beneath a $mm^2$ of cortical surface. 'cell types': cell type. Indicated in the parentheses is the main layer of projection. 'rel cell num': Relative number of cells of a cell type in a layer (relative to the total number of cells in the layer). 'cells/type': The total number of cells of a cell type beneath a $mm^2$ of cortical surface.
4.4. DISCUSSION

Horizontal projections

For simplicity we focused on the clusters in the primary layer of innervation and used the 2D distribution of the bouton cloud, after having the boutons projected onto a horizontal plane. The axons of spiny cells spread their boutons over an area which is roughly the size of $11 \times 300 \mu m$ cubes. For the smooth cells this area is only about $2 \times 300 \mu m$ (fig 4.22 A). The total area occupied by the clusters is in general higher than 50% of the total area (fig 4.22 B). The exceptions are the thalamic afferents and the pyramidal cells in layer 2 and 3, both of which form many though small clusters.

The horizontal displacement of the clusters from the soma can be as much as 2mm (fig 4.19), which can be far enough to innervate cells whose classical receptive fields do not overlap with its own receptive field (Albus 1975a). It is hypothesised that such far reaching axons are responsible for the non-classical receptive field features (Gilbert 1992). However, for the spiny cells the average displacement from the soma is about $800 \mu m$. Boutons of these clusters can form synapses with cells whose receptive field locations are neighbouring the receptive field location of the parent cell. The direction of the displacement will be biased towards a direction that is determined by the retinotopic map and the direction of the displacement of the cluster (Albus 1975a). The average radius of the distal clusters of spiny cells (in the primary layer of innervation) is about $190 \mu m$ (fig 4.13 D). Assuming a change in preferred stimulus orientation of 10 deg per $50 \mu m$ horizontal displacement from the parent cell (Albus 1975b), the cells contacted by the boutons in the cluster will have preferred stimulus orientations that differ by 120 to 200 deg from the preferred stimulus orientation of the parent cell, i.e. cells of quite similar preferred stimulus orientation are contacted. This result is supported by direct methods (Gilbert and Wiesel 1989; Kisvarday et al. 1996).

The distal clusters of the smooth cells are less far displaced than that of the spiny cells. However, smooth cells can form horizontal arms over laterally long distances (e.g. fig 4.5 b5 and b2/3:B) which were not recognised as clusters by the cluster algorithm. The average horizontal displacement of the distal clusters from soma is $400 \mu m$ (fig 4.13 F) and have an average radius of $130 \mu m$ (fig 4.13 D). The boutons in these clusters will probably form synapses with cells whose receptive field locations are randomly distributed in a small environment around the receptive field location of the parent cell. The preferred stimulus orientation of the contacted cells will be between 50 and 100 deg, i.e. oblique or perpendicular to the preferred stimulus orientation of the parent cell (Albus 1975a; Albus 1975b). This is in accordance with the results of Kisvarday et al. (1996).

In both cases, spiny or smooth cells, the proximal cluster contains more than half of the boutons in the bouton cloud (fig 4.13 C). This means that the strongest innervation of a cell is onto cells that are located in a small local neighbourhood. For spiny cells this neighbourhood has a diameter of $600 \mu m$. For the smooth cells it has a diameter of $340 \mu m$ (fig 4.13 D). The receptive field location of the contacted cells are in both cases randomly scattered around the receptive field location of the parent cell and contact cells of preferred stimulus orientation differences up
to 34 deg for the smooth cells and 60 deg for the spiny cells (Albus 1975a; Albus 1975b).

The direction of the horizontal displacement from the soma of the distal clusters was correlated with the preferred stimulus orientation and direction of the cell (fig 4.20 and fig 4.21 C and D). For the total sample of cells no correlation could be detected. This is also the case when only the pyramidal cells or the spiny stellate cells are considered separately. However, for the layer 6 pyramidal cells and the basket cells in layer 4 the preferred stimulus orientation tended to be perpendicular to the direction of displacement of the clusters (8 of 10 clusters for the layer 6 pyramidal cells and all clusters for the basket cells (fig 4.21 C). For these clusters no systematic arrangement relative to the preferred stimulus direction of the cell could be detected (fig 4.21 D, occurrence of white and black dots in the perpendicular segment). However, the results are only suggestive because of the small sample size (5 layer 6 pyramidal cells and 2 layer 4 basket cells).

Cortical cells with elongated axons were observed for several species, such as cat, monkey and tree shrew. For the monkey it was reported that the axis of longest elongation is perpendicular to the ocular dominance columns or the border of V1 and V2 (Malach et al. 1993; Blasdel et al. 1995; Yoshioka et al. 1996). For the tree shrew the axis of largest elongation for bouton clouds of superficial cells was shown to be parallel to the preferred stimulus orientation (Fitzpatrick 1996; Bosking et al. 1997). For the cat a systematic analysis was made for the feedback projections of the layer 6 pyramidal cells to the dLGN. It was revealed that the boutons in the dLGN are elongated and the axis of longest elongation are perpendicular or parallel to the preferred stimulus orientation (Murphy et al. 1999). For the superficial cells in cat V1 it was shown the their boutons tend to be elongated along the preferred stimulus orientation (Schmidt et al. 1997). However, it was already reported by Gilbert and Wiesel (1983) that some of the axons were elongated with axis of largest elongation either perpendicular or parallel to the preferred stimulus orientation of the cell. Our data shows that such a correlation could also hold for the layer 6 pyramidal cells and the smooth cells. However, it does not seem to be true for the superficial pyramidal cells or the spiny stellate cells in layer 4.

For boutons within a radius of 500 μm no correlation was found between bouton distribution and preferred stimulus orientation in the tree shrew (Fitzpatrick 1996). We were looking for such a correlation for the proximal clusters of our cells (fig 4.20 and fig 4.21 A and B). No striking correlation could be detected, neither for the whole sample of cells nor for individual cell types. However, some tendencies could be recognised. The smooth cells, for example, lacked cluster orientations parallel to the preferred stimulus orientation. In contrast, spiny cells lacked cluster orientations perpendicular to the preferred stimulus orientation.

Asymmetric axonal fields, in the sense that the horizontal bouton distribution has a strong bias in a given direction, were observed in the cat cortex for layer 5 pyramidal cells projecting to layer 6 (Gabbott et al. 1987). If the direction of asymmetric of the axonal field is similar to the preferred stimulus direction of the cell, the excitation of the layer 5 pyramidal cell, when stimulated with a bar moving in the preferred direction of motion, would excite the contacted layer six
cells ('priming'). The response sensitivity of these cells would then be increased and could serve as an estimate of the future position of the object. Such facilitating effects were experimentally demonstrated (Nelson and Frost 1985; Ts’o et al. 1986). For a priming mechanism, one would expect the existence of distal clusters that are horizontally displaced along an axis perpendicular to the preferred stimulus orientation. Of the 18 cells with known preferred stimulus orientation 15 showed such a constellation of clusters (fig 4.20 and 4.21 D).
Figure 4.23: Laminar distribution of boutons of different cell types. A: For each cell type the relative number of boutons in the different layers were indicated by the thickness of the border of the squares. Black dot indicates the location of the soma. Horizontal stippled lines represent the lamina borders. B: Flow chart indicating the percent of boutons that are formed by the projections of the excitatory cells under $1mm^2$ of cortex. Each arrow represents a projection of a cell type to a layer (gray boxes, the labels in the boxes indicate the layer). The number close to the arrowhead indicates the percent of boutons that were formed in the layer by the projections of a given type. Projections of cell types that formed more than 10% of the boutons were indicated by bold arrows. Projections of cells that formed less than 0.5% boutons were not shown (L5 to L4: 0.3%, LGN to L6: 0.2%, L3 to L6: 0.3%).
Chapter 5

Distribution of boutons along axonal branches

The spatial distributions of boutons (and by implication, their synapses) can provide useful insights into the organisation of the connections between neurons in the cortex. For example, bouton density distributions have shown that some neurons in cortex make their synapses quite selectively (on the scale of the diameters of dendritic arborisation), forming 3D clusters of high bouton density (see chapter 4) that are likely to influence a localised population of target neurons. On a smaller scale, bouton distributions can offer insights into the manner of synapse formation between individual neurons. For example, the one dimensional (1D) arrangement of boutons along axon collaterals offers information about the possible selectivity of individual connections. In this regard, a study of mouse cerebral cortex has shown that the arrangement of boutons on axonal collaterals is essentially random, i.e. a Poisson process (Braitenberg and Schütz 1991; Hellwig et al. 1994). Those studies examined Golgi-stained material which stains only the local axonal field of cells. The results reported were therefore necessarily confined to more proximal axon segments.

Here we study the bouton distribution along the reconstructed axonal tree of the sample of neurons that were used in chapter 4.

5.1 Material and Methods

Bouton representation

The boutons are represented by points in the 3D space, and the axonal and dendritic collaterals by open polygons. The locations of the boutons were digitised together with the axons. So each bouton is linked to its source collateral, and this data organisation permits us to study the distribution of the boutons along the collaterals.

The boutons do not always lie exactly on the route of the axon as represented by the digitised polygon. Trivially, the boutons may lie away from the polygon because physically extended structures such as the axon are digitised as points and
lines, which have no thickness. Moreover, there are small measurement errors in the digitisation process. The third, and biologically relevant, reason for displacement of the boutons away from the axon is the existence of boutons terminaux. These are boutons connected by a small axonal stalk, or neck, to the axonal shaft. In order to analyse sequences of boutons on axonal branches we chose to project the measured points (boutons) onto the axonal polygon (fig 5.1).

![Figure 5.1: Representation of an axon. The reconstruction of an axonal branch is represented by a polygon (p) in the 3D space. Boutons on the axon are represented by points (q). q' is the projection of q onto the polygon. We analysed the sequence of the points q'.](image)

**Dendrograms**

A dendrogram of a tree is a 2D diagram that reflects the branching behaviour of the tree. Each branch point is represented by a horizontal line, which links the parent branch to its daughter branches. The length of this line is chosen to be long enough to avoid intersections with other branches. The branches (also axon collaterals) themselves are represented by vertical lines and their length is proportional to the measured length of the branch. We also superimposed the locations of boutons (projected onto the branches) onto the dendrogram. It is a property of the dendrogram that all its intersections with the same horizontal have the same displacement from the origin of the tree, when the distance is measured along the shortest axonal path from an intersection to the origin of the tree. End collaterals are branches for which no successor branches exist. The remaining branches we call inner branches.
5.2. RESULTS

Bouton density plots

The bouton density as a function of displacement from the tree origin (soma for the cortical cells and the point of entrance from the white matter to layer 6 for the thalamic afferents) were obtained by dividing the dendrogram into horizontal bands, or bins, of equal width, beginning at the origin. The bouton density in each bin was obtained by dividing the total number of boutons in that band of the dendrogram by the total length of axon in the band. We chose a band width of 100\(\mu m\). Thus, for example, all boutons in the second band of the dendrogram have a distance of between 100\(\mu m\) and 200\(\mu m\) from the tree origin, measured along the shortest path on the axonal tree leading to the tree origin.

Fit of inter-bouton distances

A histogram of interbouton intervals was computed for each neuron. The bin size of the histogram is 1.5\(\mu m\). The histogram was fitted by exponential and gamma distributions. For the fit only distances were used which were smaller than \(d = i \cdot 1.5 - 0.75\), where \(i\) is the first bin for which the bins \(i\) and \(i + 1\) contain no distances. The \(d\)'s were different for different cells, ranging from 42 to 104\(\mu m\) (mean 76 ± 17\(\mu m\)). The best fit was found by minimising the least square with the Levenberg-Marquardt method.

Poisson process with dead zones

A stationary Poisson process with dead zones (Cox and Isham 1980) was simulated in Matlab. First a Poisson process with rate \(1/\lambda\) (\(\lambda > 0\)) was created. A distance \(\tau > 0\) from a normal distribution with mean \(\mu > 0\) and standard deviation \(\sigma\) was chosen. Then beginning with the first point of a Poisson process, all following points were deleted until the resulting inter-distance was larger than \(\tau\). Selecting a new \(\tau\) and going on with the next point of the process, this procedure was repeated until the end of the process was arrived at. It can be shown that the new process created in this way has an average inter-bouton distance of \(\mu + \frac{1}{\lambda}\) (Cox and Isham 1980).

5.2 Results

The spatial distribution of boutons along axonal trees of the 30 neurons were investigated. The dendrograms (fig 5.2 to 5.5) were used for further analysis of the bouton statistics. The dendrogram captures the relevant properties of the data because it represents compactly the branching behaviour of the tree, the length of the collaterals and the location of the boutons on the collaterals, in the correct proportions. The statistics of the interbouton distances and bouton densities on the dendrograms will be particularly important here.
Figure 5.2: Dendrogram of a spiny stellate cell (ss4:B). The large circle indicates the cell body in layer 4. Axonal branches are represented by vertical thin lines. The horizontal lines are only drawn for visibility and do not represent axonal branches. The boutons are represented by the dots. Large dots indicate the boutons in one of the axonal patches of the cell (see chapter 4). The large circle indicates the cell body in layer 4. The same
Figure 5.3: Dendrogram of a layer 4 basket cell (b4:B). The large circle indicates the cell body in layer 4. Scale bar 500μm.
Figure 5.4: Dendrogram of a layer 6 pyramidal cell (p6:A). Scale bar 500\(\mu m\).
Figure 5.5: Dendrogram of a thalamic afferent (lgn:B). The curved line indicates the border from layer 6 to white matter (wm). Scale bar 500μm.
Characterisation of the axonal trees and branches

The total axonal length, i.e. the total sum of the collateral lengths, of the neurons was about 34 ± 13mm (range [10, 60], fig 5.6 A). Although the axonal arborisation of smooth cells (n = 9) are restricted to a smaller volume than spiny cells (n = 18) or thalamic afferents (n = 3), this is not reflected in the branch length. The longest branches were made by the pyramidal cells in layers 2 and 3 (n = 5) and the layer 5 pyramidal cell (n = 1). They all had values longer than 55mm. On average, the axonal tree was formed of 419 ± 261 collaterals (range [103, 1271], fig 5.6 B). The spiny cells and thalamic afferents tend to have fewer axon collaterals than the smooth cells. For example the basket cell b2/3 B had about twice as many collaterals than the maximal number of collaterals of the former group. The average number of branches of the smooth cells was 689 ± 309 (range [325, 1271]), that of the spiny cells and thalamic afferents 303 ± 126 (range [103, 587]). It follows that the branch lengths are different for the two groups. Indeed, as can be seen in fig 5.6 C the branch lengths fall into broad groups. One is formed by smooth cells. The medians of the collateral lengths of this group vary between 21μm and 45μm (27 ± 7μm). The other group is formed by the spiny cells and range from 58 to 148μm (92 ± 20μm). Within this group all spiny stellate cells in layer 4 had in general smaller collaterals than the remaining cells. The thalamic afferents tend to fall into the same group as the smooth cells.

The distribution of collateral lengths tends to be similar for axons in the same cell class. The distribution of the thalamic afferents fall between the two extreme classes of short collateral lengths (smooth cells) and long collateral lengths (pyramidal cells, fig 5.6 E, F and G). Also note the long branches of the pyramidal cell in layer 6, p6:F. This is the cell whose axon is restricted to layer 6.

Not all collaterals have the same lengths. For example the end collaterals, which make up 50% of the number of collaterals, often have a median which is different from the lengths of the inner collaterals (fig 5.6 D). An extreme example is the star pyramidal cell (sp4), whose median of the end collaterals is about 180μm longer than the median of the inner collateral. 83% of the cells have larger end collateral than inner collaterals. However, for many of them the difference is smaller than 20μm.
5.2. RESULTS

Figure 5.6: Characterisation of the axonal branches. A: The total axonal length of individual cells was computed. B: The total number of axon collaterals are shown for each cell. C: Median (1/2-quantile) of the collateral lengths of each cell. D: Difference in median of the subsets of the end collaterals and inner collaterals of each cell. E, F and G: Histogram of the number of collateral lengths for the class of spiny cells (E, without p6:F), the thalamic afferents (F) and the smooth cells (G). Bin size is 10\(\mu\)m. The shaded area indicates the standard deviation of the number of collaterals per bin for the different cells in a class. The solid line indicates a fit of a gamma function to the distribution of the pooled lengths. Parameter values \(A\) and \(B\) and goodness of fit \(r^2\) for the three fits were \(A = 0.8341, B = 159.0264, r^2 = 0.9842\) (E), \(A = 1.5706, B = 21.1260, r^2 = 0.9878\) (F) and \(A = 1.9813, B = 52.0518, r^2 = 0.9991\) (G).
5.2.1 Bouton density

For each neuron the location of the boutons on the axonal tree is characterised by a local bouton density as a function of displacement from the tree origin, measured along the axonal tree (fig 5.7). The origin of the axonal tree is at the soma for cortical neurons. For geniculate neurons the origin was chosen for convenience to be at the border of white matter and layer 6. The bouton density was determined by partitioning the dendrogram into horizontal strips of 200\(\mu m\) width. The total axonal length and the total number of boutons of a cell falling into each strip was determined and the density was calculated by dividing the former by the latter.

The layer specificity that we have already seen for the bouton distribution in 3D (chapter 4) is also reflected in the bouton density as defined above. For example the layer 6 pyramidal cells avoid layer 5 and innervate strongly layer 4. The bouton density is in fact low close to the soma and suddenly jumps to a higher level at some distance, presumably at the border of layer 5 and layer 4 (fig 5.7 A, shaded area and bold line in inset p6). In contrast, the layer 6 pyramidal cell whose axon is restricted to layer 6 (p6:F, lower stippled line in fig 5.7 A, inset p6) has a high density of boutons within 400\(\mu m\) from the soma.

The bouton density tends to be similar within a class of neurons (fig 5.7 A). However, the variation can be large. For example the layer 6 pyramidal cell p6:G (upper stippled line in fig 5.7 A, inset p6) has, compared to the other cells in this class, a very high bouton density. The spatial density of this cell was also exceptionally high (chapter 4). The class of the layer 6 pyramidal cells that project to layer 4 have an initial low density region of about 400\(\mu m\), followed by a high density region of about 1\(mm\) starting at a distance of 1\(mm\) from the soma. In contrast, the remaining pyramidal cells, together with the spiny stellate cells, have an initial region (smaller than 200\(\mu m\)) of low density, followed by a large, high density region of about 2\(mm\) (fig 5.7 A, inset ss4 p2/3 p5 p4). The smooth cells have a very high density region from the origin onward (fig 5.7 A, inset b5 b4 b2/3 db2/3). This region is short, as is the length of the longest axonal path (fig 5.2). The thalamic afferents resemble closely the density distribution of the layer 6 pyramidal cells that project to layer 4. The only difference is a possible innervation of layer 6, e.g. the two Y afferents (lgn:B and lgn:C). This causes the first bump in the density distribution (fig 5.7 A, inset lgn).

The average number of boutons per 100\(\mu m\) is, for the spiny and thalamic cells, between 4 and 10 boutons. Exceptions are 3 layer 6 pyramidal cells with values higher than 10. With the exception of the double bouquet cell (db2/3) and the layer 5 basket cell (b5) the smooth cells have more than 10 boutons in a 100\(\mu m\) bin (fig 5.7 B). At least for the basket cell b5 it cannot be excluded that the low density could be due to an incomplete filling of the axonal tree.

The obvious reason for the change in bouton density along the axonal tree can be observed in the dendrograms (fig 5.2 to 5.5). The cells tend to have their boutons distributed on the end collaterals and the inner collaterals are often only sparsely covered with boutons. In fact, the sum of the end collateral lengths range between 40 and 73% of the total axonal length (54 ± 8%) and carry between 52 and 82% of
the total number of boutons \((67 \pm 8\%)\). This is true for the smooth cells as well as for the spiny and thalamic cells. The existence of collaterals with sparsely covered boutons lower the average bouton density wherever they occur on the tree.

That the boutons tend to be located towards the end collaterals of the axonal tree can also be seen by the fact that for the spiny cells and thalamic afferents 75\% of the boutons are contained in the last \(231 \pm 96\mu m\) part of the axon. This was determined as follows. For each bouton the path was determined that leads from the bouton to the closest tip of an end collateral. For each cell the \(\frac{3}{4}\)-quantile of these lengths was determined and the average over the different cell was calculated. The quantiles ranged from \(81\mu m\) (ss4:C) to \(474\mu m\) (sp4). For the smooth cells the range was 29 to \(136\mu m\) (57 \(\pm 33\mu m\)).

**Figure 5.7:** Bouton density measured along the axonal tree. A: The bouton density as a function of distance from the soma, measured along the axonal paths, was determined. The y-axis indicates the number of boutons that are typically found in a \(200\mu m\) long piece of axon which is located at some distance from the soma (x-axis), the distance measured along the axonal paths. The density profiles are shown for four different groups of cells, as indicated by the labels at the top of the four figures. The bold line indicates the average of the density profiles in a group. The shaded area indicates the maximum (top curve) and minimum values (bottom curve) of the cell profiles. Stippled lines indicate the density of cells that were ignored for the computation of the mean curve and the shaded area (p6:F and p6:G). B: Average bouton density of each cell.
5.2.2 Interbouton distances

An important characterisation of the boutons along the axonal tree is derived from the distances between consecutive boutons. For each bouton on the tree the distance to the 'lower' neighbour', i.e. to the nearest bouton which is on the axonal path from the bouton to the root was determined. If no lower neighbour exits, the bouton was ignored. Because there are branch points, it is possible that several boutons have the same lower neighbour. Long distances were viewed as outliers and were eliminated for further analysis (see 5.1 for determining the threshold). The relative number of distances discarded ranged between 0.1% and 3.4% (0.9±0.7%). The two highest values occurred for two spiny stellate cells (> 2%). The largest interbouton distance of individual cells ranged between 84\(\mu\)m and 2\(\text{mm}\). The exceptionally huge distance of 2\(\text{mm}\) was on a thalamic afferent (lgn:B). Excluding this large distance, the average of the interbouton distances that were eliminated was 419 ± 283\(\mu\)m.

Histogram of interbouton distances

The histogram of interbouton distances (bin size is 1.5\(\mu\)m) was skewed to the left for all cells (fig 5.8). The distributions within a class tended to be similar (e.g. basket cells in layers 2 and 3). Cells of different cell classes could also have similar distributions (e.g. pyramidal cells in layers 2 and 3 and spiny stellate cells in layer 4) but this was not necessarily the rule (e.g. layer 6 pyramidal cells and basket cells).

A clear difference in the interbouton distances of different cells could be observed in the first three bins of the histogram. Either the number of interbouton distances was maximal in the first bin or in the third bin (fig 5.8). We took the ratio of the number of distances in the first bin and the third bin in order to quantify this difference (fig 5.9 A). For a ratio smaller than one we say that the cell lacks small distances. In general the thalamic afferents and the smooth cells showed a distinct lack of small distances (< 0.6), with two exceptions (b4:C and db2/3). The layer 6 pyramidal cells had the highest ratios (four of them > 2).

We classified the different cell types into four groups according to the histogram of interbouton distances (fig 5.9 C, D, E and F). This was done by eye and should serve only as a help to emphasise the differences and similarities of the distributions. The layer 6 pyramidal cells and the star pyramidal cell in layer 4 formed one group. All members except the pyramidal cells p6:F and p6:G had quite similar distributions. These two exceptions formed extreme cases. p6:F had a flat distribution while p6:G had a steep distribution. p6:F could have been placed in the group which is formed by the remaining spiny cells. This group had mainly flat distributions. Some of these cells had a slight tendency to have less small values so that the maximum of the curve was not in the first bin but in the third. For the third group, the thalamic afferents, the lack of small distances is more pronounced for all three members. No difference could be detected between the X or the Y cells. The last group is formed by the smooth cells. For these cells (except the double bouquet cell), the lack of small distances is more pronounced and the distributions are very steep.
Figure 5.8: For each cell the histogram of interbouton distances is shown (black dots). Gamma distributions with parameter $A$ and $B$, indicated in the lower right corner of each distribution (two numbers in parenthesis), were fitted to histograms which showed a lack of small distances. Exponential distributions with parameter $\lambda$, indicated in the lower right corner of each distribution (one number in parenthesis), were fitted to the data which did not show a lack of small distances. In both cases the fitted distribution is indicated by a gray thick line. Horizontal direction: interbouton distance. Vertical direction: relative number of events per bin. Bin size $1.5 \mu m$. 

\[ 5.2. \text{RESULTS} \]
Figure 5.9: Classification of interbouton distance distributions. A: Lack of small distances was quantified for each cell by the ratio of the values in the first bin and the third bin of the interbouton distance histogram. A ratio smaller than 1 is interpreted as a lack of small distances. B: Mean of interbouton distances determined for each cell (dots). C, D, E and F: Cell types of similar interbouton histograms were grouped together. Four groups were formed. The cell types in each group are indicated. The solid line indicates the mean of the histograms in each group. The upper border of the shaded area indicates the maximum and the lower curve the minimum of the histograms. Bin size was 1.5\( \mu \text{m} \). The stippled upper curve in group C is the histogram of the layer 6 pyramidal cell p6:G, the stippled lower curve indicates the histogram of p6:F. The stippled curves in group F indicate the histograms of db2/3 and b5. These cells were not used in the determination of the mean, maximum and minimum curves.
5.2. RESULTS

Average interbouton distances

Average values of interbouton distances varied considerably, between 5.1 and 14.3\(\mu m\) (fig 5.9 A), depending on the cell class. Smooth cells, with the exception of the basket cell in layer 5 (mean 7.3\(\mu m\)) and the double bouquet cell in layer 3 (mean 8.3\(\mu m\)), had small average distances (6.9 ± 0.7\(\mu m\), range [6.0, 8.3]). The spiny cells and thalamic afferents tended to have larger interbouton distances (10.1 ± 1.8\(\mu m\), range [7.6, 14.3]). A layer 6 pyramidal cell (p6:G) was not included in the spiny cell group. This cell had the smallest average interbouton distances of all the cells (5.1\(\mu m\)).

Fit of exponential and gamma distributions to the histograms

The histograms in fig 5.8 suggests fitting a gamma or exponential distribution to the data. If the cell lacked small distances (ratio < 1) we fitted a gamma distribution with parameters \(A\) and \(B\). For cells which did not show a lack of small distances (ratio > 1) an exponential distribution with parameter \(\lambda\) was fitted. About 40% of the cells with ratio > 1 could be fitted well with an exponential distribution (\(p < 0.001\), Kolmogorov-Smirnov test). This included the cells p6:C, p6:F, s4:B, s4:C, p2/3:B, p2/3:C and db2/3 (fig 5.8). Thirty percent of the cells that showed a lack of small distances (ratio < 1) had no significant deviation from the gamma distribution. These were the cells p5, p4, ss4:A, b4:B and b4:D (fig 5.8). The distribution of the interbouton distances of most basket cells and all thalamic afferents are not well fitted by the gamma distribution. In all cases the number of small interbouton distances (between 3 and 4.5\(\mu m\)) was underestimated by the fitted curves.

The distribution of the interbouton distances on different clusters

We also wanted to know if the distributions of interbouton distances are different for different clusters. We compared the cluster that contained the highest number of boutons (which is in general the local cluster around the soma, i.e. the proximal cluster) with the remaining clusters. Only interbouton distances were considered for which both boutons were in the cluster. Some clusters had significantly different distributions (\(p = 0.001\), Kolmogorov-Smirnov test). While no smooth cell had clusters of different distributions, 52% from the spiny cells and thalamic afferents had such clusters. No systematic pattern could be observed (not shown). The result is not altered if only the subset of boutons on the end collaterals were considered. But in this case a clearer picture evolves. If a cluster had a significantly different interbouton distance distribution it was either in a different layer to the soma or, if it was in the same layer, it was one of the most distal clusters (fig 5.10, black dots). Examples of distributions of significantly different clusters are shown in fig 5.11. The number of distances in these clusters ranged from 184 to 627 (388.9 ± 152.3).
Figure 5.10: For each cell the clusters are shown (big circles) in the layer of appearance. The layers are indicated by stippled horizontal lines. The gray shaded dots indicate the reference clusters. The black dots indicate clusters whose interbouton distances were significantly different from that of the reference cluster. White dots indicate clusters which were not significantly different. The small white dots indicate the cell bodies. The large distances were excluded from the analysis and only boutons on end collaterals were used.
5.3 **Discussion**

5.3.1 **Differences in bouton formation**

Our data suggests that cortical cells (including thalamic afferents) of cat V1 form, on average, a bouton every 10\(\mu\)m along the axonal tree. However, the mode of bouton formation changes for different cells and also for different parts of the axonal tree.

**Different axonal parts**

Evidence for changes in the mode of bouton formation on different parts of the axon is evident for all cell types studied. The density of boutons on axonal branches changes with the location on the axon (fig 5.7 A). The change can often be correlated with the location of the bouton in the cortex. For example the layer 6 pyramidal cells that project to layer 4 avoid forming synapses in layer 5. But also within a layer the formation of boutons can change. Often boutons are formed infrequently on an axonal branch before the branch forms a distal cluster (fig 5.2). This last observation can also be seen as the prominent preference of the boutons to be located on the end part of the axonal tree (fig 5.5). In fact the number of boutons that are formed on the end collaterals, which make up about half of the total length of the axonal tree, comprise on average about 70% of the total number of boutons. A possible explanation is that the boutons prefer to form synapses in axonal clusters which are primarily made on the end part of the axonal tree.

**Different cells**

The bouton density as function of distance from the soma changes for different cells (fig 5.7 A). However, cells of similar type tend to have similar interbouton distance distributions. For example all layer 6 pyramidal cells that project to layer...
4 will prefer to form boutons in layer 4 and avoid layer 5. This is reflected by a stereotyped shape of the density function. A low density up to 500µm (measured from the soma) followed by a region of higher density (fig 5.7 A p6). The bouton density function can also change for cells within one cell class. The density function of p6:G for example has the same shape as all the other layer 4 projection pyramidal cells in layer 6 but is scaled in y-direction, i.e. the bouton density is everywhere higher compared to the other cells (fig 5.7 A inset p6, upper stippled line).

The differences in the bouton formation for different cells is also revealed when studying the distances between consecutive boutons (interbouton distances) on an axonal tree. The mean of these distances covers a range between 5.1 and 14.3µm. That means that on average one cell forms a bouton every 5.1µm of axon while another cell only every 14.3µm. It is difficult to explain this difference. There is a weak correlation of the average interbouton distance with the cell type. Spiny cells and thalamic afferents tend to have larger average distances than smooth cells (7.5±1.8µm and 10.3±2.3µm). This could reflect the fact that the target selectivity of smooth cells is different from that of spiny cells. But even within the same cell class and for boutons within the same layers large differences exist. For example in layer 4 bouton formation on axons of layer 6 pyramidal cells occur every 5.1µm (p6:G) or every 9.5µm (p6:B).

The differences in interbouton distances could also be due to different target specificities of cells. For example a basket cell in layer 4 forms on average every 7µm a bouton on the distal part of the axon, for the spiny stellate cells it is only every 12µm (fig 5.9 B). This could have to do with the different target preferences. Although both cells prefer to contact spiny cells, the basket cells prefer to form synapses with cell bodies and dendritic shafts, while the spiny stellate cells prefer dendritic spines. This is further supported by other cell types in our sample (fig 5.12). If this correlation holds for the pyramidal cells p6:G and p6:B it would follow that p6:G prefers to target less spines than p6:B. Unfortunately we do not have the data to check this. From fig 5.12 one can also see a tendency for the interbouton distances to correlate with the layer of soma. Layer 6 pyramidal cells have a smaller interbouton distance than the spiny stellate cells in layer 4 or the pyramidal cells in layers 2 and 3. However, the correlation does not seem to hold for smooth cells.

There are two studies in which the average interbouton distance of cells in cat V1 were determined. For a layer 4 basket cell the average was 6.4µm (Kisvarday et al. 1985) and for a basket cell in layer 5 the value was 9.4µm (Kisvarday et al. 1987). This supports our finding that the basket cells in layer 5 have larger interbouton distances than the basket cells in layer 4. In our sample we found a mean interbouton distance of 10µm for the basket cell in layer 5 and a mean of 7µm for the basket cells in layer 4. For interbouton distances of thalamic afferents of the cat (or kitten) visual cortex (area 18, layer 4A) an interbouton distance of 11.2µm was determined (Friedlander and Martin 1989). Again, this is in good agreement with our values (average 10.7±0.8µm).

The distribution of interbouton distances reveals a further difference between bouton formation. All distributions are skewed to the left but display differences for very short distances. Some cells show a lack of small distances while others do
5.3. DISCUSSION

Figure 5.12: For each cell the average interbouton distance (y-axis) was correlated with the percent of contacted spines (x-axis) of cells of similar type as given in table 1.1). Correlation analysis $r = 0.80$, $p = 0.00$, $a = 5.86$, $b = 0.07$.

not (fig 5.8). The occurrence of small distances is especially pronounced for the smooth cells and the thalamic afferents while it is less pronounced for superficial pyramidal cells and absent for layer 6 pyramidal cells (5.9 A and C-F). A lack of small distances was also reported in the study of the basket cells in layer 4 and 5 (Kisvarday et al. 1985; Kisvarday et al. 1987) and for spiny cells (Martin and Whitteridge 1984a).

A lack of small distances can be interpreted as the existence of a 'dead zone', i.e. a small neighbourhood that is formed on the axon around each bouton in which no other bouton can be formed. The existence of a dead zone can be understood if one remembers that the boutons are physical objects of about 1$\mu$m in diameter, aligned on an axonal branch of about 0.1$\mu$m diameter. It follows that the centre of two boutons can never be closer than 1$\mu$m. Indeed, the observed lack of small distances is of that order ($< 3\mu$m). However, other effects such as limitations given by the growth mechanisms for boutons could also play a role. The reason why we do not see a lack of small distances for some cells could have to do with our representation of the boutons. In order to analyse sequences of boutons we projected the location of the boutons terminaux onto the polygon that represents the axonal branch (fig 5.1). In this case the physical extent of the bouton does not imply a dead zone (fig 5.13 A).
If the lack of small distances is due to our representation of the boutons, one would expect a correlation between the relative number of boutons that are of type terminaux and the lack of small distances observed in the distributions. We investigated if this is true for our sample. The relative number of boutons of type terminaux are shown in fig 5.13 B. Most cells have less than 40% of these boutons. Exceptions are the layer six pyramidal cells that project to layer 4 (60±10%, range [50,70]). For the spiny cells this agrees with the findings of other studies. It was found that layer 6 pyramidal cells have many boutons terminaux (McGuire et al. 1984; Martin and Whitteridge 1984a). For the thalamic afferents, star pyramidal cells and basket cells boutons en passant were the most common (McGuire et al. 1984; Freund et al. 1985; Martin and Whitteridge 1984a; Ahmed et al. 1997). For a layer 3 and layer 5 pyramidal cell the axon was covered with both types of boutons (Kisvarday et al. 1986; Martin and Whitteridge 1984a). We also found some exceptions to this scheme. One spiny stellate and a thalamic afferent had many boutons terminaux (ss4:C, 75%, Iq:n:A, 80%). Iq:n:A is, in contrast to the remaining thalamic afferents in our sample of type X. Of the spiny cells ss4:A projects strongest to layer 3.

The scatterplot shows that the higher the relative number of boutons terminaux is, small distances will occur more frequently (fig 5.13 C). This favours our hypothesis. However, the cells ss4:C and Iq:n:A d which were ignored for the correlation analysis do not fit into this scheme. A similar correlation was observed by Martin and Whitteridge (1984). They compared the interbouton distances between rows of boutons en passant and rows of boutons terminaux in a row (at least 5 in a row). The study showed that the boutons terminaux formed more small distances than the boutons en passant. They used a similar method to ourselves for the measurement of the distances between boutons, i.e. they used the projections of the boutons onto the axonal shaft.

5.3.2 A model for bouton placement

Poisson process

It was argued that a possible mechanism for bouton placement along axonal branches is randomness, i.e. that the boutons on all collaterals are placed according to the same Poisson process (Braitenberg and Schüz 1991; Hellwig et al. 1994). The model was proposed for cells of the monkey cortex. We would like to discuss how far this model applies to our sample of neurons.

It is clear that the model can not be true for the whole axonal tree because of the tendency of the boutons to be located towards the end of the tree. Randomness would imply that all collaterals form about the same density of boutons. However, the model could be true for the end part of the axonal tree. Indeed, the distribution of the interbouton distances between boutons on the end collaterals are in general the same for different clusters (fig 5.10). This indicates that the same mechanism of bouton formation is at work at different locations on the end part of the axonal tree (stationarity).
5.3. DISCUSSION

Figure 5.13: Dead zones. A: Schematics that shows that the physical extent of the boutons terminaux do not necessarily imply a dead zone. Shown are bouton terminaux (a and b). They are represented in the analysis as points projected onto the axon (a' and b'). The distance between a' and b' can be arbitrarily small. Also shown are two boutons en passant (c and d) and their representation (c' and d'). A lower limit for the distance between c' and d' is given by the diameters of the boutons. B: Relative number of boutons that are of type terminaux. (A Correlation between the lack of small interbouton distances (measured by the ratio of the number of distances that are smaller than 1.5\mu m and the number of distances between 3 and 4.5\mu m) and the relative number of boutons terminaux. Correlation analysis $r = 0.87$, $p = 0.00$, $a = 0.20$, $b = 3.29$. Each dot represents a cell. For the fit only the closed dots were used. Open circles were considered as outliers (cells Iqn:A and ss4:C).

It is convenient to think of bouton placement according to a Poisson process as follows. Starting with the first bouton on the collateral, at what distance from this bouton is the next bouton to be formed? This distance is drawn from an exponential distribution. After the second bouton is formed, another distance from
the exponential distribution is determined in order to place the third bouton and so on. By construction, the interbouton distances have to be exponentially distributed. Based on the Kolmogorov-Smirnov test, some of the distances in our samples were indeed distributed in that way. However, most were not (24 of 30). The result does not change if only distances from boutons on the end collaterals were considered (not shown). For many of these cells the reason for mismatch with the exponential distribution is the lack of small distances. It is easy to see how the Poisson process could be modified to allow for a lack of small distances in the interbouton distance distribution.

A simple example of such a model is shown in fig 5.14 A. It is assumed that the boutons are produced by a Poisson process so that the interbouton distances are distributed by an exponential distribution with mean $\lambda$. It is further assumed that the length of the dead zones fluctuate around a value $\mu$ so that the distribution of the zone lengths is given by a normal distribution with mean $\mu$ and standard deviation $\sigma$. When the first bouton is produced, a length of a dead zone is randomly selected from the normal distribution. All boutons which were created by the Poisson process within that dead zone are ignored (5.13 A). Fig 5.13 B shows that the simulation of the process for the parameters $\lambda = 5$, $\mu = 2$ and $\sigma = 0.7$. The parameters were selected by a least square procedure. A grid search was used to find the parameters that fitted best to the interbouton distance distribution of the basket cell 4A. Although the deviations are significant ($p < 0.0001$, Kolmogorov-Smirnov test), the model reproduces the shape quite well. However, not all distributions could be explained in this way. An example of a bad fit is shown in fig 5.13 C (open circles). We conclude that with this simple model the data can be explained only partially.

**Long tails**

For the analysis of the interbouton distances we noticed the existence of large distances which were assumed to be outliers. Distances larger than a threshold $d$ were ignored ($d$ is the the first bin in the histogram of interbouton distances for which the bin itself and the successor bin contained no distances). $d$ changed for different cells and ranged between 42 and 104$\mu$m. The relative number of distances discarded was less than 4%. Kisvarday et al. (1985, 1987) also noticed the occurrence of large interbouton distances. In order to determine the mean of the layer 4 basket cell they ignored all distances larger than 20$\mu$m (8% of all distances). For the layer 5 basket cell they ignored distances larger than 30$\mu$m (7%). They reported that the large interbouton distances were mainly placed on the main axonal trunk were the boutons are formed only sparsely.

In contrast we noticed that for many cells the large distances were between boutons of which at least one of them was located on the end collateral. For the largest distance this was the case for 70% of the cells in our sample. This indicates that the existence of large distances could reflect something other than a low density in the initial segment of the axonal tree. In order to investigate the large distances more closely we used the log-log plot of the survival function $1 - F(x)$, where $F(x)$ is the cumulative function of the inter-bouton distances. Distances were pooled in
5.3. DISCUSSION

Figure 5.14: Poisson process with dead zones. A: Illustration of a Poisson process with dead zones. Shown is an axon (horizontal line) on which boutons (open and closed circles) are formed, starting from the left. The open circles indicate boutons which would have been formed by the Poisson process, but were prevented from being formed by the introduction of a dead zone (arrows). The sample of closed circles are the boutons that are finally observed on the axon. B: Distribution of interbouton distances created by a Poisson process with dead zones (stippled line). The parameters were selected so that the model best fits the interbouton distance distribution of a reconstructed basket cell in layer 4 (bold line). C: Another example, a thalamic afferent, for which the model does not fit as well as in B. Same notation as in C.

different groups according to the similarity of their interbouton distance histograms. The groups are basically: the layer 6 pyramidal cells, the superficial pyramidal cells and the spiny stellate cells, the thalamic afferents and the basket cells. Only interbouton distances of boutons on the end collateral were used. For comparison, the least square fit of the exponential function (fig 5.15 A and B) and of the gamma function (fig 5.15 C and D) were shown as an inset. It is obvious that the tail of the
interbouton distance distribution does not follow the fitted models. The existence of a linear part of the survival function (indicated by the gray shaded region) suggests that the long tail behaviour follows a power law, i.e. $1 - F(x)$ is proportional to $x^{-\alpha}$ for some positive $\alpha$ and for large $x$. This indicates that mechanisms other than that of a simple Poisson process (with or without dead zones) must be involved in the placement of boutons along the axonal branches.

5.3.3 Length of axonal branches

Although the total length of the axonal tree does not show a clear dependence on different cell classes, such a tendency exists for the branch lengths. For example smooth cells and thalamic afferents have more but up to five time shorter branches than the spiny cells (fig 5.6 C). It is largely unclear why this is so. A possible explanation is that cells that confine the axonal branching pattern in small volumes use branches of small lengths. This is certainly true for the smooth cell which typically has a highly compact branching pattern distributed in a small neighbourhood around the soma. It is also true for the thalamic afferents that form compact clusters. One could also expect that a cell that possess a diffuse branching pattern such as the star pyramidal cell has large branches. This is indeed true. In particular the end collaterals of sp4 are much longer than the inner collaterals (fig 5.6 D). However, there are other cells such as the superficial pyramidal cells that also form tight clusters but has long collaterals. Especially the end collaterals, which are presumably involved in the formation of clusters, are in general longer than the inner collaterals (fig 5.6 C and D).

Kisvarday et al. (1985, 1987) studied the branch lengths of basket cells and their results agree with our data. The total number of collaterals was for the small layer 4 basket cell 408 with an average length of $27.1 \pm 1.13 \mu m$. For the basket cell in layer 5 the total number of branches was $n = 853$, and average length $26.8 \pm 0.811 \mu m$. In one case the total axonal length was determined, $22.9 mm$ (small basket cell). For both cells the histogram of branch length was strongly skewed to the left.

Axonal path lengths of smooth cells are shorter than that of the spiny and thalamic afferents (see 5.3). From a functional point of view shorter path lengths allow a rapid transmission of signals to the target cells. This view is supported by the fact that axons of basket cells posses a highly myelinated axon.
5.3. DISCUSSION

Figure 5.15: Shown is the long tail behaviour of the bouton distribution of four groups of cells. The interbouton distances of cells possessing similar interbouton distance distributions were pooled (cell types indicated in titles). Only distances for which at least one bouton is located on an end collateral were used. The log-log plot of the function \( y = 1 - F(x) \), \( F(x) \) the cumulative distribution of the pooled interbouton distances \( x \), is drawn for each group (bold line with superimposed dots). In addition the best fitted exponential distribution (A and B) or the best fitted gamma distribution (C and D) is shown (thin line). The insets show the histograms of the data (dots) and the fitted curves (lines). The gray shaded rectangle indicates a region over which the log-log plot is roughly linear. 

- **A**: The interbouton distances of all layer 6 pyramidal cells except p6:F and p6:G were pooled. The linear range is given by \( \log(x) \in [3.5, 4.3] \), i.e. \( x \in [33\mu m, 73.7\mu m] \). Correlation analysis of the linear region \( r = -0.99, p = 0.00, a = 7.35, b = -3.12 \). The fitted exponential distribution had parameter \( \lambda = 7.9 \).
- **B**: The pooled cells were the spiny stellate cells in layer 4 and the pyramidal cells of layer 2 and 3. The linear range was \( x \in [33\mu m, 90\mu m] \). Correlation analysis of linear region \( r = -0.99, p = 0.00, a = 5.67, b = -2.46 \), and \( \lambda = 10.6 \).
- **C**: Pooled cells were the thalamic afferents. Linear range \( x \in [27.1\mu m, 73.7\mu m] \), correlation analysis \( r = -0.99, p = 0.00, a = 4.15, b = -2.01 \) and the estimated parameters are \( A = 1.3, B = 7.3 \).
- **D**: Pooled cells were the basket cells of layer 2, 3 and 4. Not included was the cell b4:C. Linear range \( x \in [13.5\mu m, 27.1\mu m] \). Correlation analysis of linear region \( r = -0.99, p = 0.00, a = 4.36, b = -2.56 \). The estimated parameters were
Chapter 6
Principles of connectivity

Much of the computation that is done in the cortex involves the information exchange of many thousands of neurons. The most prominent information transmission between neurons happens via synapses. Because the pre- and post-synaptic membranes of a synapse have to be in close apposition it follows that information exchange between cells is only possible when the axon of one cell is very close to the process of the target cell. So in order to carry out computations, the axons and dendrites must have a spatial extent. It seems therefore reasonable to assume that the spatial shape of the dendritic and axonal shapes allow some insights into the principle of connectivity and hence computation. It is this context, correlation between computation, connectivity and the spatial shape of the dendrites and axons, in which this work has to be seen.

On our level of analysis, an estimate of the strength of a connection between two cells can only be given by the an estimate of the number of boutons of the presynaptic cell that form synapses with the postsynaptic cell. However recent data of EM studies allows us to investigate the number of synapses that are formed on a bouton. A synapse that is formed by a bouton with the post-synaptic target ('active zone') can be recognised under the EM by an accumulation of synaptic vesicles in the presynaptic bouton, rigid appositions of the pre- and postsynaptic membranes in conjunction with a widening of the extracellular space and the presence of pre- and postsynaptic membrane specialisations (see for example Buhl et al. 1997). Although the determination of the number of synapses can be problematic because the active zones are not always well separated and can be of quite complicated shape (fig 6.1) the general picture that evolved is that the number of active zones per bouton of cortical cells in cat V1 range between 0 and 2 and only in rare cases the number is larger. An overview of the number of synapses per boutons is given for different cell types in table 6.1. From this it follows that the assumption of 1 synapse per bouton is valid for the spiny cells. For the thalamic afférents and smooth cells the average number of synapses per bouton is larger, ranging from 1.03 to 1.39 for basket cells and from 1.3 to 1.64 for the thalamic afferents.

One could assume that the size of the bouton reflects the total number of synapses per bouton. The bigger the bouton, the more synapses there are on the bouton. Size estimates were made for boutons in layer 4. Kisvarday et al. (1985)
estimated the diameter of boutons in a range of 1 to 2\(\mu m\). A systematic measure of the cross sectional area of boutons based on EM serial sections were made by Ahmed et al. (1994, 1997). When converted to diameters, the mean value for the layer 6 pyramidal cells is 0.18\(\mu m\), for the spiny cells 0.64\(\mu m\), and for the thalamic boutons 0.98\(\mu m\). Boutons of basket cells were different in size when forming synapses with soma (0.91\(\mu m\)) or shafts (0.77\(\mu m\)). Diameters of layer 5 pyramidal cell boutons (in layer 5 and 6) were measured by Gabbott et al. (1987) and ranged from 0.5 to 3\(\mu m\), most of them having an intermediate diameter. However, no strict correlation between the number of synapses and the bouton size could be detected. The thalamic afferent boutons are the biggest and also have the highest number of synapses per bouton. But the layer 6 pyramidal cell boutons are smaller than the boutons of the spiny stellate cells. However, the spiny stellate cells have on average the same number of synapses per bouton than layer 6 pyramidal cells.

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Table 6.1: Synapses per bouton. 'ref': Reference number, 'type': Cell type. 'bou': Number of boutons investigated. '0', '1', '> 2': Percent of the boutons that had 0, 1 or > 2 synapses per bouton. 'syn/bou': Average number of synapses per bouton.

### 6.1 Connectivity principles at different levels of magnification

We would like to understand what the rules are that determine that two cells in the cortex are connected. Principles of connectivity are established at least at two levels of magnification. a) Principles that determine the location and shape of the neurons, i.e. where the cell body, the axon, the boutons, the dendrites and the spines of each neuron are. These principles are more likely to act on a scale between some \(\mu m\) and some mm and can be investigated with the LM. In this work we attempted to find such principles by investigating the dendritic fields and bouton clouds of physiologically identified cells (chapters 3 - 5). b) Principles that explain the synaptic target selection, i.e. with what elements, such as dendritic shaft, dendritic spine, cell body or axonal shaft, that are in close apposition to a bouton or axonal shaft of a cell a synapse is formed. These principles act on a scale that is equal or smaller than a \(\mu m\) and must therefore be done with the
6.2. RANDOMNESS VERSUS SPECIFICITY

EM. Typically an investigation of these principles involves a close analysis of the synaptic junctions or the analysis of postsynaptic targets of identified cells. Often a 3D reconstruction of serial sections, such as shown in fig 6.1 are required.

Figure 6.1: Reconstruction of serial electron micrographs. A and B: Electron micrographs of BDA (biotinylated dextran amine) and PHA-L (Phaseolus vulgaris leucoagglutinin) labeled boutons found in layer 6 of area V5 of the monkey. A: Bouton filled with mitochondria forming two asymmetric synapses (solid arrowheads) with the same target neuron. One of the targets is clearly a spine (sp; note the spine apparatus) which can be traced back to the parent dendrite (d). The second synapse forms on a region of the dendritic shaft that projects slightly into the neuropil. Serial section reconstruction showed this projection to be a sessile or ‘neckless’ spine. B: A spine (sp) containing spine apparatus forms an asymmetric synapse with the labeled bouton which shows a complex postsynaptic density (solid arrowheads) within the spine. Scale bar = 0.5μm in A and B. C: 3D view of the digitised contours of the bouton shown in B. The thin line indicates the bouton contours, the bold line spines. D: Same structure as in C. Thin line indicate the bouton contours. Bold lines indicate the postsynaptic densities. Axis units are nm.

6.2 Randomness versus specificity

We will give two extreme and opposed hypothetical principles. The first principle leads to very specific connectivity rules on both magnification levels. It is achieved
when each bouton of a cell can form a synapse with exactly one cell in the cortex at a particular location on the target dendrite. Although one could imagine that the wiring of the cells is done according to this principle, it seems to be highly unlikely. In fact the consequence would be that a cell needs to find the correct location of about 5000 cells out of 27 million cells.

The other principle involves no specificity on both magnification levels. This is the case when for any cell each location in the cortex has the same probability of being occupied by one of its boutons and the bouton forms a synapse with a randomly chosen substructure in the close neighbourhood. From this it follows that each cell in the cortex has the same probability of being contacted by the cell. It is obvious that this is also not true. The fact that cortical cells have a restricted dendritic and axonal extent speaks already against this hypothesis. In fact, cells prefer to contact targets that are within a sphere, centred at the soma, of a radius ranging from 300µm to 3mm, i.e an average volume of 13mm³. For comparison, the cat V1 has a volume that is roughly 50 times this volume. So cortical cells innervate only their local environment and cells far apart will connect with probability zero.

It is likely that truth will lie somewhere between these two cases. There will be some specificity but also randomness on both magnification levels. An example of such a model was formulated already very early (Uttley 1954; Sholl 1956). In their model randomness on the larger scale (a) is introduced by the description of the location of the axonal and dendritic trees by 3D probability distributions. For each location in the 3D space a probability was assigned that a small piece of axon or dendrite of a cell could be found at that location (Uttley 1954). Selectivity is introduced by the choice of the probability distribution which is different for the different cell classes. From these distributions the probability that an axon and a dendrite are at a given location in close apposition can be determined. On the smaller scale (b) the only principle used was randomness, in the sense that any close apposition between a dendrite and an axon leads to the formation of a synapse. We know now that at least this last principle is not used in V1. Although a close apposition of processes between cells is a precondition for forming a contact, it is a quite unreliable indicator for synaptic contacts.

That randomness is involved in cortical wiring seems plausible. As pointed out by Szentagothai and Arbib (1974), randomness can easily be accepted when one considers the complex, irregular and often widespread arborisation pattern of axonal and dendritic trees which are highly intertwined. On the other hand it is also pointed out by one of the authors that specificity in neural connections can be observed on different levels (Szentagothai 1978b). A number of cell types are selective not only concerning the kind of neurons with which they establish synapses but also in respect to the part of the dendrite they contact (e.g. axo-axonic cells contact the axon initial segment of pyramidal cells only).
6.3 Principles of synaptic target selection

We will first focus on the lower magnification level (b) and investigate possible principles of target selection in the light of recent accumulated data (table 1.1) which stem from the analysis of post-synaptic targets of identified cells with the EM. Let us focus on a bouton of a cell and consider all the substructures such as dendritic shaft and dendritic spines as well as the cell bodies and axons that are in close apposition to this bouton. The bouton will form a synapse with one of these elements. What are the principles that govern this selection process? Is it randomness so that each element has the same probability of being contacted or is it a highly specific rule that allows the formation of a synapse with exactly one predetermined structure.

Different classes of cells have different distributions of contacted targets. For example the boutons of axo-axonic cells exclusively contact the initial axon segment of pyramidal cells, the boutons of spiny cells and thalamic afferents contact predominantly (80%) spines while boutons of basket cells prefer to contact the cell body and the dendritic shaft (table 1.1). There is also a weak correlation of the target specificity of spiny cells and the layer of soma (fig 5.12). The closer the soma to the pial surface is, the larger is the percent spines contacted. For example 30% of the targets of layer 4 projecting pyramidal cells in layer 6 are spines. For the spiny stellate cells in layer 4 it is 75% and for the superficial pyramidal cells it is 90%. The correlation is not strict. One exception are for example the layer 5 pyramidal cells that innervate the layers 5 and 6 (80%, table 1.1). For the basket cells the inverse correlation seems to hold. The closer to the soma, the lower is the percentage of spines contacted by basket cells. 10% of the targets of layer 5 basket cells are spines. It is 30% for basket cells in layer 4 and 40% for basket cells in layer 5.

In order to understand the principles that lead to the different preferences of the boutons it would help to know about the relative number of elements (dendritic shafts and spines, axon segments, cell bodies) that are in close apposition to the bouton. The sources of specificity as expressed by the different target preference could be simply a reflection of the fact that boutons of different cells have different elements in their neighbourhood. This could, for example, be a consequence of a different composition of the elements at different locations in the neuropil. However, it is probably reasonable to assume that the composition is the same within a layer and therefore this cannot be used as an explanation because cells innervating the same cortical layer can have very different target specificities. A more plausible explanation could be that the boutons assure that they grow only in environments that contain certain elements. For the spiny cells that would mean that their boutons can only be found in environments that contain many spines while the basket cell boutons would be located in environments containing many dendritic shafts. Such a mechanism would involve a specific path of the axon through the correct environments of the neuropil or a specific activation of boutons at certain locations. How reasonable is such a mechanism?

Density analysis of boutons along axonal trees (chapter 5) shows that an axonal
tree can have regions of different bouton densities. For example the layer 6 pyramidal cells that project to layer 4 have, when averaged over collaterals in the same region, a low bouton density until the axon arrives at layer 4 where the density increases dramatically (fig 5.7). This suggests that there must be mechanisms that prevent bouton formation in certain regions of the cortex or triggers them in others. The question is if a similar mechanism can also be seen on a smaller scale, i.e. that the boutons show a clustered appearance along a single branch.

This was already investigated for boutons on axons of Golgi stained pyramidal cells in the mouse cortex (Hellwig et al. 1991). They found that the distances between boutons on a branch were exponentially distributed and concluded that bouton placement is a Poisson process, i.e. no clustering in bouton placement occurs. This supports the view that a mechanism as was described above does not exist for boutons that are formed along branches. For our sample of cells, most of the boutons preferred to be located rather distally than proximally on the axonal tree (section 5.2.1). Although we found a rather similar interbouton distance distribution as reported for the mouse, it is not a Poisson process. Large distances occurred too often than one could expect from a Poisson process (5.15). However, we did not find a pronounced clustering of the boutons along branches. This favours the idea that boutons along axonal branches do not aim to contact specific sites. However, the possibility that the cell forms boutons at very specific locations can not be completely ruled out. As long as the position of the substructures relative to the axon is unknown, it is difficult to draw conclusions about avoidance or seeking of particular sites. In addition, it remains to be seen what the significance of the over-representation of the large distances means.

The preference for target specificity does not necessarily imply that the cells are specific for inhibitory or excitatory cells. In fact, direct estimation of the numbers indicate that for the smooth cells as well as for the spiny cells between 6 and 18 percent of the contacts are onto smooth (i.e. inhibitory) cells. This would be expected if boutons form synapses on both inhibitory and excitatory cells with the same probability.

6.4 Principles of location and shape

While on the synaptic level principles are needed that describe the selection of elements in close apposition to a bouton, the overall shape refers to principles that describe the selection of locations in cortical space. This is conveniently described by the density distribution (or equivalently by a probability distribution), i.e. by the amount of axon, dendrite or the number of boutons that can be found in a small volume of cortical space.

Layer specificity of cortical connections

Depending on the cell type, the vertical component of the distribution of the bouton clouds often shows a strong preference for a particular cortical layer (fig 4.11, 4.12 and fig 4.23 A). As a consequence, the relative amount of boutons contributed by
a cell type changes for different layers. An estimate for the boutons of the spiny cells and the thalamic afferents is given in fig 4.23 B. The figure shows that most boutons in the cat primary visual cortex are involved in establishing projections between layers 6 and 4 and between layers 4 and 2 and 3 as well as for the self-innervation of layer 4 and the layers 2 and 3.

The scheme in fig 4.23 B reflects the wiring between cells only partly. Under the assumption that a dendrite selects the boutons for synapse formation randomly (i.e. unspecific sampling of input), the relative contribution of the synapses formed by the different cell types is reflected on the dendritic tree. Thus one can expect that the influence of, for example, layer 4 on layer 6 is increased, because of the sampling of synapses by the apical dendrites of the layer 6 pyramidal cells that project to layer 4.

Clustered innervation of layers

Within the main layer of innervation a further preference for location, expressed in the clustered distribution of the boutons, can be observed (fig 4.20). To a good approximation the location of the boutons within a cluster are normally distributed (fig 4.9). Because boutons are formed on axonal branches, their location is restricted to the course of the axonal branches within the volume. The description of the bouton locations by a normal distribution becomes therefore inaccurate at a certain level of magnification and the distribution of the boutons along a branch becomes relevant. This distribution was investigated in chapter 3.

The formation of clusters can vary radically for different cells, even within the same cell class (fig 4.20). However, there is a tendency, although not significant, that layer 6 pyramidal cells form few clusters (on average 3 ± 2 clusters per cell) but the clusters have large diameters (490 ± 164μm), while the spiny stellate cells, the superficial pyramidal cells and the thalamic afferents tend to form many cluster (5 ± 2) which have a smaller diameter (348.7 ± 123.9μm) and the basket cells form few clusters (2 ± 1) and also have small cluster diameters (274.4 ± 72.4μm).

While a functional correlation for the layer specificity is only poorly understood, recent investigations of the bouton clouds of superficial cells suggest that the formation of clusters reflects a preference to connect with cells of similar orientation preference (e.g. Malach et al. 1993; Kisvarday et al. 1997; Bosking et al. 1997). Using bulk injections in the cat, it was estimated that the number of boutons formed by superficial cells that contact cells of similar orientation preference as the parent cell is about 60% (Kisvarday et al. 1997). The remainder (40%) project to cells with different orientation preferences.

Evidence that the boutons of our cells contact cells of similar orientation preference cannot be given because the underlying functional maps were not determined. However there are two observations that suggest that there are connections between cells of different orientation preference. The largest clusters in our sample were made by the layer 6 pyramidal cells. About 25% of the clusters had a diameter larger than 600μm, fig 4.20, p6:A to G), followed by the clusters of the pyramidal cells in the layers 2 and 3 (about 5%). Because the typical diameter of an iso-
orientation column is only about 500\(\mu m\) (Huebner et al. 1997) it seems likely that cells of different orientation preference are contacted. In addition, many cells formed clusters for which the distance between clusters was much smaller than 1\(mm\) (fig 4.19 C) which is the typical distance between neighbouring iso-orientation columns of the same orientation preference (Huebner et al. 1997). Again this suggest that connections between cells of different orientation preference are not uncommon.

It is not clear if the interpretation of the cluster formation for the superficial pyramidal cells can be applied to deep pyramidal cells. In fact it was hypothesised that the boutons of the layer 5 pyramidal cells form clusters to innervate cells of similar direction preference (Gabbott et al. 1987). Certainly, for a better understanding of the horizontal component of the bouton distribution more experiments must be carried out that investigate the relationship between bouton clouds and functional maps.

**Correlation of bouton clouds with preferred stimulus orientation**

A potential source that could explain the specificity of the shape of the cell's dendrites and axon is that of a possible correlation with receptive field properties. As discussed in chapter 3, no such correlation was shown to exist for the dendrites. For the bouton cloud of superficial cells of the cat, however, just such a correlation was reported. The boutons tended to be aligned on an axis parallel to the preferred stimulus orientation (Schmidt et al. 1997). In our sample the clusters of the layer 6 pyramidal cells tended to be aligned on an axis that is perpendicular to the preferred orientation preference of the cell (more than 80\%, fig 3.1). Interestingly this is quite the opposite to the correlation found for the superficial cells by Schmidt et al. (1997). In addition we did not find any correlation for the clusters of the superficial cells. It is not clear if this is due to our small sample size or if there is a less trivial explanation.

**Long range horizontal connections**

Only spiny cells (including thalamic afferents) displace their boutons by large distances in horizontal direction from the soma. With few exceptions the range of displacement was between 1.1\(mm\) and 1.8\(mm\). For the smooth cells the largest horizontally displaced bouton never exceeded 850\(\mu m\) (range 200 to 850\(\mu m\)). The same observation can be made when the distribution of the horizontal displacements from the soma is considered. Although a considerable number of spiny cells (9 of 19) had a \(\frac{3}{4}\)-quantile as small as the largest \(\frac{1}{2}\)-quantile of the smooth cells, only spiny cells had very large \(\frac{4}{5}\)-quantiles. Taking into account the fact that spiny cells prefer to contact the spines of other spiny cells (table 1.1), these findings suggest that a horizontal long range connection greater than 800\(\mu m\) will be an excitatory projection and prefers to contact excitatory cells.

The widespread horizontal distribution of the bouton clouds of the thalamic afferents speaks against a fine structured retinotopic arrangement of synapses onto a dendrite, unless wiring is to some extent specific. In fact, two thalamic afferents whose axonal shafts are displaced by more than 2\(mm\) from each other can
theoretically still form neighbouring boutons. If a dendrite forms synapses with randomly selected boutons, the synapse could be formed with one or the other of the two thalamic afferents. Thus a retinotopic order on a dendritic branch can not be obtained.

The functional limitations of a retinotopic arrangement of synapses was demonstrated in the case of Livingstone’s model which attempted to explain the emergence of direction selectivity (chapter 3). As a simulation study showed, even the most favourable retinotopic arrangement of synapses on a dendrite could explain the response curve to different stimulus velocities, which is normally observed in cortical cells of cat V1, only partially. It is likely that direction selectivity emerges from the collective computation of cortical networks.

Towards a quantification of cortical circuits

From vertical considerations of the bouton clouds it was possible to give theoretical estimates about the proportions of boutons that are contributed by a cell type to a given lamina. In a similar way the horizontal component of the bouton distributions will allow us to refine our predictions and characterise the cells that converge onto a dendrite more precisely. Future work will involve the study and quantification of the wiring in cortical networks, based on the biologically realistic data as was described in this work.
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