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Maturation of Neurons in Neocortical Slice Cultures. A light and electron microscopic study on in situ and in vitro material

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With 7 Figures and 1 Table

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Abstract: Using light and electron microscopic methods, we investigated the development and morphology of neurons in neocortical slice cultures. Slices taken from the visual cortex of 6-day-old rats and cultivated for 14 or 20 days were compared with in situ material of corresponding age (P 20 and P 26). Maturation and differentiation of pyramidal and non-pyramidal cells kept in vitro were found to have progressed considerably. In the light microscope the neurons exhibited a morphological appearance strikingly similar to that of the neurons of the neocortex in situ at the same age. The fine structure of the tissue in vitro also had a mature appearance, corresponding in most respects to the material in situ. Synapses and dendritic spines were well-developed. Sometimes a spine apparatus was contained in the sections and occasionally a myelinated fiber could be seen. GABA-immunoreactive cells making symmetric synaptic contacts were also present. Despite these similarities, some quantitative differences could be observed. In slice cultures, only 52% of the synapses were located on spines (78% in situ). In vitro, a larger proportion of synapses (30%) showed a postsynaptically concave curvature than was the case in situ (12%). The areal density of synapses in vitro reached only about 70% of that in situ. This was probably a side-effect of the larger size of dendritic and axonal profiles on electron micrographs of in vitro-material. The most striking difference was that large synapses and synapses containing a large amount of synaptic vesicles were considerably more frequent in vitro than in situ.

Key words: development, plasticity, GABA-immuno-histochemistry, connectivity, morphology

Introduction

The neocortex is characterized by its laminated structure, the morphology and orientation of its cells and by its intrinsic and extrinsic fiber connections. During ontogeny the neurons pass through three successive developmental stages, termed proliferation, migration and differentiation. In brief, pyramidal (ANGEVINE and SIDMAN, 1961; BERRY and ROGERS, 1965; MILLER, 1985) and non-pyramidal neurons (WOLFF et al., 1984; MILLER, 1985) of the rat visual cortex are generated in the ventricular zone between prenatal days 13 and 21 (E13–E21) in an inside to outside pattern. The migration of cells appears to be completed about seven days after birth (BERRY and ROGERS, 1965; HICKS and D'AMATO, 1968; PARNAVELAS et al., 1978), just when the lamination of the neocortex is fully developed (MILLER, 1981). Finally, pyramidal (JURASKA and FIVKOVÁ, 1979; PARNAVELAS and LIEBERMAN, 1979; MILLER, 1981; MILLER and PETERS, 1981; NICOLAI, 1981) and non-pyramidal (PARNAVELAS et al., 1978) cells reach their adult shape around the end of the third postnatal week. It is not yet completely clear to what extent the proliferation, migration and final morphological appearance of neurons is determined by the genetic properties of the cell, by complex cell to cell interactions (RAKIC, 1972; LO TURCO and KRIEGS-

TEIN, 1991), by the arrival of afferent fibers (see RAKIC, 1988; SHATZ et al., 1988) or by sensory stimuli (e.g. WIESEL, 1982).

In this study, slice cultures of the occipital cortex of the rat were prepared at postnatal day 6. At that time the neurons have not yet fully matured and some of the afferent (WISE and JONES, 1978; LUND and MUSTARI, 1977) and efferent (STANFIELD et al., 1982; THONG and DREHER, 1987) fiber systems of the neocortex have not yet fully reached their final arrangement. Slice cultures are therefore a good model for the study of the maturation of neurons in the absence of efferent and afferent fibers. This makes them attractive for questions of plasticity and development. In addition, slice cultures can be used for studies of reorganization with respect to those properties which have already developed at the time of explantation and also with respect to later experimental manipulations.

A further motive for a detailed anatomical investigation of neocortical slice cultures is their use in physiological studies. The similarity or dissimilarity in structure helps in judging to what extent physiological results obtained on such cultures may also be relevant for the neocortex in situ.

In the present study the slice cultures were kept in vitro for 14 or 20 days and compared to in situ material of corresponding age (P 20 and 26) and of postnatal

day 6. The comparison was made by way of the Golgi-method, conventional electron-microscopy and immunohistochemistry on the electron microscopical level.

A previous light microscopic study has already shown that the main features of neocortical tissue (such as lamination and the typical morphology of the cell types) also develop in slice cultures (CAESER et al., 1989). This is also true for the main features on the electron microscopical level (CAESER and SCHÜZ, 1989; WOLBURG and BOLZ, 1991). The present study goes beyond these findings. It describes the morphology and development of the neurons in vitro in more detail, focussing not only on similarities but also on differences with respect to the tissue in situ. We also include a description of how GABA-immunohistochemistry for electron microscopy and the Golgi-method can be adapted to slice cultures. Some of the results presented here were part of a doctoral thesis (CAESER, 1989).

Material and Methods

Culturing procedure

For cultivation of neocortical explants, the roller tube technique originally described by GÄHWILER (1981) was used. Briefly, neocortical slices (350 µm) including the visual cortex were cut in the frontal plane. All slices were prepared from 6-day-old rats and kept in vitro for 14 or 20 days before histological processing. After decapitation the brains were quickly removed, rinsed in ice-cold Gey's balanced salt solution (GBSS, Gibco, with 6.5 mg/ml glucose) and stored at 4 °C for between 30 and 60 min. The slices were transferred onto cleaned glass coverslips (12 × 24 mm) and embedded in a plasma clot. The clot was formed by 20 µl of heparinized chicken plasma (lyophilized; Difco) which was coagulated by 20 µl of a thrombin solution (0.2 mg/ml, 20 NIHU/ml, Hoffmann La Roche). Following coagulation of the plasma, the slice cultures were placed in plastic culture tubes (16 × 160 mm, Nunclon) and kept in a roller drum incubator at 36 °C in dry air, without CO₂/O₂ control. The explants were fed once or twice a week with culture medium (0.8 ml) consisting of 50% Eagle basal medium (BME), 25% Hanks balanced salt solution (HBSS) and 25% horse serum, containing 0.1 mM glutamine and 6.5 mg/ml glucose. All medium components were obtained from Gibco. To reduce extensive glia cell proliferation, mitotic inhibitors (5-fluoro-2-deoxyuridine, cytosine-b-d-arabino-furanoside, uridine; Sigma) were added to the culture medium for 24 h to give a final concentration of approximately 10 µM.

Golgi impregnation

Golgi impregnated cells were studied in four brains of 6-day-old rats and five brains of 20-day-old rats, all of which were prepared according to the method of COLONNIER (1964). The 6-day-old rats were decapitated, the brains were immediately removed and fixed by immersion in 2% K₂Cr₂O₇ and 5% glutaraldehyde. The 20-day-old rats were fixed by intracardial perfusion after lethal anaesthesia with Ketavet and Rompun. All brains were kept in

fixative for 4–5 days and then transferred to 0.5% AgNO₃ for 3–6 days. After dehydration in acetone/ethanol, they were embedded in celloidin and cut frontally into serial sections 90 µm thick.

The cultures (30) were also impregnated according to the Golgi-Colonnier method, though in a slightly modified way. Due to the limited thickness of the tissue, the cultures were fixed in K₂Cr₂O₇ for 1–2 days only. They were then rinsed several times in water to wash off the excess K₂Cr₂O₇. A tiny grain of silver nitrate was then placed on the edge of the culture using a broken micropipette. The glass coverslips with the cultures were then placed horizontally in a humid chamber and kept in darkness for 24–48 hours at room temperature. The cultures were then examined under the microscope. Those containing impregnated cells were dehydrated in ethanol, transferred to terpineol and xylene and finally covered with entellan. For documentation, labelled cells were photographed or drawn with the aid of a drawing tube.

Crucial points for a successful stain with the Colonnier-technique on cultures are 1) the humidity of the tissue when the grain of silver nitrate is put onto it, 2) the humidity of the chamber in which the culture is kept, and 3) the position of the crystal. Best results are obtained when the crystal is put at the very edge of the culture close to the pial surface. The tissue must not be too wet, otherwise the silver grain dissolves immediately, but the chamber must be kept as humid as possible so that the tissue does not get too dry with time. The silver nitrate then gradually dissolves and diffuses into the tissue, forming a dark precipitate close to the starting point. Well stained neurons can be found surrounding the dark precipitate. An example is shown in Fig. 3a.

Alternatively, slice cultures were also stained by the section-Golgi impregnation procedure (HARRIS et al., 1980; FREUND and SOMOGYI, 1983; FROTSCHER and LÉRÁNT, 1986) which was adapted to the slice cultures by A. Münster in our laboratory. These cultures were fixed with 1% paraformaldehyde, 2.5% glutaraldehyde and 1 mM CaCl₂ in 0.1 M phosphate buffer (pH 7.3–7.4) for 30–60 min. Cultures were subsequently washed in phosphate buffer (PB), carefully removed from the glass coverslips and washed in several changes of (PB) for 120 min. Postfixation was performed in 1% OsO₄ in phosphate buffer (pH 7.3–7.4) for 60 min. The cultures were then rinsed three times for 10 min in PB, assembled in a pile of 6 to 12 slices separated by small pieces of parafilm. For staining we embedded the cultures in agar (5%), trimmed the agar and treated the pile with a solution of 3.4% K₂Cr₂O₇ and 0.2% OsO₄ for 3–4 days. After a short rinse in bidistilled water they were placed in a solution of 1.2% AgNO₃ for 24 hours.

Conventional electron microscopy

For electron microscopy we tried out several types of fixation. Best results were obtained by fixation in 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.3) for 2 h. After several washes in buffer, the explants were carefully removed from the glass coverslips and transferred into buffer solution for 1 to 2 hours. Postfixation was carried out for 1 h with 1% OsO₄ in cacodylate buffer. The explants were then rinsed several times in buffer, dehydrated in ethanol solutions of increasing strength and embedded in araldite between a glass coverslip and an acetate foil. During the dehydration procedure the explants were block-stained for 60 min in 70% ethanol saturated with uranyl acetate. Appropriately sized pieces were cut out of the explants and postembedded in araldite. Semithin sections were examined under the light microscope and any cultures showing signs of necrosis were discarded. Thin sections were then cut on an ultramicrotome, mounted on Formvar coated grids, contrasted with lead citrate and examined with a Zeiss electron microscope.

Fourteen cultures were investigated. As in the case of the Golgi-preparations, they had been explanted at postnatal day 6; 11 of them were kept in vitro for 14 days, three of them for 20 days. This material was compared to electron microscopic sections of the visual cortex of three 20-day-old rats and of one 26-day-old rat. The 20-day-old rats had been prepared according to PALAY and CHAN-PALAY (1974); the 26-day-old rat had been perfused with 2% glutaraldehyde in cacodylate buffer and was further treated as described above for the cultures.

Some of the differences which were evident between slice cultures and cortex in situ we wanted to demonstrate by quantitative estimates. This concerned the density of synapses, the percentage of synapses on spines and the curvature of the synaptic membrane. Differences with respect to these parameters could be seen in all cultures regardless of whether they had been kept in vitro for 14 or 20 days and in spite of slight variations in fixation. Measurements were made on electron micrographs from five cultures, three of which had been kept in vitro for 20 days and two for 14 days. The micrographs had been taken at random from the neuropil of layer II/III and were compared to electron micrographs of layer II/III from the visual cortex of the rat. Counts were made on 45 culture samples altogether, each of which had an area of 16 to 50 μm^2 . These were compared to a total of 38 samples taken from two 20-day-old rats and one 26-day-old rat.

Combined GABA-electron microscopy

Cultures for EM-immunohistochemistry were fixed for 20 min in a phosphate buffered solution (0.1 M, pH 7.4) containing 4% paraformaldehyde, 0.15% glutaraldehyde and 0.2% picric acid. After being washed (two 10 min rinses) in PB, the cultures were carefully removed from the glass coverslips and washed in 0.05 M Tris buffer (2 \times 10 min; pH 7.4). Cultures were then transferred into 0.05 M Tris buffer containing 0.1% Na-metaperiodate for 60 min. Following several washes in Tris buffer (3 \times 10 min) the explants were incubated floating freely in a solution containing rabbit anti-GABA serum at 4 °C for 20–22 hours under gentle agitation. GABA antiserum (Dianova) was diluted 1:100 in 0.05 M Tris buffer (pH 7.4) containing 1% bovine-serum albumine (BSA). The cultures were subsequently washed in Tris buffer and treated with anti-rabbit IgG (Vector) for 30 min. After washing in buffer the cultures were incubated in avidin-biotin-peroxidase complex (Vector) for 60 min. Cultures were then washed in PBS (3 \times 10 min) and postfixed in a phosphate buffered solution (pH 7.4) containing 1.5% glutaraldehyde. Following this, explants were rinsed several times in PB (2 \times 10 min) and in Tris buffer (2 \times 10 min). For visualisation of the immuno-labeling, the explants were treated with 0.05% 3,3-diaminobenzidine (DAB, Sigma) diluted in Tris buffer (0.05 M) with 0.004% H_2O_2 for 5–10 min on a rotator. After rinsing in Tris-saline (2 \times 10 min) and PB (2 \times 10 min) the cultures were transferred for 60 min into a phosphate buffered solution containing 0.5% OsO_4 and 0.8% potassium-ferricyanide for intensification of the staining. Following a final wash in phosphate buffer the cultures were dehydrated in a graded series of ethanol (5 min each step) and embedded as described above, but without en-bloc staining with uranyl acetate.

Results

Light microscopy of the immature visual cortex

By postnatal day 6 the cellular organization (Fig. 1a) and the layering (not shown here) of the neocortex

had already developed. Pyramidal cells, located in both deeper (Fig. 1b) and more superficial (Fig. 1c) layers displayed the basic components of neurons. Nevertheless, the morphology of cells at this stage was still very different from that observed two weeks later (Fig. 2). The dendritic tree of the pyramidal cells resembled an inverted radish: the cell body was not yet clearly demarcated, tapering smoothly into the thick apical dendrite which in many cases had already reached the first layer and bifurcated there (Figs. 1a, b). The oblique branches extending from the main dendritic trunk and the basal dendrites were still short, sparsely ramified and very thin (Fig. 1b, c). Only very few spines (Fig. 1d) and some hairy-like processes emanated from the dendrites. At this developmental stage, the axons appeared more mature than the dendrites. The main axon already reached far into the white matter, it gave off quite a number of collaterals (Fig. 1c), some of which were already very long.

On the basis of their orientation and dendritic branching pattern, non-pyramidal cells could also occasionally be identified in our Golgi material. An example is shown in Fig. 1e. Further classification of non-pyramidal cells at that age was, however, difficult.

Light microscopy of the mature visual cortex

During the next two weeks, the morphology of the neurons changed dramatically, the result being an adult-like appearance of neocortical structure (Fig. 2a). Typical examples of pyramidal cells located in deeper and superficial cortical layers are shown in Figs. 2b and 2c. The transition between the apical dendrite and the cell body is now more clearly delineated (Fig. 2b). The side branches of the apical dendrite and the basal dendrites have become more numerous, and especially longer and thicker (Figs. 2b, c). Third- and fourth-order dendritic branches could be observed. The dendrites were now densely covered with well-developed spines (Fig. 2d). Dendritic growth cones and filopodia could no longer be observed, indicating that the maturation of dendrites was completed.

In addition to normal pyramidal cells, inverted pyramidal cells and various types of non-pyramidal cells (Fig. 2a, arrow; Fig. 2e) could also be observed. They showed an adult-like branching pattern and could now be divided more easily into subgroups. A large proportion of the non-pyramidal cells observed in our material were Martinotti-cells which are characterized by their long ascending axon (Fig. 2e, arrow).

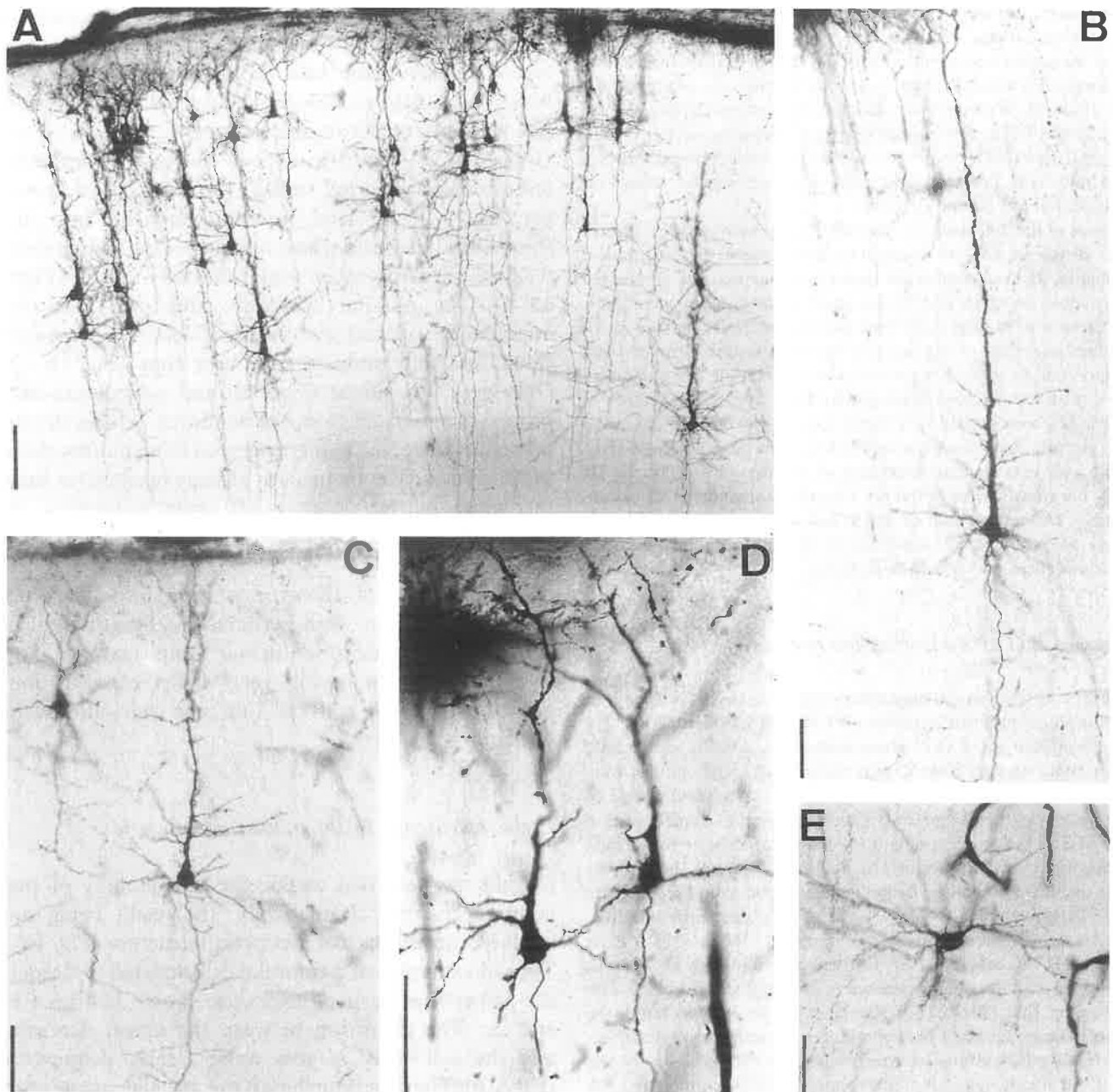


Fig. 1. Golgi impregnation of the immature rat neocortex at postnatal day 6, the time of explantation. (A) Overview, demonstrating the morphology and orientation of nerve cells in different cortical layers. Bar: 100 μ m. (B, C) Pyramidal cells located in layer V (B) and in layer II/III (C). The ascending apical dendrites are sparsely ramified. The axons descend toward the white matter and show several collaterals. (D) Two pyramidal cells of layer II/III at higher magnification, showing an almost complete lack of spines. (E) Non-pyramidal cell, located in a deeper cortical layer. Bars in (B) to (E): 50 μ m.

Light microscopy of the slice cultures

When examining neocortical explants taken from a 6-day-old rat and cultivated for 14 days, it was evident that not only the gross cellular organization of the neocortex was preserved (Fig. 3a) but also differentiation and maturation had continued considerably. For instance, pyramidal cells located in deeper (Fig. 3b) and in superficial (Fig. 3c) cortical layers were remarkably similar to those of corresponding age in situ

(Figs. 2b, c). The apical dendrites of pyramidal cells had retained their orientation toward the pial surface. Some of the cells located in deeper layers terminated at middle height of the explant, others reached the first layer. The apical and basal dendritic fields were quite complex. Dendritic branches of third and fourth order could be observed. The dendrites were now densely covered with well-developed spines (Fig. 3d) and dendritic growth cones or filopodia could no longer be detected. The density of spines was well

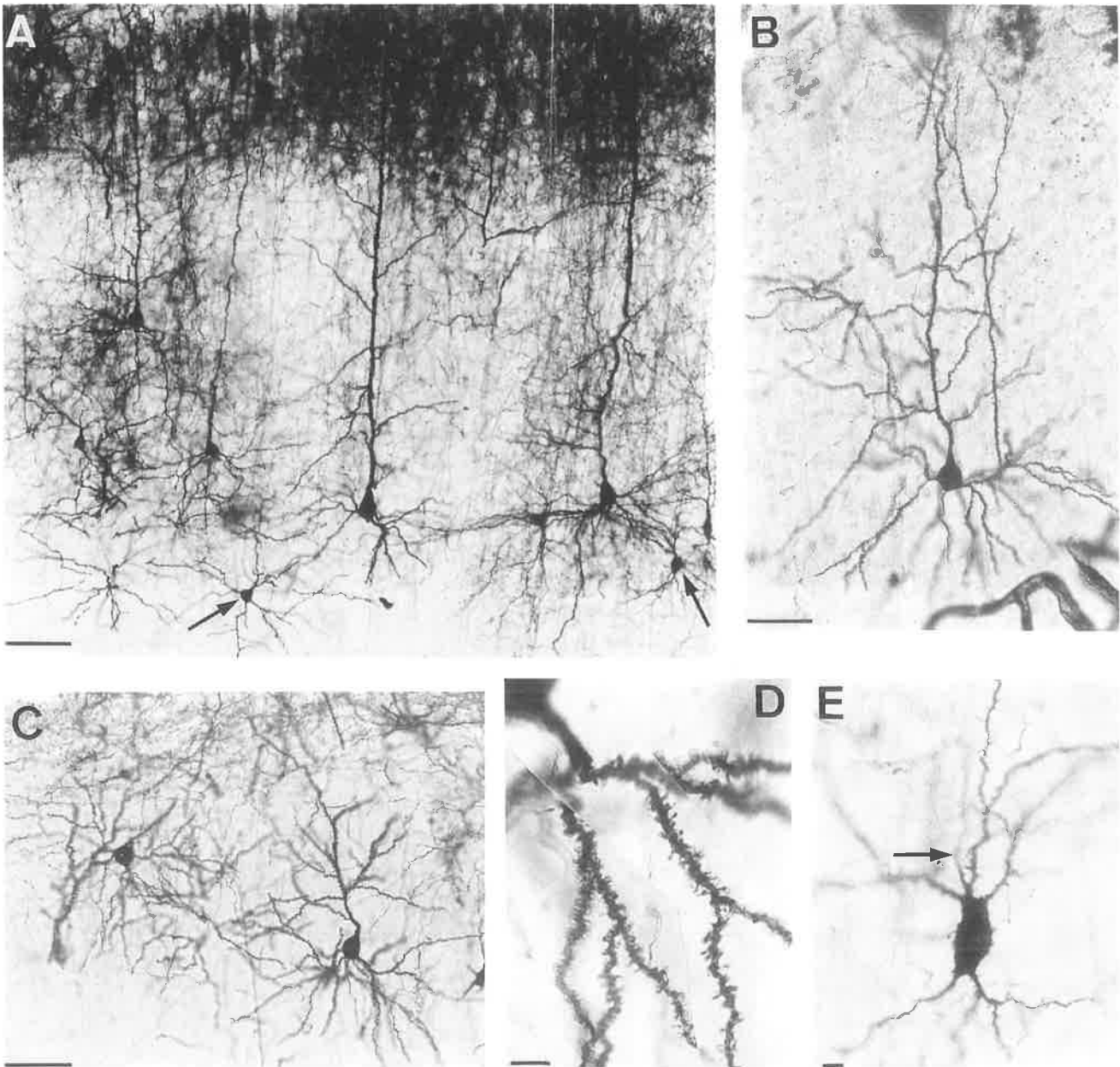


Fig. 2. Golgi staining of the rat neocortex at postnatal day 20. (A) Overview, showing many pyramidal cells and some non-pyramidal cells (arrows). Bar: 100 μ m. (B, C) Pyramidal cells located in layer V (B) and layer II/III (C). The dendrites are now extensively branched. (D) Basal dendrites of a pyramidal cell showing the high density of spines at that age. (E) Martinotti cell, showing dendrites which are sparsely covered with spines and an ascending axon (arrow). Bars in (B) and (C) 50 μ m, in (D) and (E) 5 μ m.

within the normal range, however, very high densities such as are frequent in situ at that age (Fig. 2d) did not occur in culture. The main axonal trunk descended in most cases toward the white matter (Figs. 3b, c). A distinct white matter was sometimes preserved and axons of pyramidal cells could be followed into it. Some of them could be traced back into the gray matter, others left the explant as seen in preparations stained with the fluorescent dye di I (unpublished observation). On their way down, the axons gave off numerous collaterals (Figs. 3b, c). Inverted pyramidal cells could also be seen in culture. Typically for this

type of cell, the axon left the cell body in an upward direction but then made a turn of 180 degree and ran towards the white matter.

Non-pyramidal cells in culture displayed the characteristic features of this heterogeneous group of cells. In particular, they had only few spines and their axonal tree did not enter the white matter but made only local ramifications (Fig. 3e). Some of them could be identified as Martinotti-cells (Fig. 3f).

In situ the dendritic field of rat pyramidal cells is about 180 to 480 μ m in diameter. It is obvious that the flattening of the tissue to a thickness of less than

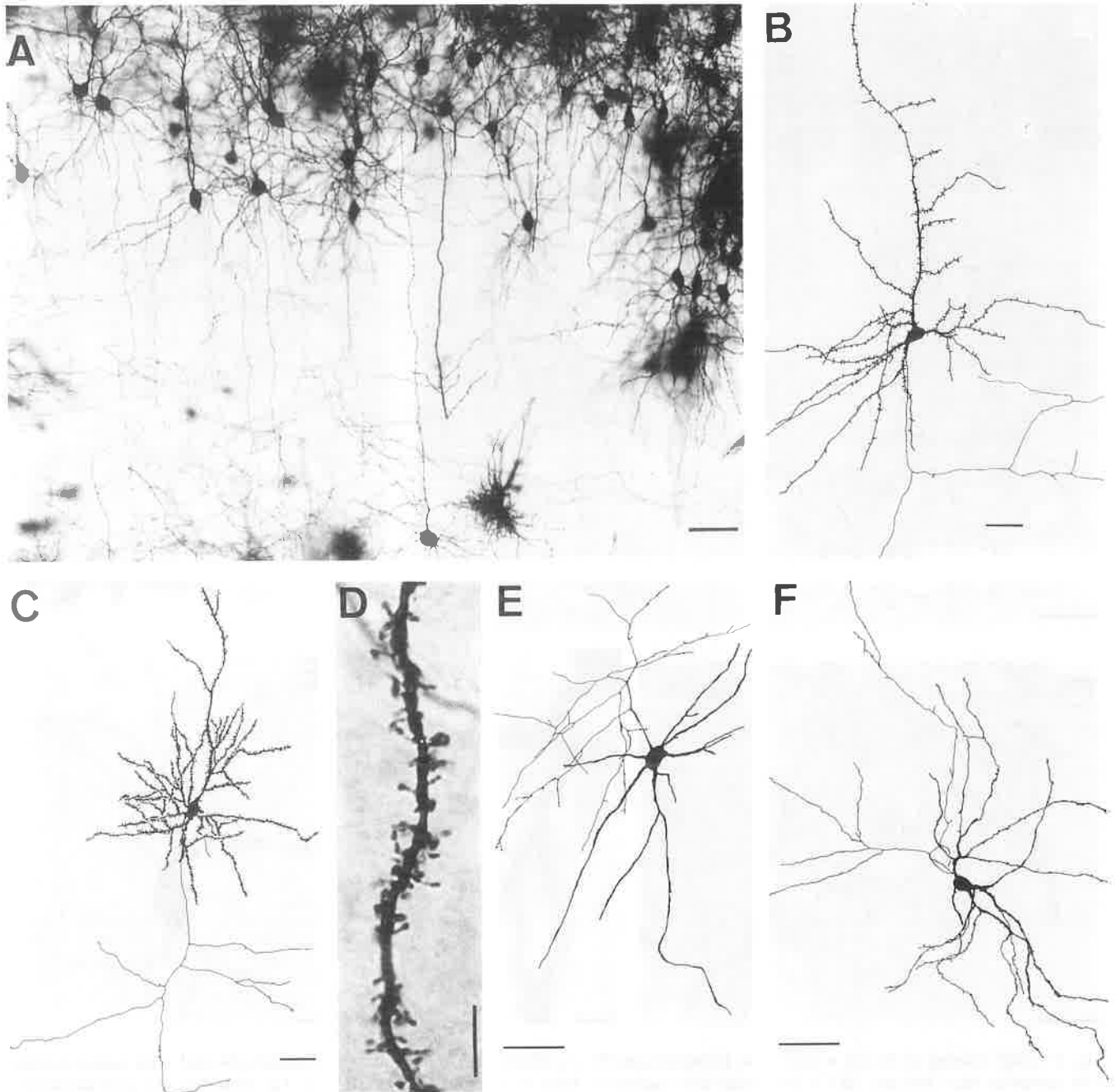


Fig. 3. Morphological organization of neocortical explants taken from 6-day-old rats and kept in vitro for 14 days before Golgi-impregnation. (A) Overview, demonstrating the gross cellular organization of the explant. Bar: 100 μ m. (B, C) Camera lucida drawings of pyramidal cells in deeper (B) and superficial (C) cortical layers. The dendrites are extensively ramified, the axons show several branching points, the main stem descends toward the white matter. (D) Basal dendrite of a pyramidal cell, demonstrating the density and maturity of spines. (E, F) Camera lucida drawings of two non-pyramidal cells, both located in deeper cortical layers. The neuron in (E) has no spines and the axon forms a dense local branching pattern. In (F) a Martinotti cell is shown with few spines and an ascending axon. Bars in (B), (C), (E), and (F) 50 μ m, in (D) 5 μ m.

100 μ m must have some effect upon the geometry of the dendritic trees. If the dendrites ramify as strongly as in situ, the dendritic trees should appear much denser in culture since the branches would be pressed into a smaller volume. However, the opposite was the case.

The density of the dendritic branching pattern tended to be rather lower than in culture, indicating that the formation of dendritic branches was somewhat reduced. In contrast, the average length of dendritic branches of cultured cells tended to be larger than in situ.

Electron microscopy of the in situ material

For electron microscopy we only used material from rats of 20 and 26 days of age (Fig. 6a). The postnatal maturation of the cortical fine structure up to that age has been described extensively by MILLER and PETERS (1981) and will be shortly reviewed here. The maturation of pyramidal cells from day 3 to day 21 is characterized by an increase in the size of the nucleus and in the amount of cytoplasm surrounding it, by a condensation of the nucleolus, by the occurrence of

invaginated nuclear membranes, by an increase in endoplasmatic reticulum and a more orderly packing of its cisternae. Growth cones and filopodia on dendrites disappear and the number of spines and synapses increases, reaching or even exceeding its adult value by about day 20 (SCHAPIRO et al., 1973; MILLER, 1981). Immature synapses are characterized by a small number of synaptic vesicles and by membrane specializations which do not yet permit a morphological classification of synaptic contacts (BLUE and PARNAVELAS, 1983). During the first weeks, the number of

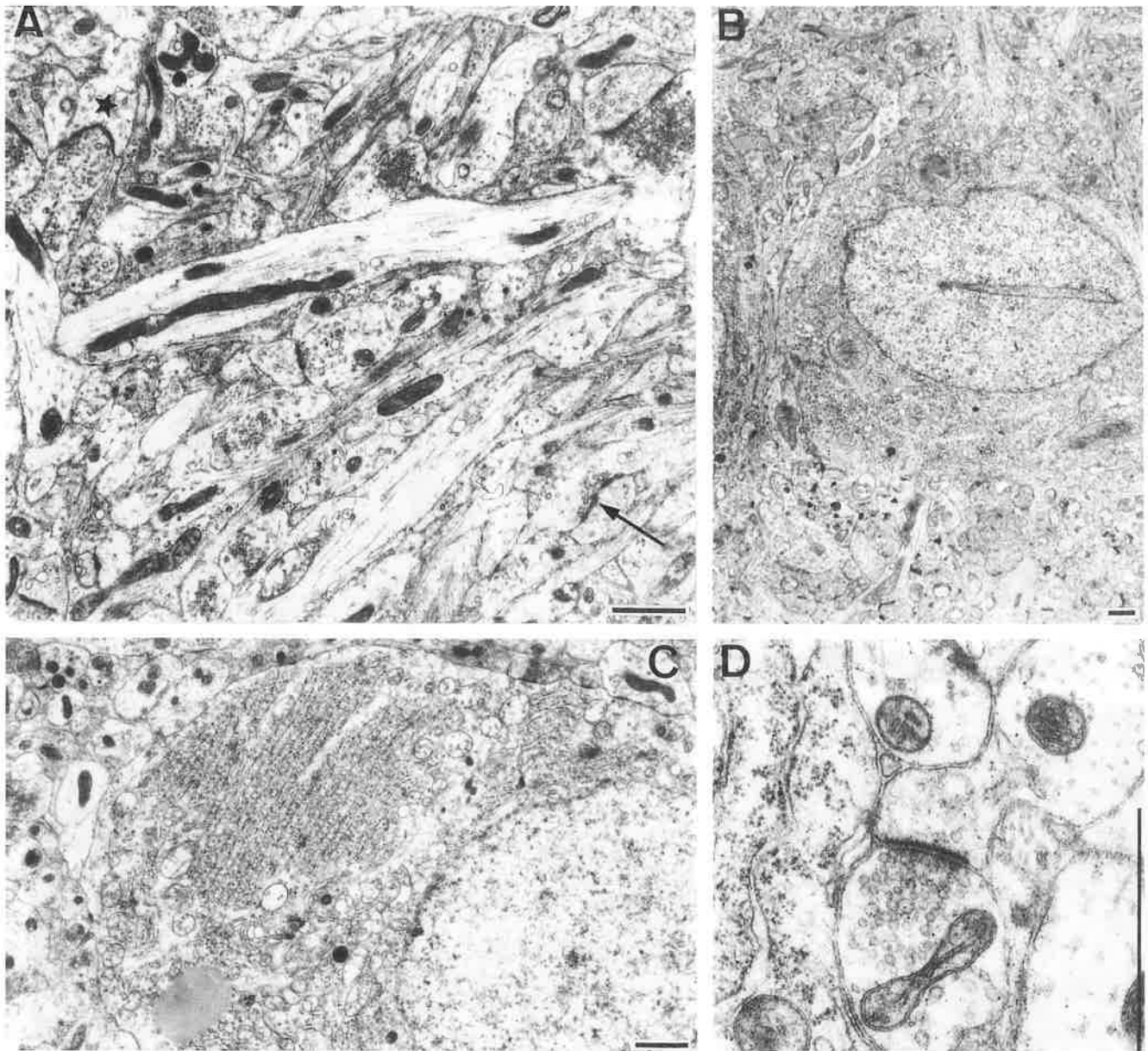


Fig. 4. Electron micrographs showing the degree of maturity of the fine structure of neocortical tissue taken from a 6-day-old rat and cultivated for 14 days. (A) Overview, demonstrating the density of the tissue. No signs of degeneration are observed. Asterisk: presynaptically convex synapse; arrow: presynaptically concave synapse. (B) Cell body. Note the richness in cytoplasm and the large nucleus with the folded nuclear membrane. (C) Example for the occurrence of highly organized endoplasmatic reticulum. (D) High-power photomicrograph showing a dendritic spine, carrying an asymmetric synaptic contact. Bars: 1 μ m.

vesicles increases considerably and it becomes possible to distinguish two morphologically different types of synapses on the basis of their membrane specializations (COLONNIER 1968): an asymmetric type in which a thickening is apposed to the postsynaptic membrane and a symmetric type in which this thickening is lacking.

Electron microscopy of the in-vitro material

The fine structure of the tissue after 14 days in vitro (Fig. 4) had a mature appearance. It corresponded in most respects to the material in situ. In spite of the extensive cell death, which is partly responsible for the flattening of the explants, the neocortical tissue was well reorganized, very dense and no longer showed signs of degeneration (Fig. 4a).

The soma, dendrites and axons contained the complete set of organelles characteristic for nerve cells. The nucleus was large and sometimes folded (Fig. 4b)

and a well-developed nucleolus was occasionally visible. The soma was rich in cytoplasm, contained many ribosomes and a highly organized rough endoplasmic reticulum (Fig. 4b, c). The dendritic spines carried synapses (Fig. 4d) and sometimes a spine apparatus could be seen (Fig. 6b, arrow). Other dendritic appendages typical for immature tissue such as growth cones or filopodia were not visible after 14 days in vitro. In addition to nerve cells astrocytes could be observed. This conforms to observations of GFAP immunopositive cells on the light microscopic level (unpublished results). Myelinated fibers could occasionally be observed, indicating that oligodendrocytes were also present.

Well-developed synaptic contacts were located on dendritic spines (Fig. 4d, 6c), dendritic shafts (Fig. 5a), and cell bodies (Fig. 5c). Both types of synapses were present in roughly the same proportion as in situ. As in situ, the asymmetric type was predominantly located on dendritic spines (Fig. 4d) and dendritic shafts, while the symmetric type was mainly located on somas (Fig. 5d) and proximal dendrites.

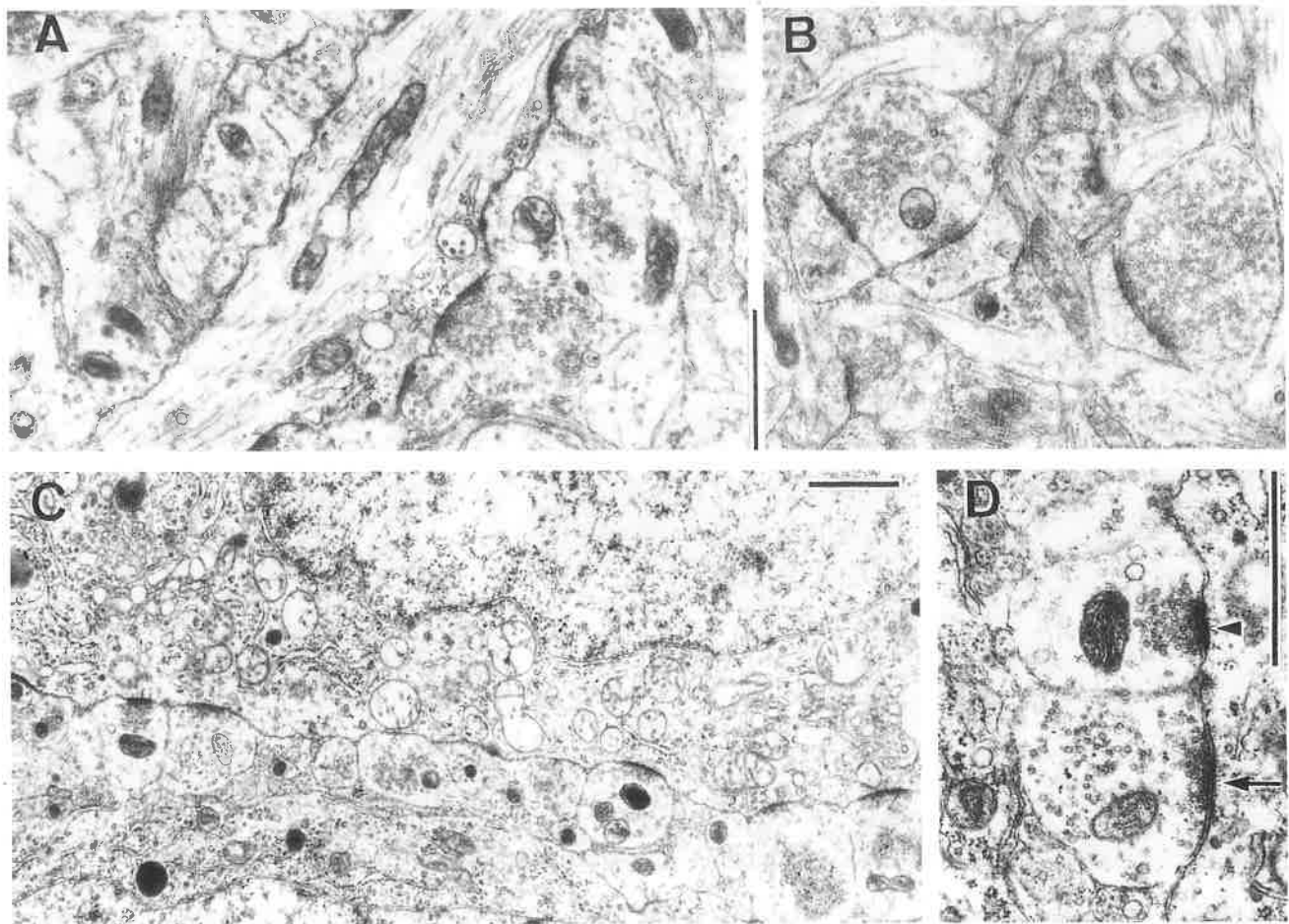


Fig. 5. Electron micrographs showing the synaptic organization in vitro. Dendritic profiles (A) and cell bodies (C) are often densely covered with synaptic contacts. The synapses are often large and very rich in vesicles (B). In (D) an example of a symmetric (arrow) and an asymmetric synapse (arrowhead) are shown. Bars: 1 µm.

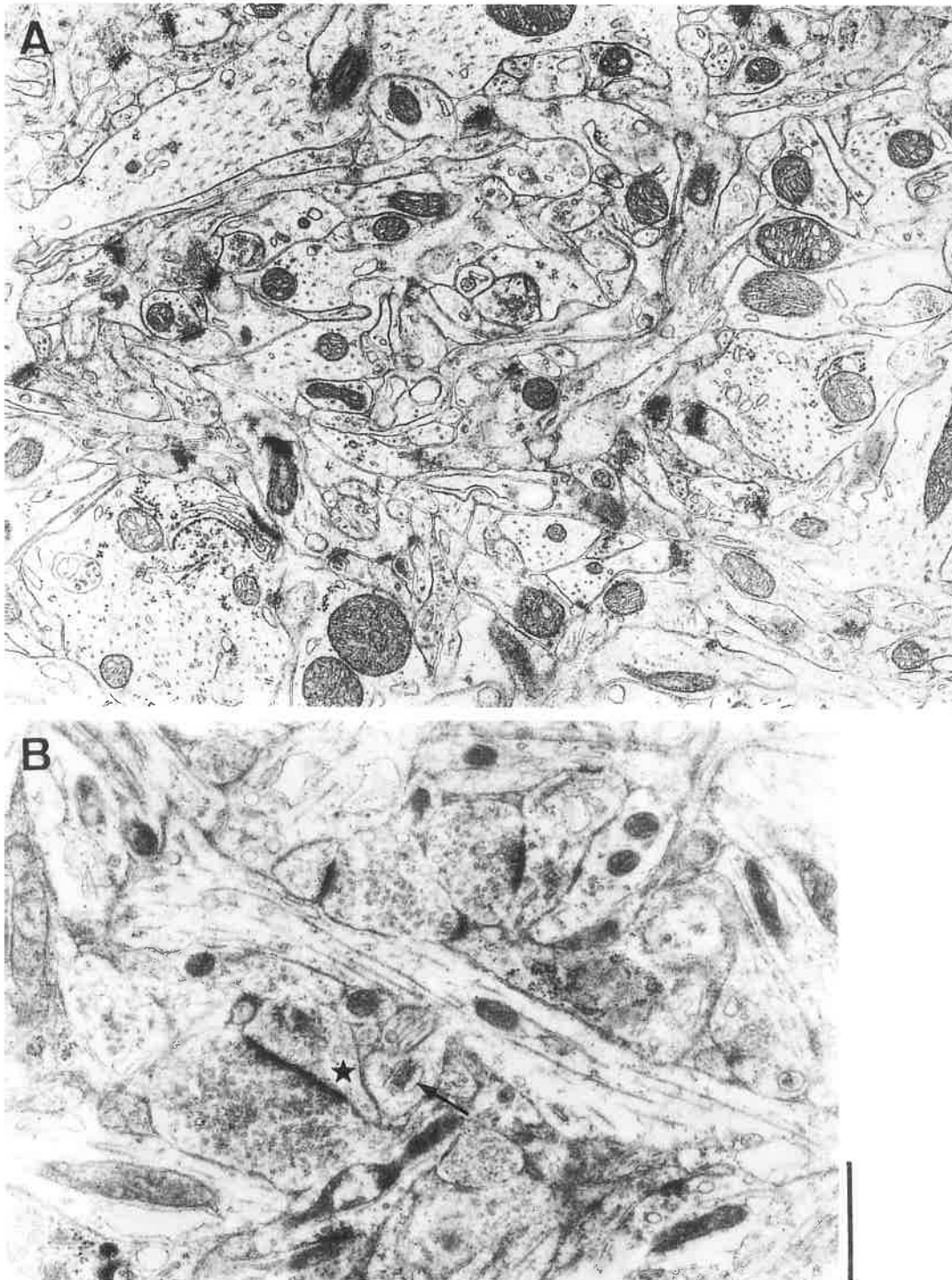


Fig. 6. Electron micrographs of the neuropil in situ (A) and in vitro (B) of corresponding age (P20) are shown at the same magnification. Note the difference in the size of the various profiles between both kinds of tissue. In (B) a spine apparatus can be seen (arrow). The asterisk marks an example of a flat synapse. Bar: 1 μm .

In summary, we can say that the fine structure of the neocortex in vitro had reached a degree of maturity very close to that in situ and that in most respects the two kinds of tissue did not differ from each other.

However, a more detailed comparison revealed some interesting quantitative differences regarding density, location and shape of synapses (Table I).

The most striking difference concerned the size of the synapses. The synapses in culture were often very large and very rich in vesicles (Figs. 5b, 6b). Synapses with a postsynaptic thickening of a length of 0.7 μm and above were frequent in culture but hardly found in situ.

Not only the synapses but the dendritic and axonal profiles on the whole tended to be larger in vitro than in situ (Figs. 6a and b).

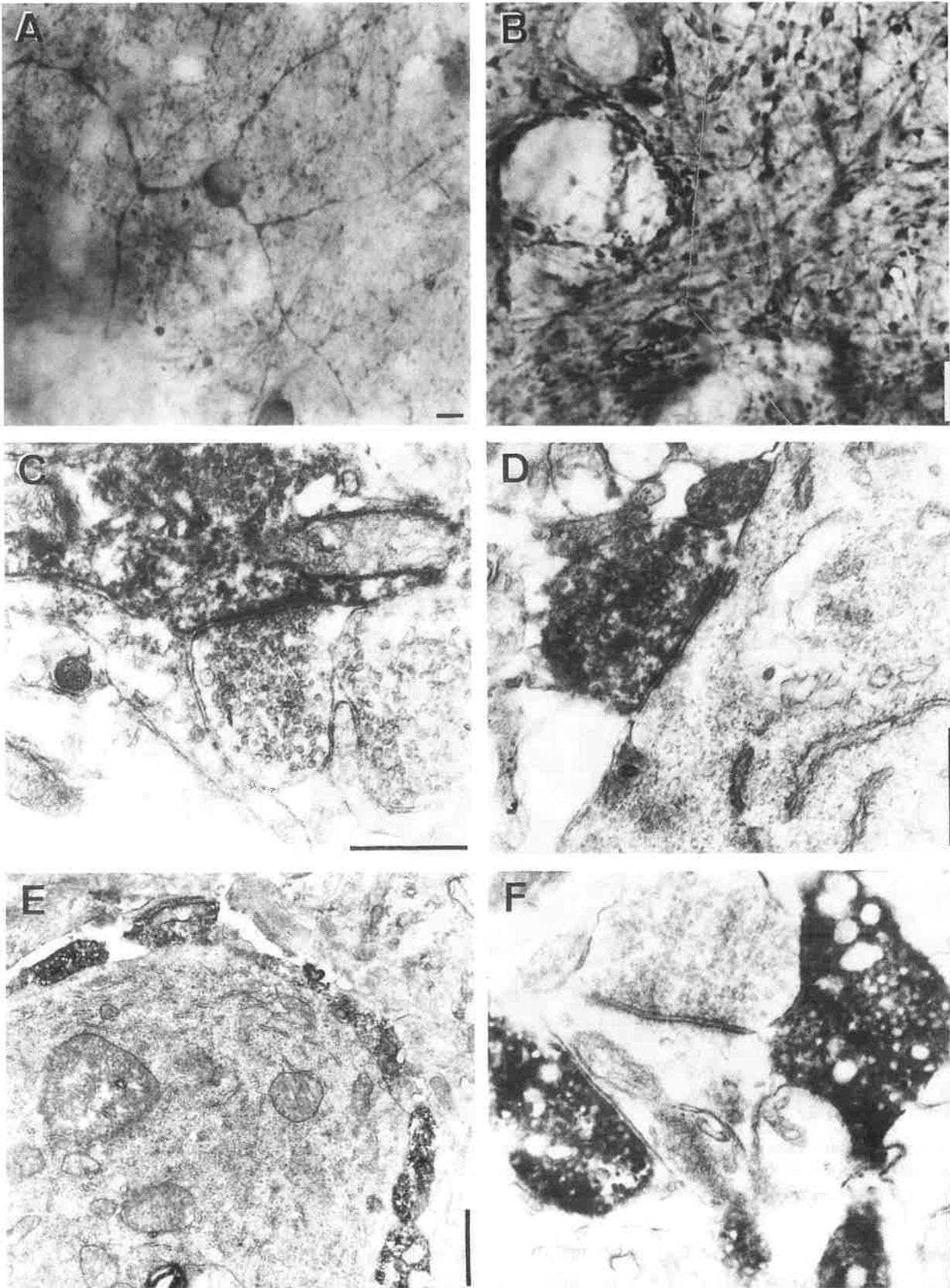


Fig. 7. (A and B) Lightmicrographs of GABA-immunoreactivity in neocortical slice cultures. Bars: 5 μ m. (A) Multipolar GABA-immunoreactive neuron. (B) High-power light-micrograph, demonstrating GABA-ergic puncta surrounding non-immunostained cells, presumably pyramidal cells. (C to F) Electron micrographs. (C) GABA-immunoreactive dendrite receiving an asymmetric synaptic contact from a non-immunostained profile. Bar: 0.5 μ m. (D) Immunostained profile making a symmetric contact onto a non-immunostained cell body. Bar: 0.5 μ m. (E) GABA-ergic synaptic terminals surrounding a non-immunostained cell body. Bar: 1 μ m. (F) Dendritic profile receiving a symmetric contact from a non-immunostained terminal and presumably also a symmetric contact from a GABA-immunostained terminal. Bar: 0.5 μ m.

Since a larger size of profiles implies a lower density of elements per area, it was not surprising to find that the density of synapses *in vitro* did not quite reach that *in situ*. In the rats, we counted an average of 26.3 synapses/100 μm^2 , while in the cultures it were 18.7 synapses/100 μm^2 , with no overlap between the two groups. The difference may be assumed to be even larger in reality because of the larger size of synapses *in vitro* which makes them dip into the section from a larger distance than *in situ*.

Another difference *in vitro* was that in slice cultures, dendritic shafts (Fig. 5a) and cell bodies (Fig. 5c) were often densely covered with synapses, while *in situ* the vast majority of synapses is located on dendritic spines. Our *in vitro* material showed only 52% of the synapses to be situated on spines while *in situ* the percentage was 78% and thus close to former counts in the mouse cortex (SCHÜZ and PALM, 1988).

A further difference was noted with respect to the curvature of the synaptic membranes (Table I), which is of interest since this feature has been related to the stage of maturation of synapses (DYSON and JONES, 1980) and to the amount of external stimulation. Synapses can be "presynaptically convex", meaning that the postsynaptic element is indented by the presynaptic terminal (e.g. Figs. 4a*, d), or they can be "presynaptically concave", meaning that the presynaptic element is indented by the postsynaptic one (Fig. 4a, arrow). In "flat" synapses the pre- and postsynaptic membranes form a straight line (Fig. 6b). In our *in vitro* model, 30% of synapses were of the presynaptically convex type, whereas *in situ*, only 12% showed this anatomical feature. In both kinds of material about 50% of the synapses were of the flat kind.

Table I

	<i>in vitro</i>	<i>in situ</i> *
density of synapses per 100 μm^2	18,7	26,3
synapses on spines	52 %	78 %
presynaptically convex synapses	30 %	12 %
flat synapses	52 %	51 %

GABA-immunohistochemistry

Most non-pyramidal cells in the neocortex are known to be GABA-ergic (e.g. HOUSER et al., 1984). In order

to gain more information about these cells *in vitro* we applied GABA-immunohistochemistry to the cultures. As described in an earlier paper (Caeser et al., 1989), various types of GABA-ergic neurons could be observed in all cortical layers at the light microscopic level. A typical GABA-immunoreactive multipolar cell is shown in Fig. 7a. Bipolar cells, fusiform cells and cells with horizontally oriented dendrites could also be observed. As *in situ*, numerous GABA-immunoreactive terminals could be seen, forming dense pericellular plexuses around non-immunoreactive cell bodies, presumably pyramidal cells (Fig. 7b).

In the electron microscope, GABA immunolabeling could be detected in perikarya, dendrites, axons and synaptic terminals. In GABA-immunoreactive perikarya (not shown here) the precipitate of the reaction product was distributed diffusely in the cytoplasm. GABA-immunoreactive dendrites (Fig. 7c) were always spineless and received non-immunostained asymmetric synaptic contacts and occasionally immunostained symmetric synaptic contacts. As *in situ*, GABA-immunoreactive terminals formed symmetric synaptic contacts. They were located on perikarya (Fig. 7d) and dendrites (Fig. 7f) of non-immunostained nerve cells, presumably pyramidal cells. Corresponding to the observations in the light microscope (Fig. 7b) GABA-ergic terminals surrounded non-immunostained cell bodies, forming multiple synaptic contacts (Fig. 7e).

Discussion

At postnatal day 6, the time of explantation, the main cell types of the neocortex have already formed, but still exhibit a very immature morphology (Fig. 1; PARNAVELAS et al., 1978; JURASKA and FIVKOVA, 1979; MILLER, 1981; MILLER and PETERS, 1981; BLUE and PARNAVELAS, 1983; MILLER, 1988). Our study shows that both pyramidal and non-pyramidal cells differentiate and mature *in vitro* (Figs. 2, 3). In most respects, the slice cultures (Figs. 3–5) cannot be distinguished from the tissue *in situ* of corresponding age (Figs. 2, 4–6). This is true for both the light and electron microscopical appearance and is in accordance with results already reported (CAESER and SCHÜZ, 1989; CAESER et al., 1989; WOLBURG and BOLZ, 1991). Similar observations can be made in explant cultures of the neocortex using the Maximov technique (SEIL et al., 1974) and in hippocampal slice cultures (ZIMMER and GÄHWILER, 1984; FROTSCHER and GÄHWILER, 1988; FROTSCHER et al., 1990; CAESER and AERTSEN, 1991). This indicates that differentiation and maturation of the neocortex is controlled by intrinsic properties to a large extent.

In contrast to results obtained in explant cultures, the orientation and morphology of neocortical cells in dissociated cultures is clearly different from that observed *in situ* (DICTER, 1978; KRIEGSTEIN and DICTER, 1983; HÜTTNER and BAUGHMAN, 1986). In dissociated cultures, cells do not show the same degree of differentiation and it is rather difficult to recognize the various cell types. This can partly be explained by the different culturing procedure and by the earlier time of explantation. Results obtained on isolated cells *in vitro* have demonstrated, that neurons of the central nervous system develop dendritic and axonal processes on the basis of their genetic capacity (DOTTI and BANKER, 1987; DOTTI et al., 1988), but that additional factors appear to be necessary for a more complete maturation of cells and fine tuning of synaptic contacts. For instance, co-cultivation of neurons and astrocytes improves the survival and maturation of hippocampal cells *in vitro* (BANKER and COWAN, 1979; BANKER, 1980), presumably due to biochemical factors, a release of growth factors or cell-to-cell interactions. This fits to findings of proliferation and differentiation of glia cells in slice cultures (STEINDLER and HARRINGTON, 1987; CAESER and AERTSEN, 1991; DEL RIO et al., 1991). Furthermore, the functional development of neocortical circuitry appears to be improved when neocortical tissue is co-cultivated with subcortical regions (LEIMAN and SEIL, 1986).

There is evidence that not only the morphological but also the neurochemical differentiation is largely determined by the intrinsic capacity of the tissue and occurs without major contribution of afferent and afferent fiber connections and incoming sensory information. To summarize, neurotransmitter substances generated in the neocortex *in vitro* are GABA (DE JONG et al., 1987; CAESER et al., 1989; ANNIS et al., 1990), glutamate (ANNIS et al., 1990) and vasoactive intestinal polypeptide (VIP; GÖTZ and BOLZ, 1990). Furthermore the transmitter synthesizing enzymes choline acetyltransferase (ChAT; ANNIS et al., 1990) and tyrosine hydroxylase (TH; OSTERGAARD et al., 1991) can be detected *in vitro*.

Although the similarities between the slice cultures and the tissue *in situ* are impressive, a closer look also revealed some differences on the electron microscopic level. Some of them may have trivial reasons. For example, the larger size of dendritic and axonal profiles *in vitro* might be due to the fact that — unlike tissue *in situ* — the cultures have plenty of space to expand. It is also clear that the lower density of synapses *in vitro* is, at least in part, simply a reflection of the resulting lower density of profiles.

An interesting finding is, however, the lower ratio of axo-spine synapses as compared to axodendritic synapses in culture. We can only speculate on this

phenomenon. There is strong evidence that spines grow out at points where a synapse has already been established on the dendritic shaft (COTMAN et al., 1973; WESTRUM et al., 1980; MILLER und PETERS, 1981; MATES and LUND, 1983; SCHWEIZER, 1990; SCHWEIZER and SCHÜZ, 1990). It may be that the second step — the outgrowth of a spine — is not as readily performed under the condition of a slice culture and that the culture is delayed in maturation with respect to this property.

It has been argued that the curvature of the synaptic junction might be affected by the amount of external stimulation. More synapses with a presynaptically concave curvature have been found under the condition of enriched environment (WENZEL et al., 1977; WESA et al., 1982). The synapses in culture speak in favor of this assumption. Synapses with a presynaptically convex curvature were more frequent in the cultures than *in situ*. This seems to be even more the case in cultures treated with TTX (VAN HUIZEN et al., 1987), indicating that curvature might be related to the activity of the synapse. This assumption has also been made on the basis of developmental studies (DYSON and JONES, 1980).

Thus, the structure of neocortical tissue is somewhat affected by the rearing conditions in a slice culture. It is an open question whether these differences on the synaptic level are simply due to the absence of afferent fibers or the result of a complete deprivation of external stimuli. Co-cultivation of neocortical explants with subcortical regions, for instance the thalamus (YAMAMOTO et al., 1989; BOLZ et al., 1990; MOLNAR and BLAKEMORE, 1991), may provide an answer to this question.

Another point to be discussed is the use of slice cultures for physiological and pharmacological studies. Due to their flatness and organotypical organization they lend themselves well to various histological and electrophysiological techniques. They are, for example, ideal for the investigation of the spatio-temporal spread of excitation using the optical recording technique (CAESER and AERTSEN, 1989). Furthermore, the longevity of these kinds of cultures makes them an attractive model for studies of neurodegenerative diseases. Survival times of more than one year have been observed (STAIGER and AERTSEN, unpublished results). The far-reaching similarity in structure between the slice cultures and the tissue *in situ*, deviating only slightly from each other in some quantitative measures, supports the view that slice cultures are a good *in vitro* model for the neocortex.

Certainly, many interesting questions still remain open with respect to connectivity within a slice culture, for example the question of the length of feedback loops. An individual neuron in culture no doubt

connects to a larger percentage of the neurons in the whole network than is the case in the cortex. The culture is thus closer to the situation of a fully connected network than even the smallest cortex is. However, this is not so clear when comparing a slice culture to a small piece of cortex, corresponding in size to a slice culture. For such a comparison it would be relevant to know in more detail how the culturing procedure influences the growth of the dendritic and axonal trees and thus the number of neurons an individual neuron can reach. As we have observed, the dendritic trees tend to be less dense but somewhat more extended in culture. In the more two-dimensionally arranged culture, this may be an attempt to maintain the high number of different synaptic neighbours a pyramidal cell has in situ (BRAITENBERG, 1978). It would be even more important to know whether the severing of the long-range connection of a pyramidal cell is compensated by a sprouting of the local axonal tree within the culture. This could lead to a higher degree of connectedness than in a volume of cortex of similar size in situ. Such a sprouting could also lead to more multiple contacts and therefore to a stronger connectivity between individual neurons. Strong connectivity between individual neurons might be an explanation for the high spontaneous activity which has been observed in some neurons in slice cultures (CAESER et al., 1989).

Such considerations on connectivity may also offer an explanation for the somewhat puzzling finding of conspicuously large synapses rich in vesicles in culture. There had been reason to assume that such synapses are the result of external stimulation and perhaps learning (WEST and GREENOUGH, 1972; GAREY and PETTIGREW, 1974; VRENSEN and DE GROOT, 1974; DIAMOND et al., 1975; SCHÜZ, 1981). At first sight, the occurrence of such synapses in culture cells this hypothesis in question. However, if the neurons are connected more strongly onto each other in vitro than in situ, HEBB's rule (1949) of coincident activity may often be fulfilled in spite of a complete lack of external stimulation and a kind of internal "learning" may occur.

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