SYNAPTIC CHARACTERISTICS OF IDENTIFIED PYRAMIDAL AND MULTIPOLAR NON-PYRAMIDAL NEURONS IN THE VISUAL CORTEX OF YOUNG AND ADULT RABBITS. A QUANTITATIVE GOLGI-ELECTRON MICROSCOPE STUDY

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Abstract—The visual cortex of 20 day old rats and rabbits has been considered as mature on the basis of the observations that the dendritic arborization and the overall synaptic population have almost reached their adult stage in these animals. In the present study we have investigated the visual cortex of 20 day and 7 month old (adult) rabbits in order to determine whether this apparent adult appearance also holds for the synaptic organization of individual neurons. Neurons mainly located in layers III and IV of the primary visual cortex (area 17) were Golgi-impregnated, gold toned and deimpregnated and were then, after embedding in plastic, sectioned serially. The number and length of synaptic profiles, and the length of the neuronal boundaries were analysed in every tenth section. From these counts and measurements the size distribution of the synaptic discs, the number of synapses per 100 \( \mu \text{m}^2 \) neuronal surface and the receptive surface expressed as the percentage of the total neuronal surface covered with synaptic contacts were estimated using stereological methods.

At both ages studied, the density of synapses was significantly higher for the non-pyramidal neurons than for the pyramidal neurons. Differences in the amount of receptive surface were parallel to the differences observed for the number of synapses per 100 \( \mu \text{m}^2 \). At day 20 the receptive surface of the non-pyramidal neurons was significantly larger than that of the pyramidal neurons. The receptive surface of the non-pyramidal neurons in the adult stage was not only larger than that of the pyramidal neurons in the adults, but also larger than that of the day 20 non-pyramidal neurons.

From our results the following conclusions can be drawn: (1) The synaptic input received by the pyramidal neurons is mainly established at day 20 of postnatal life, i.e. prior to the establishment of adult visual behaviour. (2) The non-pyramidal neurons complete their maturation in a later stage than the pyramidal neurons. (3) Medium to large sized synaptic contacts are newly formed after day 20 and are mainly added to the synaptic population on dendrites of non-pyramidal neurons. (4) The specific increase in the number of synapses on non-pyramidal neurons is discussed in relation to intracortical inhibition which is thought to be important for the fine regulation of visual function during development.

The visual cortex is probably the most intensively studied part of the cerebral cortex and its development have been the subject of thorough investigation. A wealth of morphological and physiological data have been accumulated, but the relation between them is still not clear. It is now generally accepted that vision results from the integration of the activities of the individual neurons in the visual pathway and is among other factors dependent on the number, the location and the strength as well as the inhibitory vs excitatory character of the individual synapses along the neuronal surface. Therefore a number of investigators have concentrated on quantifying synapses with respect to these parameters. Most of these studies were restricted to general estimates of the number of synapses per area, per reference volume or per neuron, but the identity of the neurons with their characteristic dendritic and axonal properties remained obscure. Classical Golgi studies revealed that the morphology of non-pyramidal neurons is much more diverse. The classification of the latter depends both on the presence of the axonal and dendritic arborizations. Many of these results in the visual system awaits detailed morphological knowledge of

Abbreviations: \( B \), Boundary length of identified neuronal profiles, including spines; \( D \), Diameter of synaptic discs; \( E(D) \), Average diameter of synaptic discs calculated from \( f(L) \) using the "unfolding" procedure; \( E(L) \), Average length of synaptic profiles; \( E(L_p) \), Average length of synaptic profiles including interruptions; \( E(\sigma_p) \), Mean surface area of synaptic discs; \( f(D) \), Relative frequency distribution of \( D \); \( f(L) \), Cumulative frequency distribution of \( D \); \( F(D) \), Cumulative frequency distribution of \( \sigma_p \); \( L \), Length of synaptic profiles; \( L_p \), Length of synaptic profiles including interruptions; \( n \), Number of neurons analysed; \( n_s \), Number of synaptic profiles in one micrograph on the identified neuron; \( N_p \), Number of synaptic profiles on identified neuronal profiles (\( B \)); \( N_s \), Number of synapses per 100 \( \mu \text{m}^2 \) neuronal surface; \( N_s \), Number of synaptic discs on identified neuronal surface in a reference volume; \( P \), Adult pyramidal neurons; \( P \), Day 20 pyramidal neurons; \( R_s \), Receptive surface or percentage of the total neuronal surface covered with synapses; \( S \), Adult non-pyramidal neurons; \( S \), Day 20 non-pyramidal neurons. \( S_p \), Surface area of synaptic discs; \( S_r \), Estimated surface of identified neurons in a reference volume.
specific cell types and their synaptic organization. Two techniques are now available to obtain this knowledge: horseradish peroxidase-injection and the combined Golgi–electron microscope procedure. Few studies have dealt with the morphology of physiologically characterized, horseradish peroxidase-injected neurons. However, since the introduction of the combined Golgi–electron microscope technique by Fairen et al. the different synapses and the number of synapses on identified neurons have been studied by several authors.

In the present study we have used the Golgi–electron microscope procedure to quantify synapses on identified pyramidal and aspiny and sparsely spiny multipolar non-pyramidal neurons at two developmental stages. Since it has been reported for the rabbit that the adult visual representation can be all gold toned elements of the cell under investigation were recorded by the 16th–17th day after birth, animals of 20 day and 7 month old were selected to study developmental stages. Since it has been for the combined Golgi–electron microscope procedure. Few spinous multipolar non-pyramidal neurons at two studies have dealt with the morphology of physiologically characterized, horseradish peroxidase-injected neurons. Which have been described qualitatively and quantitatively.

EXPERIMENTAL PROCEDURES

Twenty day old and adult (7 months) Dutch belted rabbits were anaesthetized with pentobarbital (Nembutal) and transcardially perfused with a solution of 1% paraformaldehyde and 1.25% glutaraldehyde in cacodylate buffer and subsequently with a more concentrated solution containing 4% paraformaldehyde and 5% glutaraldehyde in the same buffer. The skull was carefully removed and the brain was excised and processed with the Golgi–rapid procedure. After impregnation the tissue was cut into slices of about 100 μm on a Vibratome. Pieces of tissue containing the selected impregnated neurons, were cut into slices of about 100 μm on a Vibratome. The primary visual cortex was excited and processed with the Golgi–rapid procedure. After impregnation the tissue was cut into slices of about 100 μm on a Vibratome. Slices containing completely impregnated neurons, and having only few impregnated glial cells, were gold toned according to Fairen et al. for more details, see ref. 28. After osmication, dehydration and flat embedding in Epon 812, cells of interest were selected from the different laminae. The lamination of the selected neurons was determined in adjacent Nissl slices.

In this paper we present quantitative data of the neurons (except cell 1, Fig. 2) which have been described qualitatively in a preceding article (Figs 1 and 2). For each cell the depth of the dendrites within the slice was determined with the aid of a light microscope with a calibrated microscrew. Pieces of tissue containing the selected impregnated neurons were cut out and remounted on propylmerized Epon blanks. Serial sections with a thickness of about 70 nm were cut. The thickness of these sections was determined with the "small fold"-method. The series was subdivided in ribbons of ten consecutive sections each which was mounted on formvar coated 75 mesh or slotted grids. All sections were stained with uranyl acetate and lead citrate before examination in the electron microscope. In general the middle section of the successive grids was selected and all gold toned elements of the cell under investigation were photographed at a magnification of about 2200 x and printed at a final magnification of about 10,000 x. If parts of the profile were obscured by a grid bar, the preceding or the next section was used. Subsequently, all synaptic profiles on the impregnated gold toned neuronal profiles were photographed at a magnification of about 9100 x and printed at a final magnification of about 41,000 x. The exact magnification for each set of 100 micrographs was determined using a grating replica with 2160 lines per mm. In the presence of paramembranous densities and synaptic vesicles the criteria were used to identify synaptic contacts. Boutons establishing contacts with two or more separate postsynaptic bands were counted as one when they were connected with only one postsynaptic element (dendritic spine, dendritic shaft or soma). This was done because it has been shown that separate postsynaptic bands (intersynaptic profiles) were measured in low power micrographs (Figs 3a,b). The number of synaptic profiles on these neuronal profiles were estimated. In addition, the length of the profiles of the postsynaptic band and the lengths of the total synaptic profiles including interruptions were measured in high power micrographs (Figs 4 and 5). The length measurements were performed with a digitizing device (Videooplan, Kontron).

Stereological parameters used to quantify morphological properties depend on a reference volume. For isolated identified neurons such a reference volume is not defined, therefore, an arbitrary volume was used to which all parameters were related. This was possible since all parameters were obtained from the same sections. Moreover, the arbitrary volume could be ignored because of its presence in the numerator and denominator of the equation. The estimate of the membrane surface per volume was obtained by multiplying the total measured boundary by 4π/3. To determine the number of synapses per unit volume we applied a discrete "unfolding" procedure which included the modifications by Goldsmith and Cruz-Orive (refs 7, 15a and 48, chapter V) and in addition a correction for finite section thickness. The "unfolding" procedure converts the observed profile size distribution into a synapse size distribution, provided that the shape of the synapse is known. For this purpose we considered the shape of the synaptic contacts, as distinguished from the active region per se, to be approximately flat circular discs. The implications of the assumption will be considered further in the discussion. The distribution of the synaptic profile lengths, including interruptions in the postsynaptic band (Fig. 5), was unfolded separately for (i) each individual neuron, (ii) for each group of neurons and (iii) each group of neuronal components. This provided an estimate of the number of synaptic discs on identified neuronal surface in a reference volume and the probability distribution of the diameters of the synaptic discs for each identified neuron.

Since the synaptic profiles are part of the membrane traces the gold toned neuronal profiles, including spines, the number of synapses per unit surface area is:

\[ N_s = \frac{N_l}{S_v} \]  

(1)

where \( N_s \) is the number of synapses per 100 μm², \( N_l \) is the number of identified neurons, and \( S_v \) is the surface area of the synaptic discs with diameter \( D \), and \( f(D) \) is the relative frequency distribution of these synapses (already divided by \( N_s \)). It follows that the mean synaptic area \( E(s) \) is:

\[ E(s) = \sum_{i} \frac{C(x_i) \times f(x_i)}{D} \]  

(2)
Fig. 1. Composite figure of camera lucida drawings at postnatal day 20. The neurons are with the exception of cell 25 placed in their appropriate laminar position. The borderlines between the cortical layers are indicated by dotted lines.
Fig. 2. Camera lucida drawings of neurons in the 7 month old (adult) animals. The somata are not arranged in their correct laminar position. Cells 1 and 2 were situated in layer II, cells 14 and 15 in layer IV and the remaining neurons in lower layer III. The scale of 200 μm is therefore only valid for the neurons and not for the thickness of layer III.
Fig. 3. Somatic profile of a non-pyramidal neuron displaying four initial dendrites. This profile is characterized by a round nucleus having a crenellated nucleolus and an extensive rough endoplasmic reticulum. The number of synaptic profiles (n = 1) on this somatic profile and on the dendrites (n = 4) is indicated in the drawing.
Fig. 4. High power micrograph of an impregnated dendrite receiving four synaptic profiles. The length of these profiles (L) is marked by the arrow heads.
Fig. 5. To illustrate the $L$ and $L_0$ measurements through perforated synapses, two sections from a series of serial sections through one synapse are shown. The level of sectioning is indicated in the drawing. In (a), $n = 1$ and $L = L_0$, but in (b), $n = 1$ and $L \neq L_0$. This is important since $L_0$ is necessary to estimate the diameter $D$ of the synaptic discs.
Synaptic characteristics of neurons in the visual cortex

Fig. 6. Cumulative frequency distributions of the size of the synaptic discs from non-pyramidal and pyramidal neurons at day 20.

Fig. 7. Cumulative frequency distributions of the size of the synaptic discs from non-pyramidal and pyramidal neurons at 7 months.

Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Basic measurements</th>
<th>Calculated parameters</th>
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<tr>
<td></td>
<td>$n$</td>
<td>$\Sigma N_g$</td>
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<tr>
<td>Day 20</td>
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<tr>
<td>Pyramidal neurons (P 20)</td>
<td>10</td>
<td>1521</td>
</tr>
<tr>
<td>Non-pyramidal neurons (S 20)</td>
<td>4</td>
<td>1316</td>
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<tr>
<td>Adult</td>
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<tr>
<td>Pyramidal neurons (P ad)</td>
<td>10</td>
<td>1416</td>
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<tr>
<td>Non-pyramidal neurons (S ad)</td>
<td>4</td>
<td>1756</td>
</tr>
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</table>

$n =$ Number of neurons analysed.

*Mean ± SEM.

$D$: P 20 - S 20 $P < 0.001$
$P_{ad-S ad} P < 0.001$
$P_{20-S ad} P < 0.001$
$E(t): P 20 - S 20 P < 0.001$
$P_{ad-S ad} P < 0.001$
$P_{20-P ad} P < 0.001$
$S 20: S 20 P < 0.001$

$N_i$: P 20 - S 20 $P = 0.01$
$P_{ad-S ad} P = 0.01$
$S 20-S ad P = 0.01$

$RS$: P 20 - S 20 $P = 0.01$
$P_{ad-S ad} P = 0.01$
$P_{20-P ad} P = 0.01$
$S 20-S ad P = 0.01$
Fig. 8. Cumulative frequency distributions of the size of the synaptic discs from pyramidal neurons at day 20 and at 7 months.

Fig. 9. Cumulative frequency distributions of the size of the synaptic discs from non-pyramidal neurons at day 20 and at 7 months.

Table 2.

<table>
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<th>Pyramidal neurons (P)</th>
<th>Non-pyramidal neurons (S)</th>
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<tr>
<td></td>
<td>Day 20 (10)</td>
<td>Adult (10)</td>
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<tr>
<td>Apical dendrites</td>
<td>0.144 ± 0.014*</td>
<td>0.154 ± 0.014</td>
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<tr>
<td>Somata</td>
<td>0.137 ± 0.010</td>
<td>0.150 ± 0.016</td>
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<tr>
<td>(Basal) dendrites</td>
<td>0.140 ± 0.017</td>
<td>0.138 ± 0.009</td>
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<tr>
<td></td>
<td>Day 20 (4)</td>
<td>Adult (4)</td>
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<tr>
<td>Apical dendrites</td>
<td>0.140 ± 0.017</td>
<td>0.119 ± 0.014</td>
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<tr>
<td>Somata</td>
<td>0.119 ± 0.014</td>
<td>0.114 ± 0.017</td>
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<tr>
<td>(Basal) dendrites</td>
<td>0.140 ± 0.020</td>
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(1) Number of neurons analysed.
*Mean ± SEM.

P 20 apical dendrites-P 20 basal dendrites P < 0.05
P 20 somata-P 20 basal dendrites P < 0.05
P ad apical dendrites-P ad basal dendrites P < 0.05
P ad apical dendrites-P ad somata P < 0.001
P 20 apical dendrites-P ad apical dendrites P < 0.001
P 20 somata-P ad somata P < 0.001
S 20 somata-S 20 dendrites P < 0.001
S ad somata-S ad dendrites P < 0.001
S 20 somata-S ad somata P < 0.001
S 20 dendrites-S ad dendrites P < 0.001
Table 3.

<table>
<thead>
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<th>Pyramidal neurons (P)</th>
<th>Non-pyramidal neurons (S)</th>
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<td>Day 20 (10)</td>
<td>Adult (10)</td>
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<tr>
<td></td>
<td>Day 20 (4)</td>
<td>Adult (4)</td>
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<tr>
<td>Apical dendrites</td>
<td>15 ± 2*</td>
<td>13 ± 1</td>
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<tr>
<td>Somata</td>
<td>13 ± 2</td>
<td>13 ± 2</td>
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<tr>
<td>(Basal) dendrites</td>
<td>21 ± 5</td>
<td>19 ± 3</td>
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</table>

*Number of neurons analysed.

*S = Non-pyramidal neurons

P < 0.05

Table 4.

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<th>Receptive surface (RS)</th>
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<td>Pyramidal neurons (P)</td>
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<td>Day 20 (10)</td>
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<tr>
<td></td>
<td>Day 20 (4)</td>
</tr>
<tr>
<td>Apical dendrites</td>
<td>2.1 ± 0.3*</td>
</tr>
<tr>
<td>Somata</td>
<td>1.8 ± 0.3</td>
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<tr>
<td>(Basal) dendrites</td>
<td>2.8 ± 0.3</td>
</tr>
</tbody>
</table>

*Number of neurons analysed.

*S = Non-pyramidal neurons

P > 0.05

Multiplying (1) and (2) yields the receptive surface:

\[ RS = N_s \times E(s) \] as a percentage of the total surface area of the neuronal membrane. The Smirnov-test was used to determine whether differences in the frequency distributions of synapse diameter and surface area were significant. For this test the distributions must be transformed into their cumulative form (see Figs 6-9). Differences in the receptive surface and the number of synapses per 100 \( \mu m^2 \) were analysed with the Mann-Whitney-test. Differences were considered significant if \( P < 0.05 \). Thus differences in \( D \) (Tables 1 and 2) are differences in their cumulative distributions, whereas differences in \( N_s \) and \( RS \) are differences between individual neurons (Table 1) and differences between the components of individual neurons (Tables 3 and 4). No correction was made for tissue shrinkage since it has been shown that after the third week of postnatal life the effects of fixation can be ignored.

RESULTS

Size of synaptic active zones and synaptic discs

The number of neurons analysed \( (n) \) and the basic measurements \( \{\Sigma N_s, \Sigma B, E(L)\} \) of the different groups of neurons are summarized in Table 1. How these basic measurements were obtained is illustrated in Figs 3-5. Although the amount of neuronal boundary analysed for each individual neuron varied considerably, the total boundary did not differ much for the different groups of neurons in the young and adult animals. Thus, the measurements were not biased by overemphasis of distinct parts in one or the other group. Day 20 pyramidal neurons (P 20) and day 20 non-pyramidal neurons (S 20) had an overall mean synaptic profile length \( [E(L)] \) which was significantly smaller than the synaptic profile length measured in the adult pyramidal (P ad) and non-pyramidal (S ad) neurons (Table 1). Because the distribution of \( L \) is only a good estimate for the diameter of synaptic discs if perforations are absent (Figs 4 and 5) we have estimated the diameter distribution \( f(D) \) of the synaptic discs from \( L_D \) measurements (Fig. 5). Comparison of the diameter distribution obtained by a discrete “unfolding” procedure revealed a significant difference between P 20 and P ad \( (P < 0.001) \) and between S 20 and S ad \( (P < 0.001) \) (Table 1). Although the mean diameters of synaptic discs of P 20 and S 20 were almost equal (Table 1), the corresponding frequency distributions showed a highly significant difference \( (P < 0.001) \). A significant difference was also observed when P ad and S ad were compared, whose mean synaptic disc diameter were 417 and 411 \( \mu m \) respectively \( (P < 0.001, \text{Table 1}) \). The increase in the mean diameter of the synaptic discs \( [E(D)] \) is directly related to an increase in the mean size of the synaptic discs \( [E(s)] \) of both pyramidal and non-pyramidal neurons (see Experimental Procedures). The average size of the synaptic discs increased for both types of neurons between day 20 and the adult stage \( (P < 0.001, \text{Table 1}) \). It is obvious from Table 1 and the cumulative distributions of P 20 and S 20 neurons (Fig. 6) and P ad and S ad neurons (Fig. 7) that the difference between the two types of neurons at the same age is not simply a matter of larger or smaller synapses on one or the other cell type. The difference is best described as a preponderance of large and small synapses on pyramidal neurons and more medium-sized synapses on non-pyramidal neurons. This phenomenon was more...
suggests that synapses on non-pyramidal neurons were more homogeneous than synapses on pyramidal neurons. As can be seen in Fig. 8 these distributions have not reached their 100% level.

The mean synaptic disc area of the different components of pyramidal and non-pyramidal neurons at day 20 and in adulthood is presented in Table 2. For the pyramidal neurons there was a significant difference between the synaptic size distributions of apical dendrites and basal dendrites at each age ($P < 0.05$). The synaptic discs on the apical dendrites of P20 were larger than those on the basal dendrites and in their turn larger than those on the somata ($P < 0.05$). The difference in synaptic size between apical dendrites and somata was also seen in the adult tissue ($P < 0.001$). In addition, the synaptic discs on the apical dendrites and somata were significantly larger in adult animals than the corresponding synapses in day 20 animals ($P < 0.001$). The difference between the distributions of the synapses on apical dendrites and basal dendrites in the day 20 old animals ($P < 0.05$) was more pronounced in the adult animals ($P < 0.001$, Table 2). Although the mean size of the synapses on the distinct components of non-pyramidal neurons did not differ much, the differences in the synaptic size distributions ($F(6, 51)$) were more prominent than for the pyramidal neurons. This suggests that synapses on non-pyramidal neurons were more homogeneous than synapses on pyramidal neurons. Besides a highly significant overall difference between the frequency distributions of S20 and S20 dendrites ($P < 0.001$, Table 1) a significant difference was also found between the distributions of S20 and S20 dendrites ($P < 0.001$) and between S20 and S20 dendrites ($P < 0.001$).

The results with receptive surface area were similar to synaptic density: significant differences between synaptic discs on subsets of both cell classes and maturational differences for the non-pyramidal neurons but not for the pyramidal neurons. For the non-pyramidal neurons the receptive surface of the dendrites at both ages was significantly larger than that of the somata ($P = 0.01$). The RS-values of the somata showed a non-significant increase from day 20 to the adult stage. On the other hand the receptive surface of the dendrites increased significantly ($P < 0.01$, Table 4).

For pyramidal neurons the basal dendrites in both young and adult animals tended to have a larger receptive surface area than the somata and apical dendrites. This difference was significant for the somata vs basal dendrites comparison in the young animals ($P < 0.01$, Table 4).

In summary, when the total population of synapses and consequently the number of synapses per 100 $\mu m^2$ and the receptive surface are differentiated according to their neuronal position (dendrites or somata), these parameters hardly change for the pyramidal neurons and increase for the non-pyramidal neurons between day 20 and the adult stage.

**DISCUSSION**

The present study shows that the apparently adult appearance of the visual cortex at postnatal day 20 as found for the dendrites and perikarya is certainly not real for the synaptic organization of multipolar non-pyramidal neurons. They exhibit a dramatic increase in the number of synapses between day 20 and

**Table 1** and the results for the different components of pyramidal and non-pyramidal neurons in the Tables 3 and 4. The non-pyramidal neurons showed a synaptic density which was significantly higher than that of the pyramidal neurons and a receptive surface which was also significantly larger than that of the pyramidal neurons both at day 20 and in the adult stage (Table 1, $P < 0.01$). The synaptic density ($N_s$) and the receptive surface ($RS$) on pyramidal neurons did not change significantly between day 20 and 7 months (adult animals). The various components of the pyramidal neurons, likewise, showed no changes with age. The difference in synaptic density between somata and basal dendrites of pyramidal neurons at day 20 ($P < 0.01$) persisted into adult age ($P < 0.05$, Table 3). Synapses were significantly more frequent on basal dendrites than on apical dendrites in the adult sample ($P < 0.05$, Table 3). The dendrites on non-pyramidal neurons at day 20 received three times more synapses than the somata (50 vs 17, Table 3). This difference was also observed in the 7 month old animals (83 vs 27, Table 3). Thus the increase in the number of synapses on non-pyramidal neurons was mainly due to the increase in the number of synapses on the dendrites (Table 3).
adulthood. This increase is less obvious for the pyramidal neurons.

Non-pyramidal neurons

Most studies dealing with the developmental aspects of cortical organization have focussed on pyramidal (type I) neurons. Only during the last years have non-pyramidal (type II) neurons attracted serious attention. Major reasons for studying this diverse population of neurons, which is predominantly located in layer IV of the visual cortex, is that these neurons play an important role in the transformation of primary visual input from the thalamus to the cortex and because of the fact that there exist obvious differences between pyramidal and non-pyramidal neurons at the light microscopy level. A general conclusion from prenatal studies is that neurons in deeper layers of the cortex are formed earlier than the neurons in the upper layers and that the differentiation of the deeper located cells also starts earlier. This could imply that pyramidal neurons in layers V and VI reach their maturity at an earlier developmental stage and those in the upper layers II and III at a later developmental stage than the non-pyramidal neurons in layer IV. It has been suggested by Jacobson that cortical type II neurons would complete their differentiation later than type I neurons. However, detailed Golgi and electron microscope studies revealed that non-pyramidal neurons do not complete their differentiation significantly later than pyramidal neurons. The differentiation of the dendritic tree in the rat and in the rabbit and the ultrastructural components of non-pyramidal somata are mainly completed at the end of the third postnatal week.

For all the non-pyramidal neurons analysed in the present study, the substantial increase in both synaptic density and mean synaptic surface area resulting in a similarly considerable enlargement of the total receptive surface from day 20 to adulthood, shows that non-pyramidal neurons change substantially during this period with respect to their synaptic organization (Table 1). At day 20 a large fraction of the synaptic population consisted of small synapses, e.g. about 30% of the synapses had a surface area of 0.050 μm² (Fig. 9). The majority of these small synapses disappeared during maturation. At present it is not possible to decide whether they were replaced by larger synapses (i.e. turnover or that they simply enlarged their surface by growth. Indeed a combination of these processes may have occurred. However, neither possibility can account completely for the increased number of medium-sized synapses, ranging from 0.07–0.20 μm², in the adult stage. If we do not discriminate between somata and dendrites about 23 of these synapses were added per 100 μm² of membrane area during maturation (Fig. 9 and Table 1). It is therefore obvious that new synapses must have been formed. The formation of synapses with a specific size had already been suggested by Müller et al. in a systematic study of the size of the synaptic discs using semithin (0.5 μm) E-PTA sections. There was a difference in synaptic size between the upper and lower cortical layers up to 4 weeks postnatally. This difference was also observed for adult animals and it was not affected by dark rearing. However, the increase in synaptic size after 20 days as observed in this study was not found in the above mentioned studies. This is probably due to the staining properties of OsO₄ and E-PTA, which stain different components of the synapse.

On the average the dendrites of non-pyramidal neurons had larger and more synapses per 100 μm² membrane surface area (about threefold) and consequently a higher receptive surface than their somata at 20 days (Tables 2, 3 and 4). This situation persisted into adulthood, although the absolute values of these parameters increased significantly. The size distributions of the day 20 and adult synapses on the somata had similar shapes (not shown) which suggests that the addition of new synapses was in accordance with the shift of the entire size distribution. In contrast, on the dendrites the disappearance of small synapses during maturation is not proportional to the strong increase in the number of medium-sized synapses. In Golgi preparations Mathers observed a considerable increase in the number of spines on layer IV non-pyramidal neurons after day 15, and considered this increase as a second wave of synaptogenesis. Since it is well known that the spiny non-pyramidal neurons form only a minor component of the cell population in cortical layer IV of the rat and of the rabbit, we can predict that the second wave of spine formation will only slightly reflect this second wave of synaptogenesis. The present study shows that the second wave is much more pronounced for the spine-free and sparsely spiny non-pyramidal neurons from 20 days to adulthood than was deduced from spine analysis. This rise becomes even more dramatic if we take into account that concomitantly the total dendritic length increases by 67%. As reported by Davis and Sterling, partly reconstructed non-pyramidal neurons in the adult cat comprise a heterogeneous population of cells regarding their synaptic organization which varied between 7/100 and 48/100 μm² on the somata and between 9/100 and 65/100 μm² on the initial dendrites. In young rabbits we found between 8/100 and 27/100 μm² on the somata and between 26/100 and 67/100 μm² on the dendrites. In the adult animals the maximum values were higher and the range less pronounced on the dendrites. On the somata the number of synapses ranged from 7/100 to 42/100 μm² and on the dendrites from 7/100 to 91/100 μm². For clarity of presentation the data for individual neurons were not described in the Results section because the essential data would get lost in so many details. Since we know that the non-pyramidal neurons analysed in the adult animals are large multipolar neurons, our data are to a certain extent...
comparable with the reconstruction analysis of Davis and Sterling. Their relatively low number of 65 synapses per 100 \( \mu m^2 \) on dendrites of large non-pyramidal neurons in comparison with our results can either be due to the large number of synapses on distal dendrites which they could not reconstruct, or by the variation among neurons. The variation in the number of synapses on different types of neurons was also demonstrated by White and Rock who reconstructed four complete dendrites of an un-impregnated bitufted neuron and two dendrites of a multipolar gold toned Golgi-impregnated non-pyramidal neuron in the somatosensory cortex of the rat. The number of synapses on the bipolar neuron was about twice as high per unit length than on the multipolar neuron.

**Pyramidal neurons**

In adult rabbits we found for the entire pyramidal cells a slight decrease in the density of synapses and a small increase in the receptive surface between day 20 and adulthood. Neither of these differences was significant (Table 1). However, the synaptic discs on the pyramidal neurons in the adult animals had a significantly larger size (Fig. 8). The mean number of synapses per 100 \( \mu m^2 \) on the somatic surface area remained the same from 20 days to 7 months. Since Mathers showed that the somatic surface area increased markedly, it seems likely that the total amount of synapses per neuronal soma will be larger in the adult stage. The observed high variability in synaptic density is mainly responsible for the fact that the increase in the surface area of the synaptic discs on the somata and apical dendrites is not significantly reflected in the increase in receptive surface. When it is assumed that the diameter of the dendritic segments does not change with age and thus segment length is proportional to area, our synaptic density observations of the basal and apical dendrites are in accordance with the spine counts of Mathers. He reported that spine counts on basal, primary and secondary apical dendrites exceeded 85% of their adult values by 20 days of age.

Reconstruction analysis of somata and initial parts of pyramidal apical dendrites in layer IV of the cat visual cortex revealed that the number of synapses on somata (11/100 \( \mu m^2 \)) is comparable with the number of synapses on somata of pyramidal neurons in the adult rabbit visual cortex (13/100 \( \mu m^2 \)) (Tables 1 and 2). From the present results it is obvious that pyramidal neurons use a very small part of their total receptive surface for synaptic contacts (Tables 1 and 4). This means that dendritic spines are not cellular sites specifically meant to increase only the surface area of dendrites as postulated by Cajal because less than 3% of the total neuronal surface is covered with synapses. Moreover, the non-pyramidal neurons receive more synapses on their main dendritic shafts than the pyramidal neurons (this study). With respect to the specificity of the spines the postulation of Cajal seems to be correct since it has been shown in the electron microscope that almost every spine in the cerebral cortex is involved in a synaptic contact, whereas the majority of the dendrites of pyramidal neurons is devoid of synapses.

Basal dendrites of pyramidal neurons are known to occupy a large piece of cortical tissue within the layer where they are located and can therefore receive more synapses both from intra- and extracortical origin. Their profusely arborized dendritic tree and their location within the cortex suggest the higher number of synapses and the larger receptive surface area of basal dendrites than that of somata and apical dendrites of pyramidal neurons at day 20 and in the adult stage (Tables 3 and 4).

Explicit mention should be made as to the results of comparing layer II/III and layer V pyramidal neurons within each developmental period. The results were similar when the large pyramidal neurons in layer V of the 20 day old animals were omitted. This was done to verify whether neurons in the deeper layers would influence differences between neurons in the upper layers (Figs 1 and 2).

**Pyramidal and non-pyramidal neurons**

The small receptive surface of pyramidal neurons (Tables 1 and 4) and the four times larger receptive surface of the non-pyramidal neurons (Tables 1 and 4) may be specific for the visual cortex. In routine osmium-treated material H. Haug (personal communication) found in the neuropil of the cat an average receptive surface of 2.5%. Although this RS-value is the result of both pyramidal and non-pyramidal neurons it is obvious that the receptive surface of neurons in the cat visual cortex is also small.

The differences in synaptic organization found in the present study between pyramidal and non-pyramidal neurons were not observed in Golgi preparations and electron microscope studies. Although Parnavelas and Lieberman could not observe differences between both pyramidal and non-pyramidal neurons on the basis of well defined criteria, they reported a conspicuous increase in the number of cell organelles in the non-pyramidal neurons during the third and the first part of the fourth week which gave the impression that these neurons were in a highly active metabolic state. They suggested that the active state was most likely necessary for the formation of substances required for synapse formation. Their idea is in accord with our findings pointing to a second wave of synaptogenesis. Moreover, it has been suggested by Jacobson that type II neurons which arise at a later time in development than type I neurons would be influenced by sensory input either directly through synaptic stimulation or indirectly by hormonal and metabolic changes during differentiation. We did find a change in the synaptic organization of type II neurons in the visual cortex after a period of visual experience. This change was
not recognizable in the type I neurons. However, the type I neurons analysed in the layers II and III are later generated than the type II neurons in layer IV. If we consider the number of synapses on neurons in the visual cortex as an important aspect of neuronal maturation we can put forward that the type II (non-pyramidal) neurons analysed in the present study complete their maturation later than the type I (pyramidal) neurons in the upper layers (Figs 1 and 2). The increase in the number of synapses and consequently the receptive surface of the spine-free and sparsely spiny non-pyramidal neurons is characteristic for these neurons and is probably not related to the time of generation. The increase in the number of synapses on these neurons some of which may be inhibitory in character, could mean that they are influenced by a stronger synaptic input. It has been shown that inhibitory intrinsic connections made by non-pyramidal neurons are essential for cortical function. In the visual cortex these connections are needed for the development of fine regulation of function and their formation is probably dependent on visual experience.

This conclusion could be drawn because many nonselective neurons in dark-reared cats lack inhibitory inputs which, in normal animals, give rise to selectivity. This inhibitory function tempted us to speculate about the meaning of the increase in the number of synapses on the non-pyramidal neurons. We would like to propound that visual experience may induce the spine-free and sparsely spiny non-pyramidal neurons to form and receive more synapses in the visual cortex such that it can function properly. Due to the limitations of the Golgi-technique, no relevant conclusions can be drawn about the subdivisions of these non-pyramidal neurons on the basis of their axonal pattern. One has to keep in mind that the inhibitory role of the basket cells and the chandelier cells might be more selective than other non-pyramidal neurons since their inhibitory synapses on somata and axon initial segments respectively are in the vicinity of the axon hillock, the place where the action potential is generated.

Of course it is of importance to know what the ratio of type I and II synapses on these neurons is (L. J. Müller et al., in preparation), since it is thought that asymmetrical (type I) synapses are excitatory and symmetrical (type II) synapses inhibitory in function. This is more important because we know that these neurons receive both types of synapses at intervals of about 4 μm. Furthermore the present data give evidence that the functional significance of the differences in the number of synapses on neuronal perikarya in the visual cortex as given by Winfield et al., was indeed uncertain without data about the number and distribution of synapses upon other parts of the neurons (Table 3).

We would like to emphasize the fact that the newly formed synapses after day 20 of postnatal life are mainly added to the dendrites of the non-pyramidal neurons (Table 3).

Technical considerations

To quantify the number of synapses per unit neuronal membrane surface area, we assumed that the shape of the synaptic discs, as distinguished from the active zone per se, are approximately flat circular discs. If this assumption is not sufficiently in accord with the actual synaptic shapes the resulting density estimate is likely to be erroneous and we realize that a method which avoids such a shape assumption would be preferable. Such a method involving serial sections has been developed by Cruz-Orive. However, E-PTA pictures of "en face" synaptic grids on identified neurons indicate that the assumption was not unrealistic in our case, also with respect to the occasional synapses that contained perforations. We therefore feel confident that our results are consistent. Moreover, application of the method of Cruz-Orive would imply serial reconstruction of synapses at several planes of the identified neurons. That this is a prohibitively laborious procedure is illustrated by the fact that only two electron microscopic reconstruction studies on non-pyramidal neurons, one on apical dendrites of pyramidal neurons and one on 32 arbitrarily classified neurons have been published so far.

Although in the present study we have analysed 28 complete neurons, the sample is still small. However, the sample is large enough to apply statistical procedures to determine variations between pyramidal and non-pyramidal neurons at similar ages and differences between two developmental stages of the same group of neurons. Nevertheless, we have to admit that some of the neurons analysed are less complete than other neurons because of the staining properties of the Golgi-impregnation or the loss of dendrites which were cut at the surface of the 100 μm thick slices. Reconstruction of somata and initial dendrites in layer IV of the cat visual cortex revealed for the somata of pyramidal neurons and for the somata and initial dendrites on non-pyramidal neurons in the adult stage comparable results with our study.

Conclusions

The analysis of 28 complete neurons at two developmental stages in the visual cortex of rabbits indicates that only a small part of the neuronal surface of neurons is covered with synaptic contacts. The receptive surface of pyramidal neurons is smaller than that of non-pyramidal neurons. After day 20 the receptive surface does not change much for the pyramidal neurons but significantly increases for the non-pyramidal neurons. These maturational changes were also observed for the number of synapses per 100 μm² surface area. The observed increase in the average size of the synapses on both pyramidal and non-pyramidal neurons between day 20 and adulthood...
was either caused by synapse turnover, growth of the individual synapses or addition of newly formed medium-seized synapses. Because turnover of growth could not completely account for the shift in the size distributions, we concluded that numerous medium-sized synapses are added to the existing population of non-pyramidal neurons between day 20 and adulthood. These synapses were mainly added to the dendrites of the non-pyramidal neurons.

The formation of the new synapses after a period in which the majority of synapses have been formed points to a specific contribution of these later developed synapses in the complexity of visual function.

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REFERENCES


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