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On the Development of Non-Pyramidal Neurons and Axons Outside the Cortical Plate: The Early Marginal Zone as a Pallial Anlage

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Summary. The development of non-pyramidal neurons was studied in the pallium of albino rats using autoradiography after thymidine labelling (determination of "birth dates"), Golgi impregnations (differentiation of dendrites and axons) and electron microscopy including 3D-reconstructions (cytoplasmic differentiation and early synaptogenesis).

The marginal zone appears between E13 and E14 and contains glial cells, axons and preneurons from the beginning. The latter can be identified by structural criteria (contacts, cytoplasm, nuclei). The first vertically oriented pyramidal neurons (cortical plate) appear within the marginal zone not before E16, separating its contents into a superficial (lamina I) and a deep portion (intermediate and subventricular zone). Since this old neuronal population of lamina I and the subcortical pallial region can be followed until adulthood, it is proposed to call the early marginal zone a "pallial anlage". It can be demonstrated that during the whole period of neuron production (until E21) non-pyramidal neurons are added to all parts of the "pallial anlage".

The structural differentiation of non-plate neurons is described. Neurons form specific, desmosome-like contacts with axonal growth cones already on E14. Typical synapses (vesicle aggregations) have been observed two days later. In lamina I two types of neurons develop: horizontal neurons (Cajal–Retzius cells) and multipolar neurons (small spiny stellate cells). Subcortical pallial neurons retain mostly their clear horizontal orientation. Only neurons situated very close to the lower border of the cortex show dendritic branches extending into lamina VI. Axons appearing early in the neocortex originate not only from subcortical regions, but also from neurons of the paleopallium, the archicortex, the limbic cortex and the neighbouring neocortex. The tangential growth of the neocortex, as estimated from E14 onwards causes a strong dilution of the elements of the "pallial anlage" until adulthood.

The classification of neurons outside the cortical plate and the fate of the total "pallial anlage" are discussed. As a consequence of these observations some modifications of the terminology of the Boulder Committee are proposed.

Key words: Development – Cerebral Cortex – Neurons – Axons.

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Introduction

Until recently the development of the neocortex could have been summarized as follows:

1. Before cortical development there is a short period, during which a marginal zone begins to separate from the ventricular zone, but does not contain cells of its own. This early marginal zone would then represent an a-neuronal precursor of the cortical development (stage B after Boulder Committee Terminology, 1970).

2. All cortical neurons are derived from proliferating ventricular cells and migrate along the vertical processes of radial glial cells to find their final position in the cortical plate (see Rakic, 1972).

This migration is always stopped at the border between the cortical plate and lamina I. Consequently, old cells are positioned in deep layers, while the later migrating young neurons form the superficial laminae ("inside-out layering", Berry and Rogers, 1965; Hicks and D'Amato, 1968; Rakic, 1974).

In addition to this laminar gradient, there are regional temporo-spatial gradients, according to which neurons are for instance formed earlier in the lateral than in the medial parts of the neocortex.

3. The differentiation of neurons follows similar patterns, i.e. neurons in deep layers and in lateral cortical regions differentiate earlier than superficially and medially situated ones (Sidman and Rakic, 1973; Peters and Feldman, 1973; Raedler and Sievers, 1975).

The above scheme, though applicable in part to pyramidal cell development seems not to hold for non-pyramidal neurons in the cortex. The few data available suggest that at least some non-pyramidal neurons are formed, before the cortical plate appears. Later on, these oldest cells are located in lamina I as well as in deep lamina VI and even in the subcortical white matter (see Wolff, 1978; rat). The so-called Cajal–Retzius cells belong to this preplate population of neurons (Raedler and Sievers, 1975; König *et al.*, 1977: rat). They differentiate early and are regarded as cortical interneurons (Marin-Padilla, 1971). These findings contradict the general statements on a late development or differentiation of local circuit neurons, as related to efferent neurons (Jacobson, 1975; Rakic, 1975).

The present study was initiated to investigate early developmental stages and the fate of unequivocally identifiable non-pyramidal neurons residing outside the cortical plate.

Materials and Methods

The cortical development was studied in Sprague Dawley rats from the 13th day of gestation onwards. In this type of work it is important to define clearly the stages of embryonic development. Embryo day one (E1) was specified as the day sperm was first found present in a vaginal smear (in agreement with: Atlas and Bond, 1965; Hicks and D'Amato, 1966; McAllister and Das, 1977; Shimada and Langman, 1970; in contrast to: König *et al.*, 1977, and Marin-Padilla, 1971, who count the day of mating as E1, and in contrast to: Sturrock, 1974; Hinds, 1968, Derer *et al.*, 1977; and Raedler and Sievers, 1975, who count the day a sperm plug is found as E0). By this system birth takes place on E23 which is synonymous with postnatal day one (P1).

All tissue samples were transversely sectioned, till the interventricular foramina of Monroe were reached. These served as our common reference points from which cortical samples from various stages of development could then be selected for further analysis.

The terminology of the Boulder Committee (1970) was used as far as ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ) and the cortical plate (CP) are concerned. The term marginal zone (MZ) is redefined as a zone which contains non-pyramidal neurons and is the precursor

of the total pallium instead of lamina I (LI) only. The term "preneuron" is used instead of neuroblast, because these cells are not proliferating.

All the animals were anesthetized with pentobarbital-sodium. Before E18 the heads were immersed in the fixation medium. From E18 onwards the animals were fixed by intracardial perfusion. *Golgi preparations* were prepared by single and double rapid Golgi impregnations (Palay and Chan-Palay, 1974) or by the dichromate chloralhydrate method of Stensaas (1967a). The optimal time (2–3 days) of immersion in the dichromate solution varied with the developmental stage. The time in silver nitrate seemed to be less crucial. The brains were embedded in soft Epon (Epon 812 (20 ml); 2,4-6-tri(dimethylaminomethyl)phenol (0.6 ml); methylnadic anhydride (8 ml) and dibutylphthlate (12 ml)), and routine sections were cut at 70 µm.

Silver impregnations of adult neurons (Fig. 6a) were obtained by the following procedure: Deparaffinized 10 µm thick sections were incubated in an ammoniacal silver nitrate solution (4.5% NaOH, 8% NH_4NO_3, 7% $AgNO_3$) for 3 to 5 minutes. Rinsing in distilled water, 1% citric acid and 0.5% acetic acid each about 5 minutes. The subsequent physical development was stopped by 0.5% acetic acid, when the sections reached a brown colour. The developer consisted of equal parts of stock solutions A and B (A: 5% Na_2CO_3, B: 0.196% $AgNO_3$, 0.2% NH_4NO_3, 1% silicotungstic acid, 0.13% formalin).

For *electron microscopy* the animals were perfused with saline followed by the fixation procedure of Kalt and Tandler (1971). After osmification the tissue was embedded in Epon 812. Serial ultrathin sections were cut, collected on large hole Formvar coated grids and double stained with uranyl acetate and lead citrate (Reynolds, 1963).

On gestation days 13 to 21 pregnant females were injected intraperitoneally with 5 µ Ci/g body weight of tritiated thymidine (specific activity 2.0 Ci/mmol) and sacrificed after various survival times (see text). The prenatal stages were prepared according to the EM method above and sectioned at 2 µm. The postnatal stages were perfused with saline followed by 1% calciumchloride in 4% formalin, embedded in paraplast and sectioned at 10 µm. *Autoradiograms* were produced by dipping in Ilford K2 or K5 emulsion and developing in Kodak D19b after an exposure of three weeks. The prenatal material was poststained according to Richardson *et al.* (1960) and the postnatal material with cresylviolet.

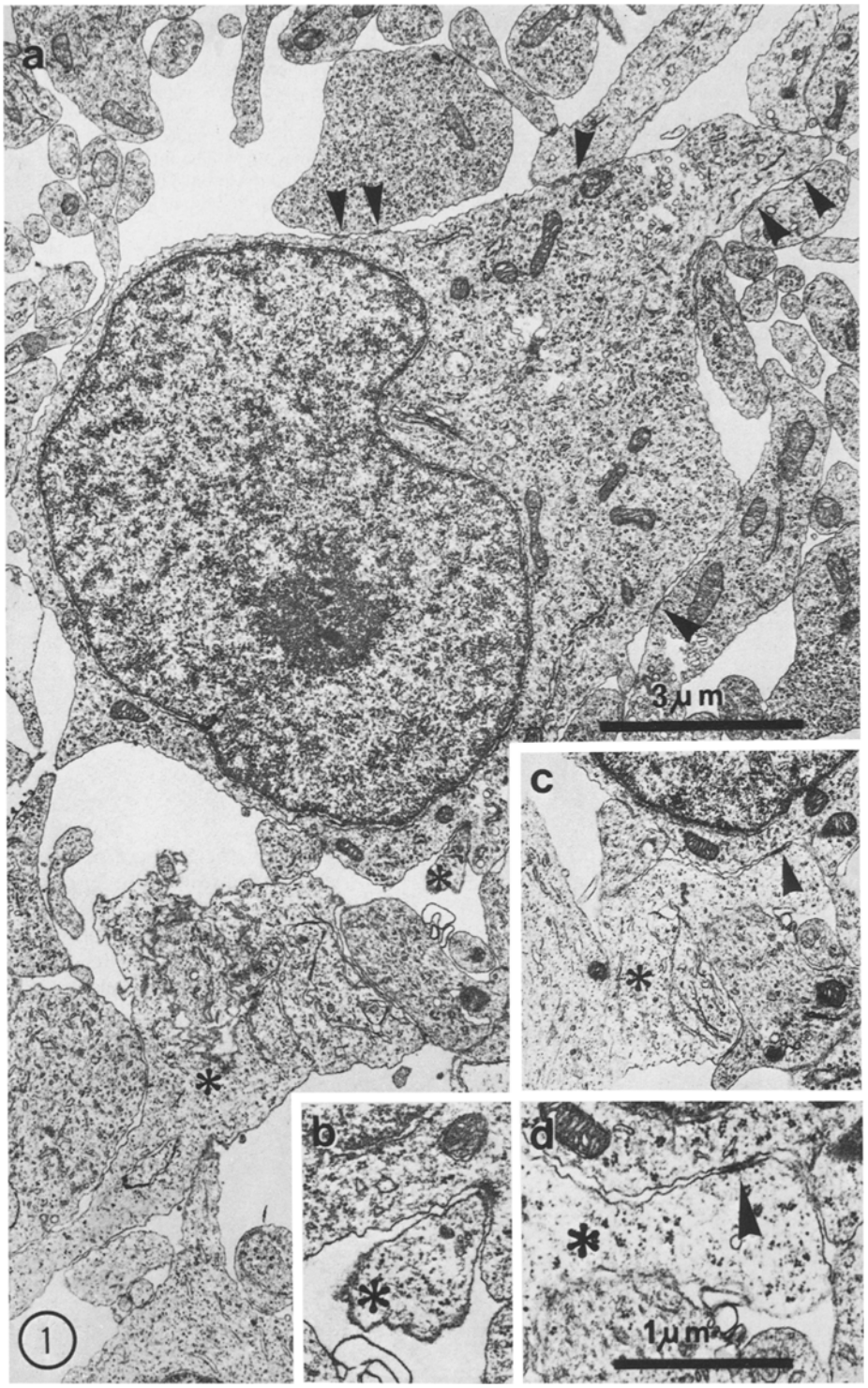
Results

1. The Marginal Zone—the Anlage of the Total Pallium

The marginal zone (MZ) of the paleopallium, the archicortex, the mesocortex (limbic cortex) and the neocortex appears between E13 and E14 following a homogenous latero-medial differentiation gradient. The MZ, lying outside the ventricular zone, consists of the marginal processes of ventricular cells, large intercellular spaces and the first differentiating cells of the pallium, which are horizontally oriented. This early MZ already contains preneurons and glial cells (Rickmann and Wolff, 1976). Therefore, the present description of the earliest neurons in the cortex was based on a clear distinction between neuronal and glial cells, including ultrastructural criteria

Table 1. Cytological discrimination between developing neuronal and glial cells

	Neuronal cells	Glioblasts
mesodermal contacts	absent	present
rough endoplasmic reticulum	narrow cisternae without contents	wide cisternae containing a dense granular matrix
chromatin	is continuously dispersed	remaining dense, or even further condensed


a
3 µm
c
b
d
1 µm
1

(Tennyson, 1965; Nossal and Radouco-Thomas, 1971; Pannese, 1974; Vaughn, 1969; Phillips, 1973; Sturrock, 1974; Wolff and Rickman, 1977) as well as EM-3D reconstructions (see Rickmann and Wolff, in preparation). The criteria contained in Table 1 show the most characteristic differences.

At late E14 immature dendrities of non-pyramidal neurons and axons were found in the MZ, sometimes forming desmosome-like contacts. These contacts may be formed even with axonal growth cones and may already contain a few small sized vesicles (Fig. 1). However, definite synapses which possessed a postsynaptic thickening as well as vesicles aggregated beneath the presynaptic membrane were found in our material at E16 for the first time. The axons emerging from neurons in the MZ belong to the least differentiated group of all axons we found. They can be distinguished from more mature axons by a greater number of free ribosomes and a more irregular diameter.

At least in the neocortex it is not before E16 that the first vertically oriented neuronal elements appear within the MZ. The strictly horizontally organized MZ precedes, therefore, the development of the corticotypic pyramidal neurons. Since this is true for the total pallium, the MZ may be called "pallial anlage". At E16 the primary uniform MZ is divided into a superficial part, the molecular layer or lamina I (LI), and a deep horizontally organized zone by the interposition of the cortical plate (CP). The best evidence for this division is provided by *autoradiography*. When 3H-thymidine was injected at E14, fully labelled cells were found in the MZ 30 h later (Fig. 2a). When 3H-thymidine was injected before E16 and the animals survived till after the formation of the CP, one finds fully labelled neurons in LI as well as in the level below the CP (Fig. 2b, c, d). Thus, the CP is bordered at both sides by elements of the "pallial anlage". Our results agree with the statement of König *et al.* (1977) that the maximal formation of Cajal–Retzius cells takes place at E14 (his E13) and that there is a decreasing formation of these cells at E15. But in addition, we found a continuous labelling of neurons of LI until E21 (Fig. 2f). Neurons being born before E16 lie in the upper half of the adult LI, while the majority of latecomers occupy its lower half. There is also a continuous addition of neurons to the cell population below the CP until E21 (Fig. 2e). Thus, the "pallial anlage" only contributes the oldest elements to this inhomogeneous population of neurons above and below the CP (non-plate neurons).

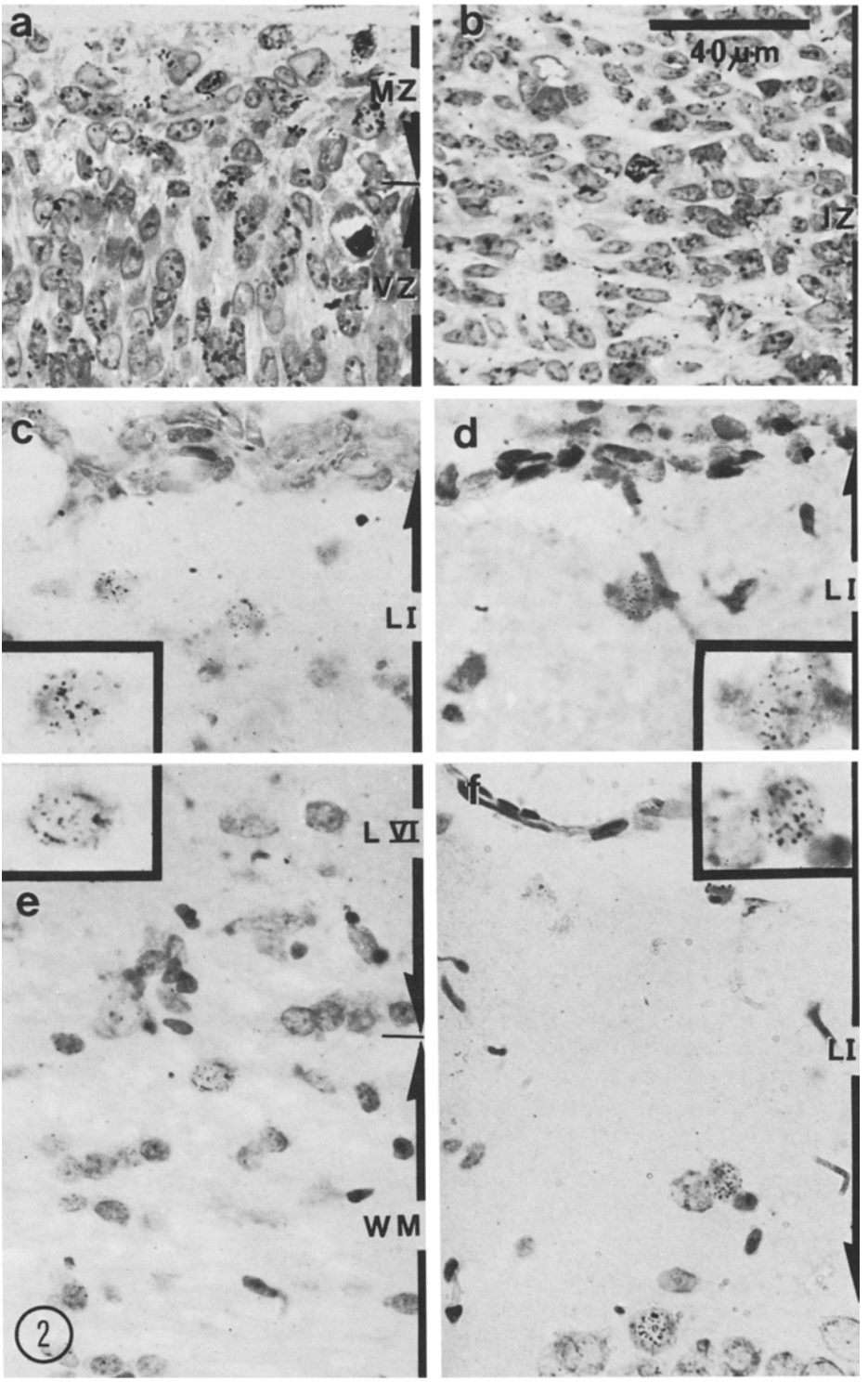
2. *Structural Characteristics of Non-Plate Neurons*

Golgi impregnations show that during embryonic stages mono- or bipolar perikarya as well as horizontally oriented dendrites and axons are common characteristics of the non-plate neurons in all regions of the pallium.

2.1. Lamina I Neurons

Two main types of neurons develop later in LI: (1) Horizontally oriented neurons, mono- or bipolar, and (2) multipolar neurons with dendrites extending in all directions.

Fig. 1a–d. Marginal zone of the neocortex at E14. A preneuron forms numerous close contacts (arrows) and one desmosomal contact with an axonal growth cone (asterisks **a** to **d**). The desmosomal contact shown at higher magnification in **b** is adjacent to a close contact in another plane of sectioning (**c** and **d**)


a
MZ
VZ
b
40 µm
IZ
c
LI
d
LI
e
L VI
WM
2
f
LI

2.1.1. Horizontal Neurons with a dendritic tree which is more extended than that of other neurons at the same medio-lateral position are abundant during the embryonic stages (Fig. 3a) in the *neocortex* and correspond to Cajal–Retzius neurons. The dendrites may extend for more than 250 μm parallel to the pial surface. The axons may emerge as well from the perikarya as from dendrites. However, there is EM-evidence also for "axon-like dendrites" (see below). The very long axons are horizontally oriented and bear collaterals and growth cones. Both axons and dendrites stay within LI. In tangential sections (E18 and adults) the dendrites of Cajal–Retzius cells show no preferential direction. The neuronal population seems to be inhomogeneous with respect to the dendritic length (Fig. 4). In postnatal stages up to P8 the Cajal–Retzius neurons are still relatively abundant. However, now the pyramidal neurons show more developed dendrites than the Cajal–Retzius cells. Towards adulthood, the Cajal–Retzius neurons become very rare. This does not essentially mean that they degenerate, because they are strongly "diluted" (see Table 2). In our preparations we do not find the extensive, comb-like ramifications of dendrites and axons extending towards the pia as described by Cajal (1891) and Retzius (1893, 1894) in man, rabbit, dog and rat, although the neuron in Figure 3b shows some of these features.

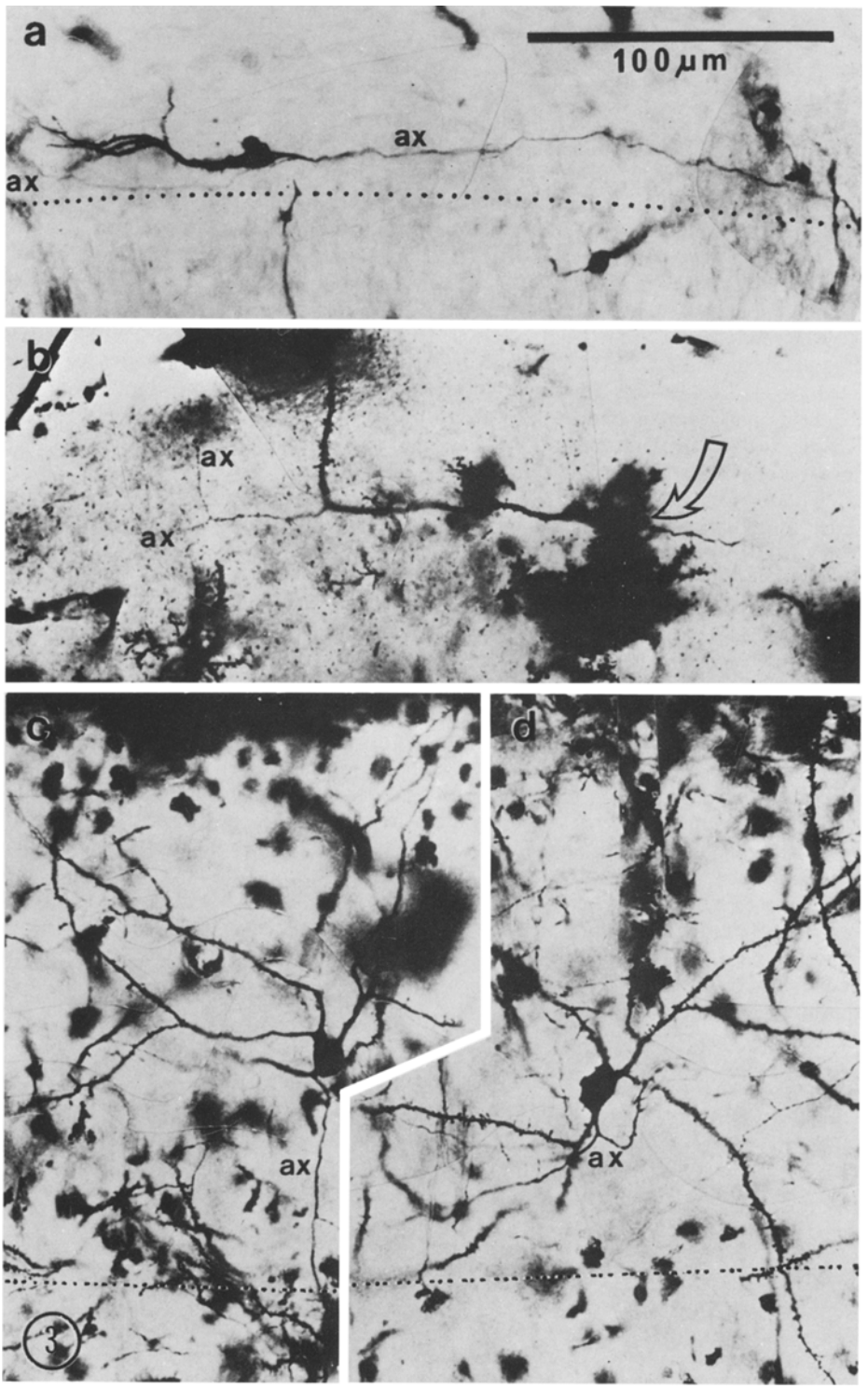
The *ultrastructure of neurons in lamina I* at E18 implies that the degree of their differentiation is inhomogeneous (Fig. 5a and b). One neuron being reconstructed from thin sections rather completely showed several horizontal dendrites varying in diameter, very thin axon-like dendrites as well as irregular cytoplasmic protrusions arising from the perikaryon and from dendrites. Several well developed synapses were present on the perikaryon and proximal dendrites. Only one axon was found emerging from the perikaryon which could be identified by a typical structure resembling that of adult initial segments (Fig. 5c, Paly *et al.*, 1968). The shape of our reconstructed neuron resembled that of Golgi impregnated Cajal–Retzius neurons in the neocortex. For further details see Rickmann and Wolff (in preparation).

2.1.2. In contrast to Cajal–Retzius neurons, horizontal cells in the lower part of LI transform into *multipolar neurons*, because their spiny dendrites tend to extend in all directions after P4 (Fig. 3c, d). Their position is identical with the position of the late labelled neurons in autoradiography (see above). Their axons and dendrities may descend into lamina II (Fig. 3d). All these criteria allow them to be classified as small "spiny stellates". Tangential sections of silver impregnated adult material (Fig. 6a) show numerous neurons in LI, lying in clusters which, however, do not seem to coincide with the underlying columns of pyramidal neurons. Most of the neurons in the tangential sections have to be related to the small spiny stellate neurons of the neocortex.

In the *limbic cortex*, the perikarya of the superficial neurons are situated deep in LI (Fig. 7b), although the dendrites and axons are horizontally oriented and stay within this lamina. Their axons preferentially run towards the neocortex.

In the *paleopallium* the corresponding neurons again occupy the deep part of LI (Fig. 7e). The axons run within LI also towards the neocortex.

Fig. 2a–f. Autoradiograms of coronal sections after labelling with 3H-thymidine (injection day/day of sacrifice). *E* = gestational day. *IZ* = intermediate zone. *LI* = lamina I, *LVI* = lamina VI, *MZ* = marginal zone, *P* = postnatal day, *VZ* = ventricular zone, *WM* = white matter. **a** to **d** *early labellings:* **a**) E14/E15: the early MZ contains labelled neurons and glial cells, **b**) E14/E18: neuron in the IZ, **c**) E15/P7 and **d**) E15/P7: neurons in LI, **e**) *and* **f** *late labellings:* **e**) neuron in the white matter E18/P10, **f**) one neuron in LI and another at the top of lamina II E20/P21. Insets showing labellel cells at twice the magnification demonstrate that the grain density of the evaluated cells is about the same


a
100 µm
ax
ax
b
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ax
c
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3
d
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2.2. Subcortical Pallial Neurons

From E17 onwards we found neurons in the subventricular zone (Fig. 7c) and intermediate zone (IZ) and later in the white matter of the *neocortex* (Fig. 7d, compare Fig. 8a–c). These neurons may form the postsynaptic elements of synapses appearing below the cortical plate prenatally (man: Molliver *et al.*, 1973; rat: Wolff, 1978), and we refer to them as subcortical pallial neurons. Neurons are scarce in the subventricular zone. Figure 7c shows an example with a horizontal dendrite and an axon traversing the subventricular zone and reaching the IZ.

Neurons high up in the IZ have a more pronounced horizontal arrangement with their main dendrites running parallel to the border between the cortical plate and the IZ (Fig. 8c). Neurons lying very close to the lower border of the cortex often have vertical dendritic branches extending into lamina VI (Fig. 8b). In general, the axons are horizontally oriented and stay below the cortex. During embryonic stages, the subcortical pallial neurons have relatively well developed dendritic branches (Fig. 8c), while at later stages the neurons of the white matter have fewer dendritic branches than the pyramidal neurons of the overlying grey matter (Fig. 8a). However, the horizontal extension of the dendrites seems to increase in parallel with the tangential growth of the cortex (Fig. 8a to c). Comparisons of coronal and tangential sections of silver impregnated material of the adult cortex show that subcortical pallial neurons are relatively numerous in the white matter (Fig. 9). They appear singly or in clusters (Fig. 6b) similarly as in lamina I.

Electron microscopy confirms the presence of neurons in the embryonic subventricular zone (Fig. 5d). Their perikarya are wrapped into glial sheaths, similar to the class of small neurons observed in the raphe nuclei, as described by Fox *et al.*, (1976). We do not yet know whether these subventricular cells are identical to the just described neurons with well developed dendrites of the Golgi preparations and whether they possess any synaptic contacts.

In the *limbic cortex* the perikarya of the early subcortical pallial neurons are situated close to the lower border of the cortical plate (Fig. 7a). Their dendrites and axons descend in the IZ and take a lateral course towards the neocortex.

In the deep part of the *paleopallium* the neurons are not so regularly arranged, and unequivocally horizontally oriented neurons are rare (Fig. 7f).

3. Early Axons

The findings on early axons presented here are mainly based on rapid Golgi impregnations of E17. Single axons could be traced backwards to their perikarya, as the neuronal system is still relatively simple and as only a few neurons are impregnated.

Table 3 summarizes the presumed sequence of appearance of these axons in the neocortex till E17. The axons of the groups (2) to (7) seem to arrive in the neocortex within one day. We cannot exclude that some fibres in LI (4) and (5) do not terminate within the neocortex, but traverse it.

Fig. 3a–d. Coronal sections of Golgi preparations of the neocortical lamina I. Dotted lines show the lamina I/lamina II border, axons and collaterals (*ax*). **a)** Cajal–Retzius neuron at E19 with one vertically oriented dendritic branch and an axon with a collateral, **b)** Cajal–Retzius cell of P18 with a vertical spiny dendrite, an axon with a collateral and the perikaryon partly concealed (arrow), **c)** to **d)** small spiny stellate neurons of P18, in **c)** the axon descends into lamina II

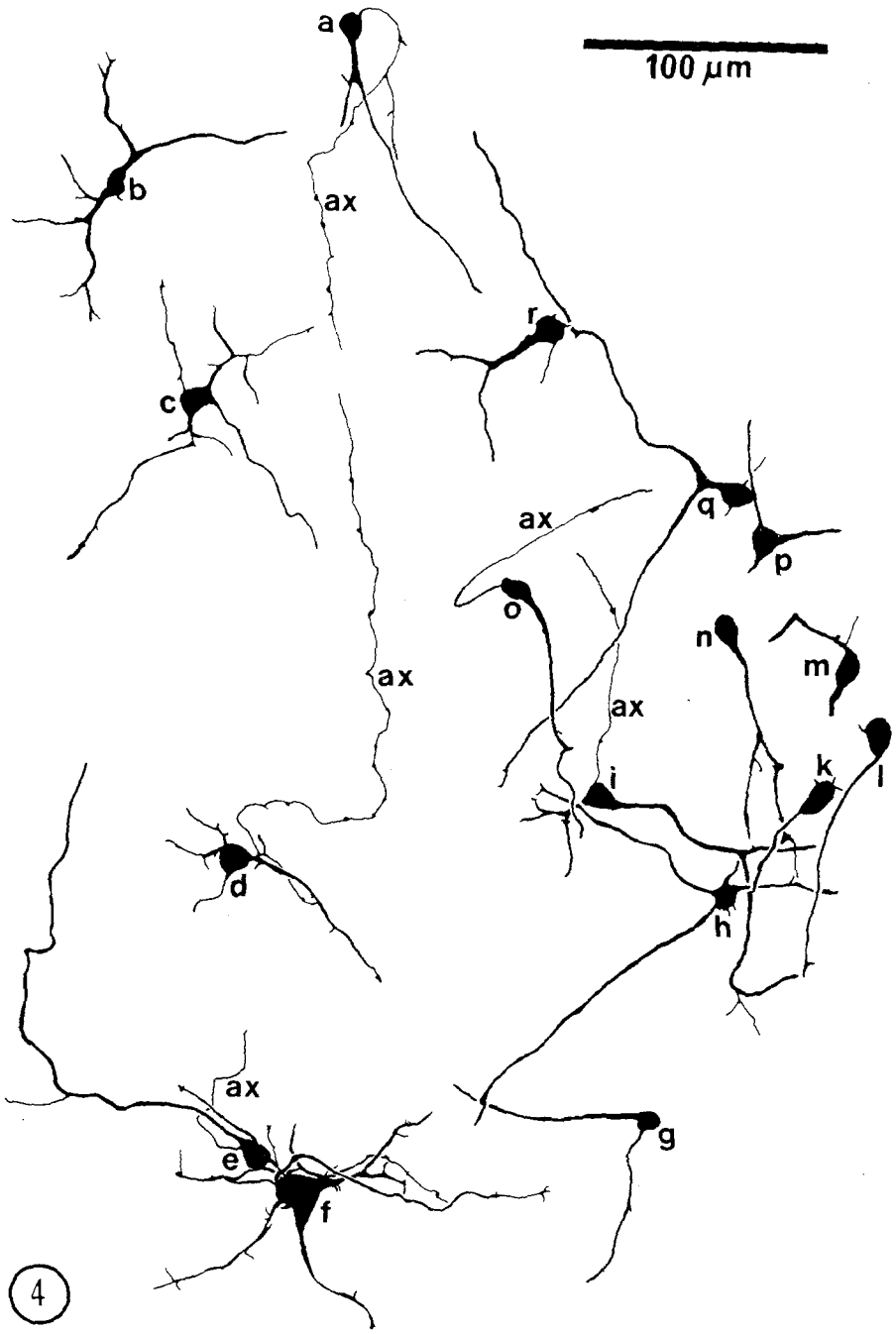


Fig. 4. Golgi impregnated Cajal–Retzius neurons, drawn from thick tangential sections of different parts of the cortex at E18, processes identified as axons (*ax*). Cells located well within the neocortical borders (*a* to *d*), cells from the neocortical to limbic border (*e*, *f*), the cluster of cells (*g* to *r*) was contained in a single section through the occipital pole

Table 2. Neocortical surface increase till adulthood

Developmental events in the neocortex	Time of ontogenesis	Estimated magnification of the neocortical surface till adulthood
arrival of the first afferents	early E14	36 : 1
most frequent birth date of neurons of lamina I	late E14	24 : 1
deposition of the first pyramidal neurons	E16	14 : 1
deposition of the last neurons	P1	6 : 1

Three aspects of the fibres listed in Table 2 ought to be mentioned separately: Axons from the caudal thalamus (1) are probably the first subcortical afferents to the "pallial anlage" and possess a peculiar course through the CP during later development (Fig. 10). Axons which run below the CP except the latest (8) do not cross the neocortical borders, while axons in LI do so. The later the time of arrival, the deeper the axon seems to run within the intermediate zone (Fig. 11).

Structural differerences between axons were found in the neocortical LI. Long axons from limbic neurons (5) have a thick diameter, a straight course and nearly no varicosities. In contrast, fibres from neocortical neurons in LI (7) are rather thin, possess a more irregular course and show many varicosities.

4. Tangential Growth of the Neocortex

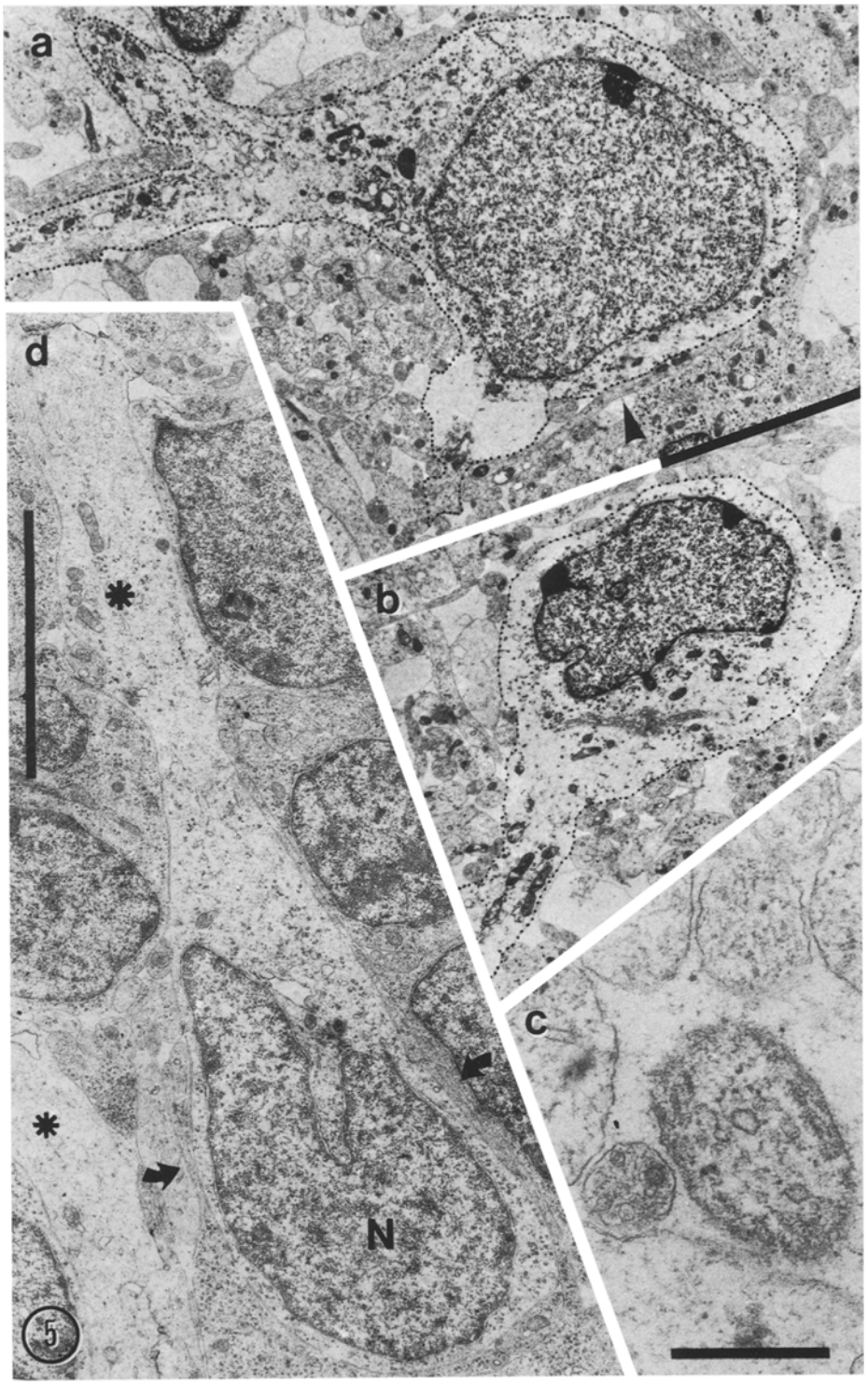
Since the tangential growth must have a strong influence on the packing density of neurons and axons, we estimated roughly the growth of the neocortical surface.

From E20 onwards we calculated the neocortical surface directly from measurements of the cortical circumference in serial sections. Before E20 in corresponding coronal sections the lengths of the neocortical surface were measured and taken to the square (E18 to E20: between the neocortical borders, E14 to E18: from the limbic to neocortical border to the edge of the basal ganglia). These sets of data were step by step calibrated starting with a calibration referring to data on the postnatal development of the neocortical surface (Eulner, Wolff, Bär, unpublished).

The results as given in Table 2 show that the earliest neuronal elements of the pallial anlage are very diluted by adulthood.

Discussion

There are numerous observations on the structure and development of neurons in lamina I (e.g. Retzius, 1893, 1894; Cajal, 1891, 1959; Noback and Purpura, 1961; Åström, 1967; Raedler and Sievers, 1975; König *et al.*, 1977). On the other hand, neurons have been observed to exist also below the cortical plate, i.e. the precursor of lamina II to VI (see Boulder Committee, 1970). Cajal (1906) and Åström (1967) found polymorphous or stellate neurons in the subpyramidal regions, and the Boulder Committee (1970) states that immature neurons are present in the intermediate zone. Drawings, but no descriptions, of such cells have been contributed


a
b
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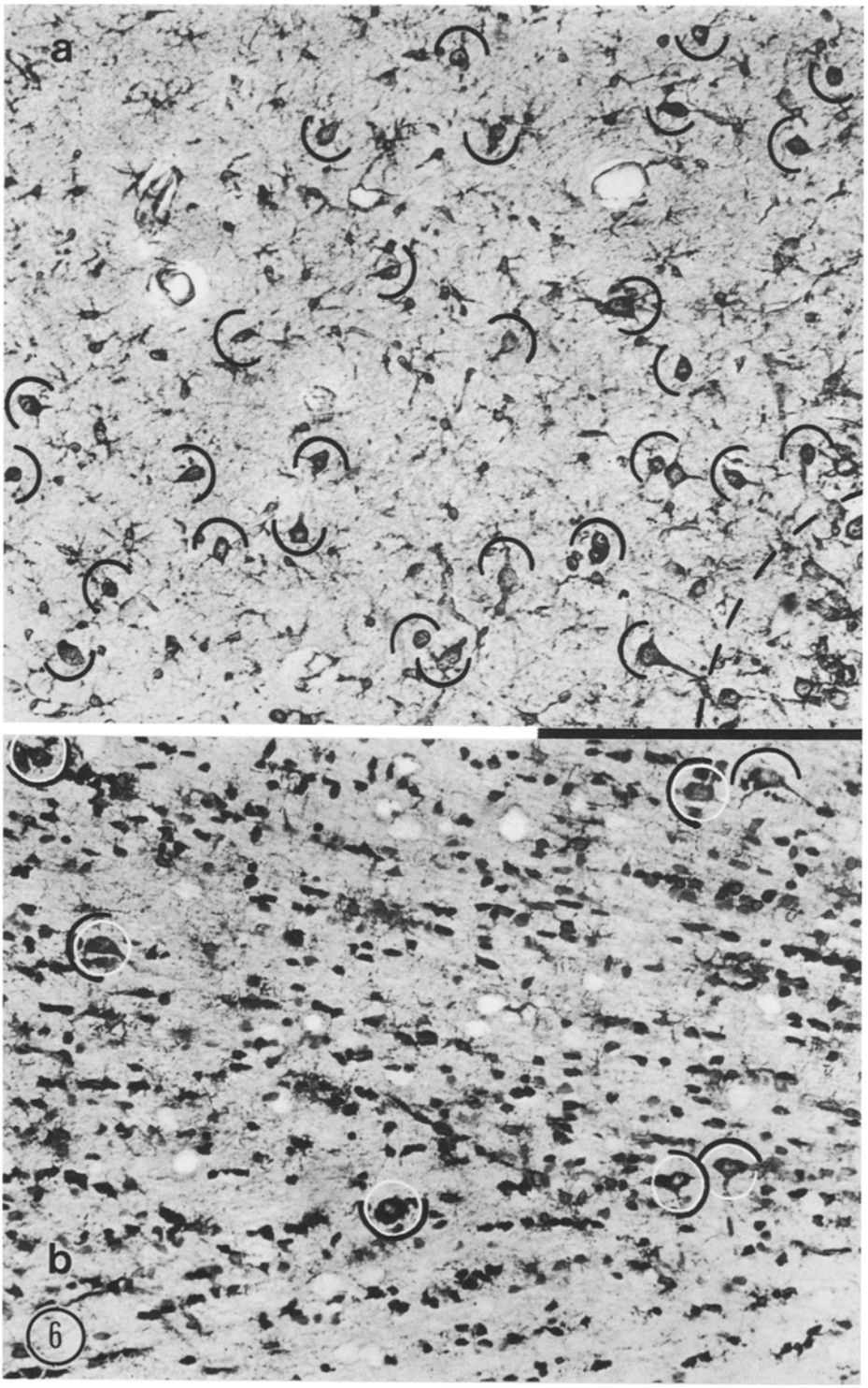
N
5

by Retzius (1893), Lorente De No (1933) and Stensaas (1967a, 1967b, 1967c, 1967d, 1967e, 1968). Our findings confirm that non-pyramidal neurons are consistent constituents of lamina I and the intermediate zone. Additionally, these neurons have been demonstrated in the subventricular zone. And this holds true not only in the neocortex and archicortex (see Figs. 6, 7 of Stensaas, 1967a), but also in the limbic cortex and paleopallium. The oldest of these neurons being generated at E14 and 15 appear in the marginal zone before the cortical plate is formed. Therefore, we propose to call this common preplate stage of the cortical development *"pallial anlage"*.

In the rat's neocortex it is not before E16 that the first vertically oriented corticotypic pyramidal neurons appear (Raedler and Sievers, 1975; rat, compare also Marin-Padilla, 1971: in cat E24). They are interposed into the primarily uniform and horizontally oriented population of neurons of the "pallial anlage" separating it into a superficial and a deep portion (lamina I and the subcortical pallial level). This is in agreement with observations on the olfactory cortex of the mouse (Derer *et al.*, 1977). The present results, therefore, suggest that the separation of the pallial anlage by a cortical plate is a common phenomenon in all parts of the pallium of different species. The neurons of the pallial analage differ not only in shape and position from those of the cortical plate, but also by the mode of their deposition. Cortical plate neurons are migrating along radial glial cells to their final position in the cortex (Rakic, 1972) producing an inside-out layering time gradient (Berry and Rogers, 1965). In contrast, the non-pyramidal neurons are continuously added to all levels of the pallial anlage. Consequently, the difference between the two groups of neurons seems to appear between their last cell division and the arrival at their final position, i.e. during migration. Precursors of non-pyramidal neurons may use long radial glial cells as lifts to lamina I and also short radial glial cells terminating at blood vessels (Wolff and Rickmann, unpublished) as lifts to the subcortical pallial level. This would also allow for the continuous addition of neurons to both sites. However, the mechanism of the termination of the neuronal migration is totally unexplained, and it cannot yet be excluded that non-pyramidal neurons are produced locally, i.e. not in the ventricular zone.

We feel that it is difficult to discriminate more than four main stages of neocortical ontogenesis instead of the five stages as outlined by the Boulder Committee (1970). The first we refer to as the "*neural primordium*" corresponds to stage A of the Boulder Committee. This stage of the neuroepithelium consists only of proliferative ventricular cells. These cells have been extensively described by Sauer (1935), Sidman *et al.* (1959), Hinds and Ruffett (1971) among others. If development does not exceed this stage, no neurons are produced and all cells transform finally into ependymal cells as, for example, in the case of the choroid plexus. The next stage of cortical development is started by the generation of non-pyramidal neurons and glial cells (Rickmann and Wolff, 1976) forming the marginal zone accompanied by the first invasion of afferent axons and blood vessels (Wolff *et al.*, 1975, Wolff, 1978). Since this earliest neuronal population is located in lamina I and the subcortical white matter of all parts of the adult pallium, we refer to this stage as the "*pallial anlage*". If development does not proceed beyond this stage, the

Fig. 5a–d. Tangential sections of the neocortex at E18. **a**), **b**) Cajal–Reyzius neurons in lamina I, note the different densities of their chromatin, the arrow points to an axon of another Cajal–Retzius neuron, **c**) typical initial segment of such an axon, **d**) subventricular neuron (*N*) enwrapped by glial lamellae (arrows), dendrites are labelled by asterisks. Bars = 5 μm in **a**), **b**) and **d**), = 0.5 μm in **c**)


a
b
6

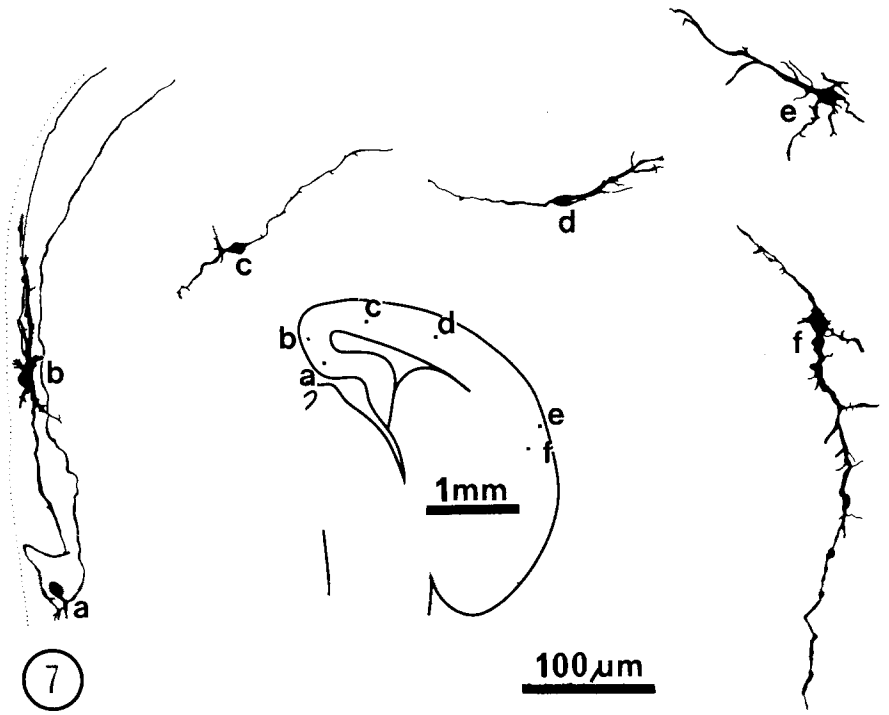
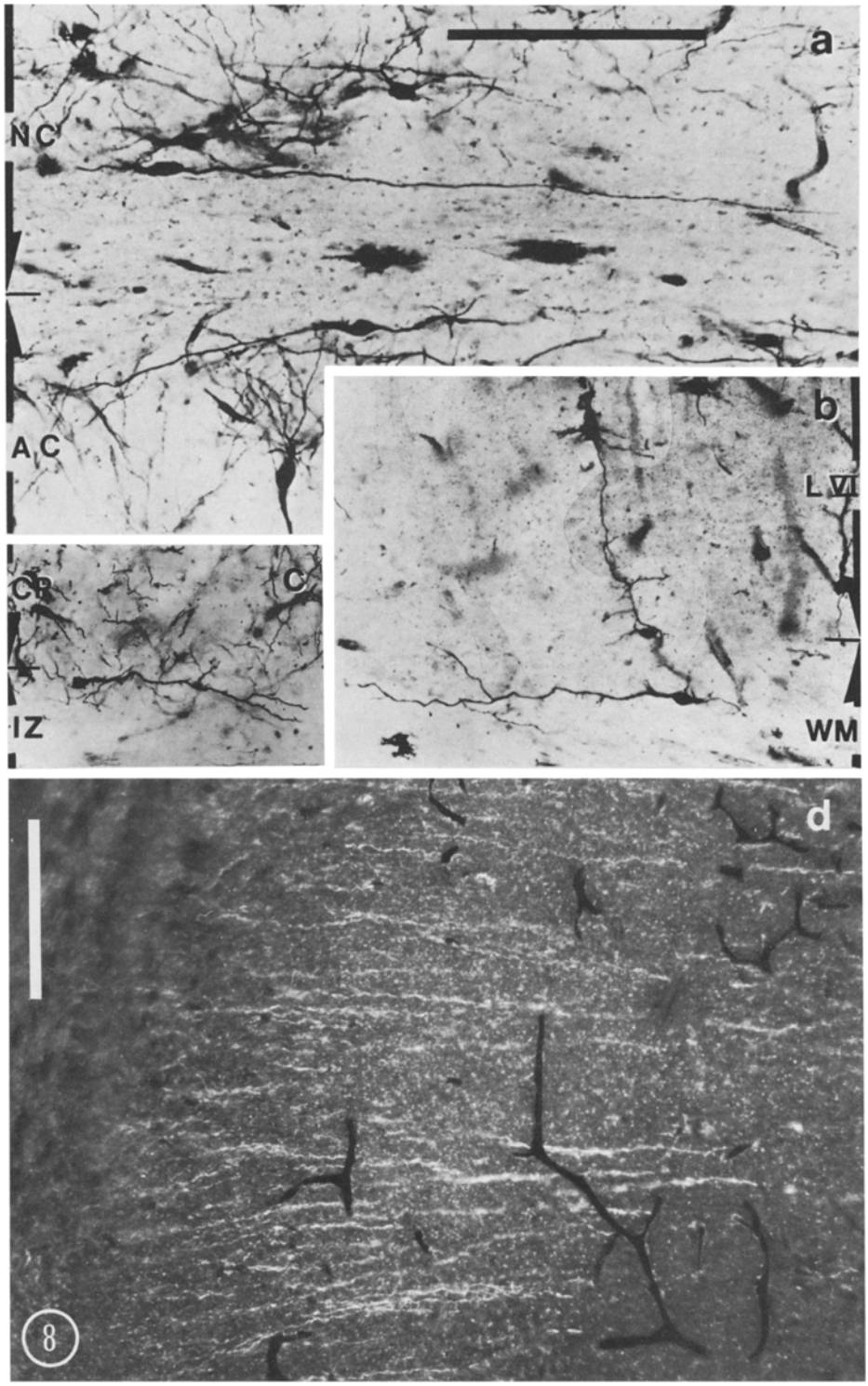


Fig. 7. Drawings of Golgi preparations of horizontal neurons. Their relative positions and orientations within the hemisphere correspond to the labelled dots in the central scheme and the dotted line marking the pial surface. *a*) subcortical limbic neuron on E17, *b*) lamina I limbic neuron on E17, *c*) subventricular neuron on E19, *d*) subcortical neuron on P5, *e*) lamina I paleopallial neuron on E19, *f*) subcortical paleopallial neuron on E17

resulting tissue should contain non-pyramidal neurons dispersed between axons. The third stage is characterized by the formation of pyramidal neurons of the cortical plate which causes the distinct layering of the "*cortical anlage*". After the cortical plate has formed, the components of the previous marginal zone (pallial anlage) are found in various zones of the cortex and subcortical pallium: lamina I, deep cortical plate, intermediate zone, subventricular zone. Consequently, the term "marginal zone" (as in stages D and E, Boulder) would now be misleading and can be replaced by "lamina I", which is the layer superficial to the cortical plate. During the final stage of "*cortical maturation*" no further neurons are added to the neocortex. We should like to include into this stage all events which accompany the modelling of cortical neurons and their connections: e.g. the growth of axon collaterals, dendrites and their branches, the formation of spines and the main part of synaptogenesis, and also the formation of glial cells, myelinization and the postnatal sprouting of blood capillaries.

Fig. 6a and b. Tangential silver impregnated sections of the adult neocortex demonstrating neurons labelled with arcs and glial cells. **a**) lamina I reaching lamina II at the dashed line, **b**) white matter, bar = 200 μm


a
NC
AC
b
L VI
WM
CP
C
IZ
d
8

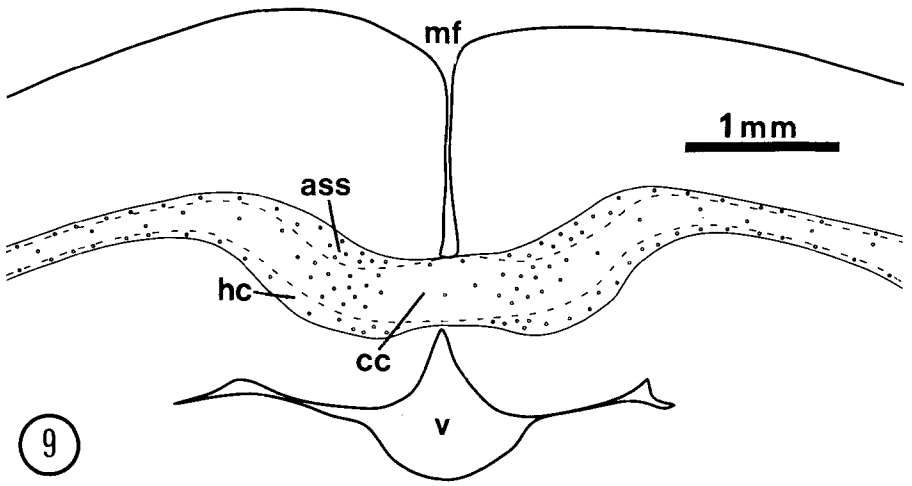


Fig. 9. Semiquantitative representation of the distribution of neurons in the adult subcortical white matter, median fissure (*mf*), U-fibre location (*ass*), hippocampal commissure (*hc*), corpus callosum (*cc*), third ventricle (*v*)

Table 3. Early axons in the neocortex

	Origin	Termination	Direction	Position	Course
(1)	caudal thalamus	neocortex	medial	lower border of the cortical plate and lamina I	tangential, may change obliquely into lamina I and vice-versa
(2)	paleopallium	neocortex	medial	lamina I	tangential
(3)	paleopallium	paleopallial to neocortical border	medial	intermediate zone	irregular
(4)	archicortex	limbic to neocortical border	lateral	intermediate zone	tangential
(5)	limbic cortex	neocortex	lateral	lamina I	tangential
(6)	neocortex	neocortex	every	lower border of the cortical plate	tangential
(7)	neocortex	neocortex	every	lamina I	tangential
(8)	limbic cortex	growth cones within the neocortex	lateral	intermediate zone	tangential

Fig. 8a–c. Horizontal subcortical neurons in coronally sectioned Golgi preparations. **a)** two horizontal neurons on P18 located at the border between the grey and the white matter of the neocortex (*NC*), respectively the archicortex(*AC*), **b)** neuron on P4 close to the lower border of the neocortex with ramifications in lamina VI (*LVI*), presumptive white matter (*WM*), **c)** neocortical subplate neuron on E20, cortical plate (*CP*), intermediate zone (*IZ*), **d)** axons from limbic neurons radiating to the neocortex within lamina I on E18, dark field, tangential section, the white magnification bar outlines the median fissure. Bars = 200 μm

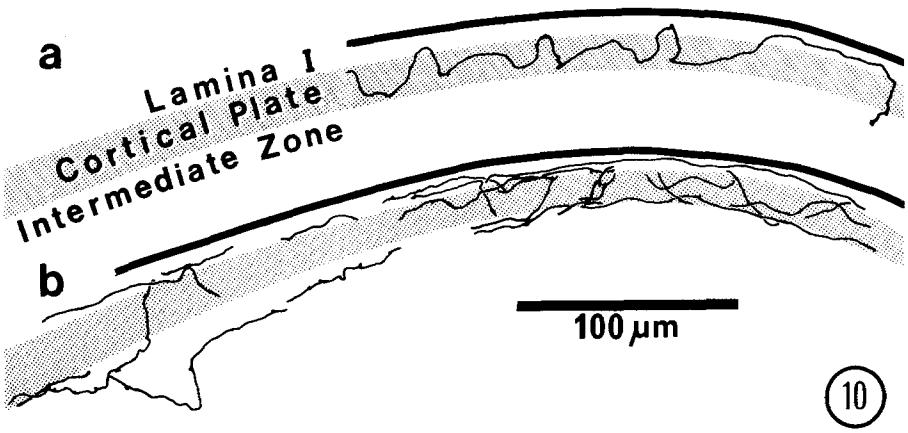


Fig. 10a and b. Drawing from a single coronal section at E17 (rapid Golgi double impregnation): fibres which had been tangentially oriented earlier are now distorted by the interposition of the CP into the MZ and thus show a sort of lattice pattern, **a**) the course of a single axon, **b**) the pattern formed by several stained axons

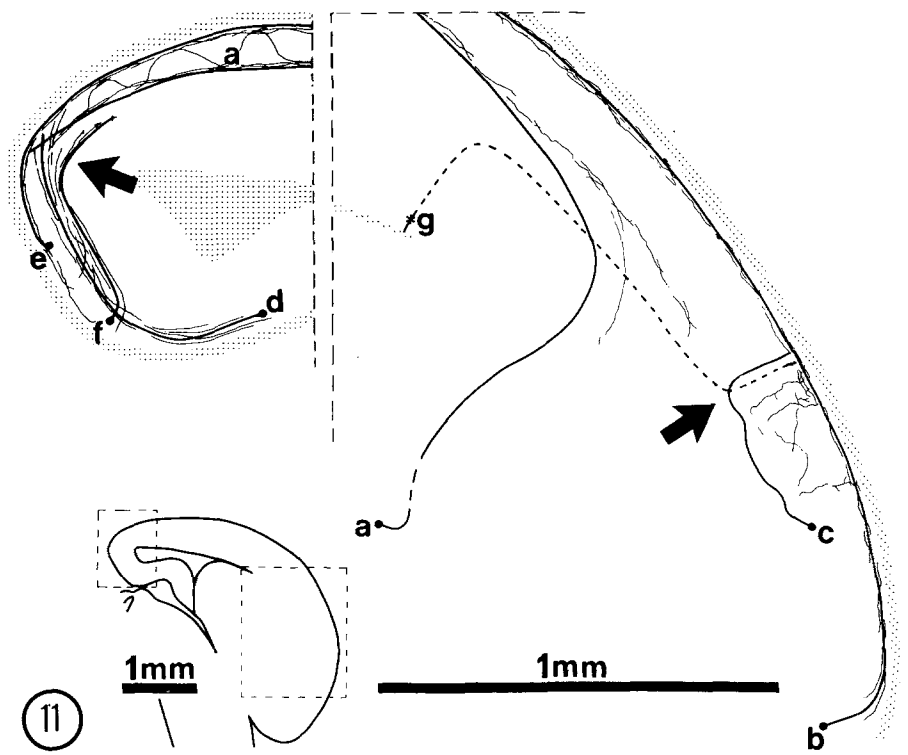


Fig. 11. Summarizing drawing of the main axon courses in the pallium at E17 (compare Table 3), dotted areas represent ventricular and meningeal spaces, arrows mark the neocortical borders, assumed first afferents from the caudal thalamus (*a*), neurons projecting either to (*c*, *d*) or across (*b*, *e*, *f*) the neocortical borders, radial glial cells seem to be concentrated at the neocortical to paleopallial border (*g*)

Origin and Fate of the Early Marginal Zone

Conflicting evidence has been presented in the literature about which type of tissue component appears first in the marginal zone (MZ) (His, 1904: "Gliazellen"; Marin-Padilla, 1971: "afferent fibres", followed by "neurons"; Raedler and Sievers, 1975: "horizontal cells of Cajal–Retzius"). According to the present observations, glial cells, preneurons and afferent fibres all appear within less than one day in the MZ of the rat's neocortex. Glial cells accumulate beneath the pia mater as non-proliferative so-called "quiescent" glioblasts (Wolff and Rickmann, 1977), i.e. in the "Siebschicht" of His (1904). Also at the outer border of the ventricular zone some proliferating glioblasts are present as they are later in the subventricular zone. The first axons are arranged roughly tangential, but occur at random in all levels of the early MZ. In contrast, the frequency of preneurons is high in the upper half of the MZ and decreases gradually towards the ventricular zone.

We were not able to trace back single corticopetal fibres to their place of cellular origin. At E17 some fibre bundles could be followed back from the neocortex into the caudal parts of the thalamus in which neurons originate early enough (E13) to give rise to the first corticopetal fibres arriving at E14 (Eitschberger, 1970, McAllister and Das, 1977). Such corticopetal connections have not yet been identified in adults. However, if no axons are added later from the same origin, it would be very difficult to demonstrate these first afferents in the adult cortex, because they are heavily diluted according to the subsequent surface growth of the neocortex (Table 2).

As proposed by P. Weiss (1941), the orientation and location of growing axons may be controlled by contact guidance. If this concept is valid for the developing neocortex, it could explain why later coming axons pose themselves preferentially into levels in which the axonal density is already high, i.e. in lamina I and in the intermediate zone.

As early as E14 desmosome-like and close contacts have been found between preneurons and growth cones of afferent axons. The initial decision of a ventricular cell to produce a preneuron which migrates out of the ventricular zone may be made without any direct influence of axons. However, all early neurons studied by EM received at least one synapse. It can, therefore, not be excluded that axons induce or at least strongly promote the further differentiation of preneurons by making contacts. Such a mechanism would allow for an inductive chain to be started somewhere in the subcortex. Subcortical afferents could induce differentiation of preneurons in the early MZ which in turn might induce the local differentiation of a corticotypic neuronal network by increasing the local density of synapses. During prenatal development preneurons are added, and axons arrive continuously in the "pallial anlage" (see below), i.e. lamina I, and subplate levels. This fact suggests that the connectivity of the preexisting neuronal elements is continuously changed.

Classification of Neurons Outside the CP

All the precursors of the neurons of lamina I (LI) appear horizontally oriented just after reaching their final position. Certain neurons later seem to loose the horizontal orientation during subsequent differentiation. These secondary differences seem to be responsible for the fact that there are at least two types of neurons in adult LI. The present observations confirm a high formation rate of LI neurons before the cortical plate builds up (König *et al.*, 1977; Wolff, 1978). Additionally, it is demonstrated that these preplate neurons are synaptically connected very early to other neurons of

the "pallial anlage". During their maturation these neurons which retain their horizontal orientation resemble both Cajal's (1959) "elongated or special cells" and the upper LI horizontal cells from the work of Sousa-Pinto and co-workers (1975). We confirm Sousa-Pinto's discription that these neurons develop spines. Axons from these neurons have been found to leave LI, only when their perikarya were located in the most lateral parts of the neocortex. Thus, Marin-Padilla's (1972) contention that the termination of these axons in deep cortical layers is a *general* characteristic of a "transient reptilian structure" of the mammalian neocortex, cannot be supported by our findings. Horizontal cells corresponding to Cajal's (1959) "triangular cells" have also been found only in the most lateral parts of the neocortex. We restrict the term "Cajal–Retzius cells" to the superficial neurons within LI, as only these resemble the descriptions of both authors and remain horizontally oriented (Cajal, e.g. 1891; Retzius, 1893).

Another fraction of LI neurons appears later, i.e. while the cortical plate builds up. After P4 they start their final differentiation into small spiny stellate neurons (Cajal, 1891: "cellules polygonales"; Noback and Purpura, 1961) which parallels the differentiation of the neuropil in lamina II and in the deep parts of LI. Thus, the statement of Meller *et al.* (1968) that "the horizontal cell changes from a primitive bipolar cell into a multipolar cell ..." seems to be valid only for the late arriving neurons in LI.

Fate of Non-Pyramidal (Non-Plate) Neurons

There has been some speculation that lamina I (LI) neurons undergo regressive change or even degeneration during postnatal development (Duckett and Pearse, 1968, Sas and Sanides, 1970). Other authors pointed out that the reduction of their packing density may be due to the increase in surface area of the cortex (Fox and Inman, 1966; Marin-Padilla, 1972). Our findings suggest that, within the neocortex of the rat, there is no principle necessity for LI neurons to die. This suggestion is based on the following arguments: (1) In agreement with König *et al.* (1977) we found that early labelled neurons in LI survive till adulthood. The continuous addition of neurons to LI, however, makes it impossible to exclude entirely a degeneration of some cells. (2) Histological preparations of adult brains show quite a number of neurons in the adult LI. A preferential degeneration of one group of neurons could not be detected in our preparations. (3) Golgi impregnations show that Cajal–Retzius neurons seem to limit or perhaps even reduce the expansion of their dendritic trees, but remain present till adulthood (compare Poliakov, 1961). The small spiny stellate cells of LI start their final differentiation rather late in ontogenesis and also do not seem to degenerate. (4) The difficulty in detecting neurons in LI from Golgi preparations of adult brains, especially the Cajal–Retzius neurons, might be caused by their dilution due to the surface growth of the neocortex. Additionally, the Golgi method might fail to stain these neurons in the adult LI.

The population of deep subcortical pallial neurons which we have described is located in the subventricular and intermediate zone, respectively in the white matter of the adult cortex. Åström (1967) has named these neurons "stellate cells". In our material, throughout all developmental stages, the horizontal diameter of their dendritic trees, as established from serial sectioning, is far longer than the vertical ones. The closer they lie to the lower border of the cortical plate, the more they resemble stellate-like dendritic trees. We called these neurons "subcortical pallial" neurons, as they may contribute the bulk of postsynaptic elements for those synapses

being formed in the presumptive white matter (Wolff, 1976; Wolff, 1978; compare also subplate synapses Molliver *et al.*, 1973; Kostović *et al.*, 1973; Kostović-Knežević *et al.*, in press), and as the term "subplate" has already been preoccupied for the deep part of the presumptive grey matter of the neocortex (Kostović and Molliver, 1974). The exact type of interaction of subcortical (pallial) neurons with arriving afferent axons below the cortex remains to be investigated.

The present paper was restricted to non-pyramidal neurons outside the cortical plate, because any confusion with pyramidal neurons could be excluded. In a separate paper we will report about the formation and differentiation of non-pyramidal neurons within the cortical plate (Cronwall *et al.*).

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