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Autoradiographic Study of Early Neurogenesis in Rat Neocortex

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Summary. The early neurogenesis of rat neocortex was analysed by means of light and electron microscopic autoradiography. It was found that the very first preneurons originate probably as early as ED 11. They are the horizontal cells of Cajal-Retzius. The peak of their formation is on ED 13 (surface index estimated on ED 17 after injection of ^{3}H-thymidine on ED 13:21, after injection on ED 12:4, after injection on ED 15:5), whereas no Cajal-Retzius cells could be found to have originated after ED 15. These cells are the developmentally most advanced of the neocortex. The cells second in date of origin and maturation are preneurons which presumably correspond to the presumptive neurons of Layer VII (VI b), and begin to originate on ED 12. The end of their formation could not be defined owing to a lack of ultrastructural differences to other, younger preneurons in later gestational stages. These two cell types are the first cellular components of the primordial plexiform layer (Marin-Padilla, 1978) or pallial anlage (Rickmann, 1977), demonstrating an outside-in gradient within this layer, and are separated by the formation of the cortical plate. This could be proven by their simultaneous labelling above and below the cortical plate after administration of ^{3}H-thymidine before ED 15. These results confirm the hypothesis of a dual origin of the mammalian neocortex (Marin-Padilla, 1978).

Key words: Cerebral cortex – Neurogenesis – Primordial Plexiform Layer – Autoradiography

Introduction

In a previous study dealing with the normal development of the visual system of the albino rat (Raedler and Sievers, 1975) we analysed the cytological differentiation of the visual cortex. In establishing ultrastructural criteria for neuronal differentiation it was found that the cells of Cajal-Retzius of Layer I, which had not been described ultrastructurally until then, were the first neurons to mature in the neocortex. This was in contrast to the generally accepted inside-out gradient of neocortical development (Angevine and Sidman, 1962; Berry and Rogers, 1967; Langman and

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Welch, 1967; Hicks and D'Amato, 1968; Brückner et al., 1976). Using the Golgi-technique, Marin-Padilla (1971, 1972, 1978) consecutively presented a new concept of the origin of the mammalian neocortex by postulating that the first step of corticogenesis is the formation of a primordial plexiform layer containing the Cajal-Retzius cells as well as the prospective neurons of later Layer VII (VI b). In a second step, this primordial plexiform layer is subdivided by the developing cortical plate. This hypothesis was shared by König et al. (1975, 1977) and Rickmann et al. (1977). The LM- and EM-autoradiographic experiments presented here were undertaken in order to analyse neocortical neurogenesis in the light of the concept of Marin-Padilla, with special emphasis on the origin and fate of the neurons of the primordial plexiform layer.

Material and Methods

Wistar albino rats were kept in temperature- and humidity-controlled cages in a shifted day-night rhythm. Females were placed with males and the day of mating determined by daily phase-contrast-microscopical examination of vaginal smears for sperms. The day of sperm-positive smears was taken as ED1. Pregnant rats on ED9, 11, 12, 13, 15 and 17 were anaesthetized with an intraperitoneal injection of Nembutal according to body weight and the abdomen incised midventrally under sterile conditions. The gravid uterus was placed on a swab moistened with sterile Ringer solution and by means of a Hamilton syringe tritiated thymidine (New England Nuclear, specific activity 6.7 Ci/mmol) was applied into each amniotic sac using different doses on both uterine horns. No lesions as described by Kennedy and Persaud (1977) were observed in the embryos after amniotic sac puncture. In single labelling autoradiographic experiments the following dosages were used after preliminary experiments: Fetuses aged nine days post conception (p.c.) received one to two microcurie each, those aged eleven days received 1.5 to 3, those aged twelve days 1.5 to 4, those aged thirteen days 2 to 4, those aged fifteen 3.5 to 7 microcurie each, those aged seventeen 5 to 10 microcurie each. This range of dosages resulted in an adequate labelling i.e. no radiotoxic effects were observed and on the other hand no increase in the number of labelled cells was counted when using higher doses. In double labelling experiments, fetuses received ^{14}C-thymidine (New England Nuclear) in a ratio of 0.6 to 1 to tritiated thymidine an hour after the first injection (Schultze et al., 1976). These experiments were performed in order to establish the precise time of cell origin: Independent of ^{3}H-thymidine-dose and duration of exposure, heavily labelled cells are assumed to be in the first cell cycle after the application of ^{3}H-thymidine. If, in double-labelled animals, these cells do not display any ^{14}C-thymidine grains, it means that they have synthesized DNA in the time interval between the two injections. It can be deduced that, in single-labelled animals, cells with a similar degree of radioactivity have the same date of origin.

After the injections, the gravid uterus was replaced into the abdomen and the incision closed with sutures.

The time intervals between injection and sacrifice are shown in the following table (Table 1):

Table 1

	Sacrifice						
Injection of ^{3}H-thymidine on ED		9	11	12	13	15	17
	after $1^{1}/_{2}$		×		×	×	×
	after 4 h		×		×	×	×
	ED13.	×	×	×			
	ED15.	×	×	×	×		
	ED17.	×	×	×	×	×	
	ED21.	×	×	×	×	×	×

At the appointed time of sacrifice the pregnant rats were again kept under Nembutal anaesthesia and each fetus in turn delivered through an incision of the uterus and the fetal membranes.

Embryos on ED 11 of pregnancy were fixed by immersion in a solution of 6% glutaraldehyde in 0.05 m phosphate buffer, all other animals being perfused with the same solution. The perfusion was performed under a stereoscope after inserting a glass capillary into the heart, up to ED 15 through the intact thoracic wall, in older animals after dissection of the thorax. After sufficient perfusion, the whole brains were prepared and left in 6% GA overnight before frontal sections of the cerebral hemispheres were taken as described previously (Raedler and Sievers, 1975). The tissue pieces were postfixed in 1% OsO_4 solution in 0.1 m phosphate buffer including 0.1 m saccharose for 2 h at 4° C, dehydrated in ethanol of increasing concentrations and embedded in Epon 812 (Luft, 1961), supplemented with PPO. Semithin sections of 1 µm of the embedded tissue were cut on a Reichert OMU 2 ultramicrotome and placed unstained on glass slides cleaned with 10% diluted $H_2SO_4/K_2Cr_2O_7$ a.p. Autoradiographs were prepared in the following way: The slides were coated with Ilford K2 emulsion (diluted 2:1 with distilled water, melted and kept at 42° C) by dipping, and after drying stored in light-proof boxes at 4° C for exposure periods of between 4 and 12 weeks. The autoradiographs were developed with an amidol (Merck) developer, fixed in 30% $Na_2S_2O_3$, washed and stained with a 4:1 solution of toluidine blue/pyronine.

For double-labelling experiments an inert layer of 10% gelatine solution was then applied. After hardening of the gelatine layer, the slides were coated with undiluted Ilford K2 emulsion (melted at 42° C, kept at 30° C), dried and stored in light-proof boxes at −20° C for exposure periods of between 4 and 12 weeks. The development was accomplished with amidol, fixation again in 30% $Na_2S_2O_3$. The sections were viewed and photographed under a Zeiss photomicroscope II. In older animals, photographic montages at 560-fold enlargement through the whole width of the cerebral cortex were made. Quantitative measurements were undertaken with a MOP/AM 01 (Kontron). The cells/area and labelled cells/area index are expressed in cells/1000 µm², the surface index in labelled cells/1000 µm.

For electron microscopic autoradiographs, tissue blocks were trimmed for relevant regions of the cerebral cortex, ultrathin sections cut with diamond knives on a Reichert OMU2 ultramicrotome and placed on gold grids. The grids were coated with an Ilford L4 emulsion (diluted 1:0.8 with aqua dest., melted and kept at 42° C) using the loop method (Fischer, 1975), stored in light-proof boxes for exposure periods of between 2 and 4 weeks, developed with Microdol X, fixed in 30% $Na_2S_7O_3$ and viewed and photographed with a Zeiss EM 9.

Results

ED 11

On ED 11 the neural wall of the rather small cerebral vesicle consists only of few rows of ventricular cells (30–40 µm). These cells are characterized by an elongated to fusiform nucleus with coarse chromatin clumps and a pericaryon bipolar in shape giving rise to a basal and an apical process. The basal process extends to the cerebral vesicle and is connected with desmosomes to the neighbouring cells. It exhibits a cilium which projects into the ventricle. The apical process reaches the pial basement membrane where it is attached with a plump triangular end-foot. The cytoplasm of the ventricular cells contains abundant ribosomes, which account for the dark aspect of these cells, several mitochondria, some in the radially oriented processes, a Golgi complex and few short profiles of endoplasmic reticulum. No other cell types and no other kinds of cell processes could be detected in the neural wall on ED 11. Furthermore, there was no significant dissimilarity between different regions. Labelling experiments, applying ^{3}H-thymidine $1^1/_2$ resp. 4 h before sacrifice revealed a heavy labelling in both the upper and lower part of the ventricular layer (Fig. 1) with the exception of most of the frequent mitoses near the ventricular border.

ED 13

The ventricular layer is now composed of about 10 cell rows (laterally) to 5 cell rows (medially) and is about 125 resp. 60 µm wide. The medial part of the cortex still consists only of the ventricular layer. In the small region devoid of cells, only radial processes of the ventricular cells can be detected ultrastructurally above and below the ventricular nuclei. In the lateral parts of the neocortex, on the other hand, the primordial plexiform layer begins to form: a small cell-scarce zone can be observed above the ventricular zone with a width of about 40 µm. This layer, the primordial plexiform layer, shows only few cells with dark polymorphous nuclei and without any recognizable pattern of distribution. Electron microscopically these cells show an undifferentiated appearance with uniformly distributed, coarse chromatin clumps in the nucleus, many ribosomes, quite a few mitochondria and very little rough ER. In addition, there are horizontally oriented cells with bipolar pericarya and large processes to be found near the pial basement membrane. Ultrastructurally, these cells display a round to oval, chromatin-dense nucleus and a large bipolar pericaryon with numerous mitochondria, Golgi complexes and microtubules and, compared with other cells of the neocortex at this stage, more rough endoplasmic reticulum. They were considered to be Cajal-Retzius cells.

Injection of ^{3}H-thymidine on ED9 resulted in no labelling at all, while the application of ^{3}H-thymidine on ED11 resulted in labelling of a few of the horizontally arranged cells of the primordial plexiform layer (Fig. 2a). The embryos injected on ED12 showed, medially and laterally, a uniform rather faint labelling of nuclei throughout the whole thickness of the ventricular layer. Only very few cells at the upper border of the ventricular layer were heavily labelled. Furthermore, a marked radioactivity was seen in some cells of the primordial plexiform layer in the lateral part of the neocortex.

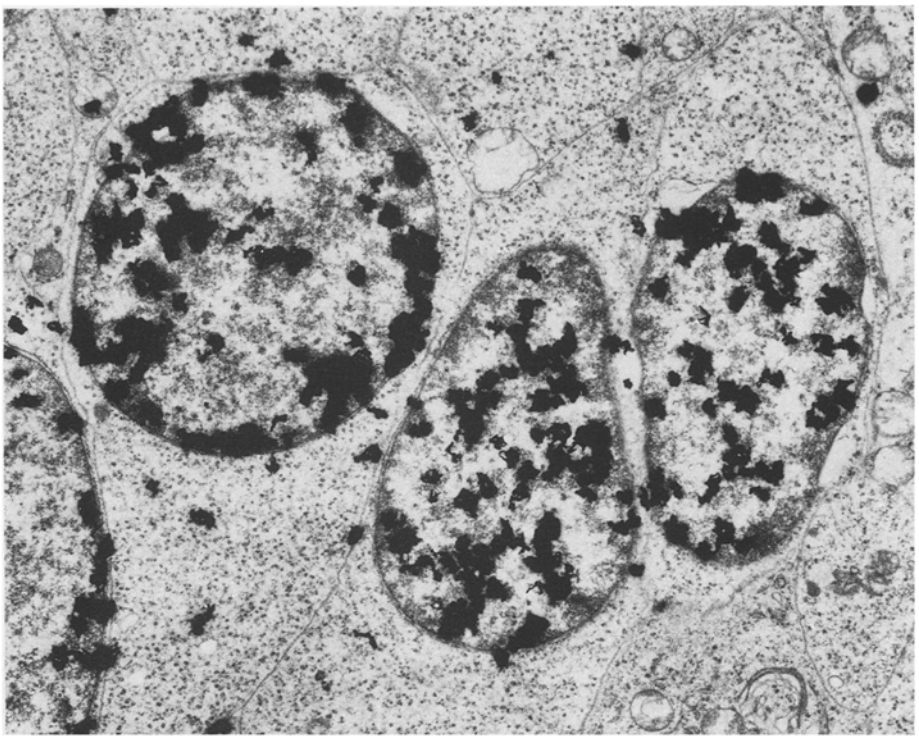
Injection of ^{3}H-thymidine 4 resp. $1^1/_2$ h before sacrifice resulted laterally in a marked labelling of the cells in the upper half of the ventricular layer (labelled cells/area index 8) while only few of the ventricle-near ones and no mitotic cells demonstrated grains (labelled cells/area index lower half 2,5) (Fig. 2 b). Very few of the polymorphous cells of the primordial plexiform layer displayed any radioactivity. The horizontal cells were not labelled at all.

Medially, a marked labelling in the upper part of the ventricular layer was detected with the exception of only few cells at its upper border.

Fig. 1. Rat neocortex after application of a high range dose of ^{3}H-thymidine on ED 11, sacrifice 4 h later. Labelled nuclei of the ventricular layer. × 6460

Fig. 2. a Lateral part of rat neocortex after ^{3}H-thymidine application on ED 11, sacrifice on ED 13. Labelled nuclei of horizontally oriented cells in the newly formed primordial plexiform layer. × 1100. **b** Lateral part of rat neocortex after application of a high range dose of ^{3}H-thymidine on ED13, sacrifice 4 h later. Marked labelling of the ventricular cells, esp. in the upper half of the ventricular layer. Cells of the primordial plexiform layer mostly unlabelled. × 560

Fig. 3. Rat neocortex after ^{3}H-thymidine application on ED 12, sacrifice on ED 15. Labelled nuclei in the primordial plexiform layer. × 875



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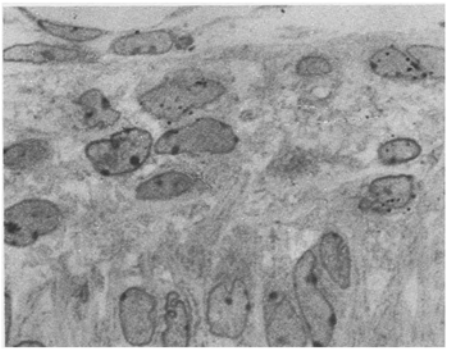

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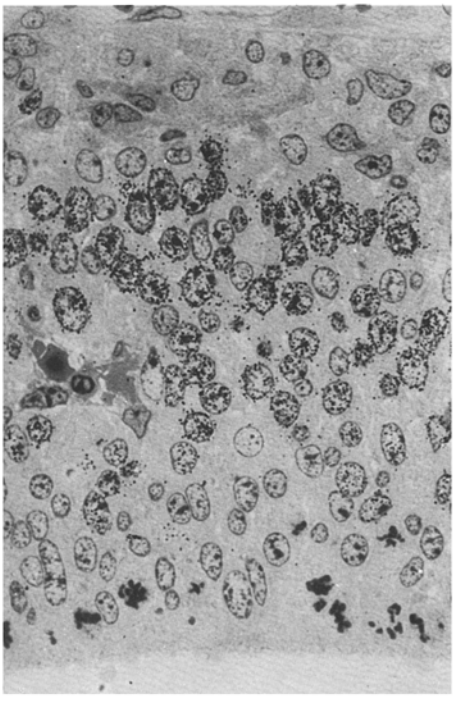

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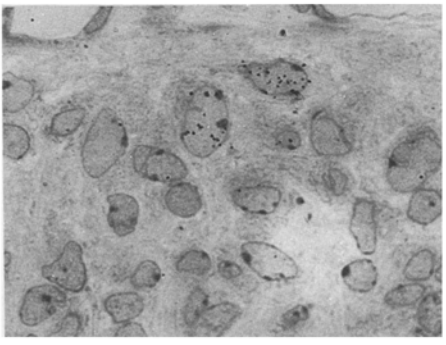


3

ED 15

At this stage of gestation there still exists a marked difference between the lateral and medial parts of the neocortex so far as regards the advance of development, although the primordial plexiform layer is now present in the whole circumference of the neural wall. Both this and the ventricular layer have considerably increased in width.

In the lateral part, the ventricular layer is now composed of about 18–20 rows of cells and measures about 155 μm. The primordial plexiform layer is about 115 μm wide. Its cellular components again are few. Directly beneath the pial basement membrane are situated horizontally oriented bipolar Cajal-Retzius cells with long slender processes. Ultrastructurally, these cells possess a round to oval nucleus with uniformly distributed chromatin and abundant cytoplasm with numerous well-developed organelles. The second cell type in the primordial plexiform layer is characterized by a small, dark polymorphous nucleus and sparser cytoplasm. Besides, in the deeper part of the primordial plexiform layer there are very few cells to be found with a large, round to polymorphous nucleus. Electronmicroscopically, these cells display a light nucleus containing uniformly distributed chromatin with only few chromatin clumps and a primitive nucleolus. Their abundant cytoplasm contains numerous mitochondria, some Golgi complexes and microtubules, and few profiles of rough endoplasmic reticulum. Furthermore, the pericarya of these cells have lost their bipolar shape and exhibit several cell processes. In the most lateral part of the neural wall, the very first cells of the cortical plate can be observed inside the primordial plexiform layer. They are identifiable by their appearance in little groups with very small internuclear spaces. The cell axes, as well as their relatively large and light oval nuclei, are radially arranged; no definite cell rows are yet recognizable.

In the medial part of the neocortex, the ventricular layer now comprises about 12–15 cell rows (125 μm). The primordial plexiform layer is still relatively small (35 μm) and shows cells of the first two types described above. The cells/area index is 7 as compared with a cells/area index of the ventricular layer of 11. Injection of ^{3}H-thymidine on ED 9 and ED 11 produced no labelling. Injection of ^{3}H-thymidine on ED 12 caused only very few ventricular cells to become sparsely labelled. On the other hand, numerous horizontal cells of the primordial plexiform layer (Fig. 3) and fewer of the polymorphous and large light ones are heavily radioactive. This applies to both the medial and especially the lateral parts of the neural wall. In the cells of the cortical plate (laterally) no grains could be detected. If injection occurs on ED 13 a sparse labelling can be found in the nuclei of some ventricular cells as well as in some of the cells of the newly forming cortical plate of the lateral neocortex. Heavily labelled cells are detectable exclusively in the primordial plexiform layer. These comprise the horizontal cells, the small polymorphous cells and the cells with the sacrifice with ^{3}H-thymidine, most cells in the upper half of the ventricular layer sacrifice with ^{3}H-thymidine, most cells in the upper $^{1}/_{2}$ of the ventricular layer (labelled cells/area index 7) showed heavy labelling without significant regional differences. In the primordial plexiform layer only very few of the cells with the small dark polymorphous nuclei were radioactive. The cells of the cortical plate showed no labelling.

ED 17

On this day the layers described above have further increased in width. Again, there are obvious differences between the medial and the lateral part of the cortex. Medially, the developmental stage now more or less resembles that of the lateral cortical part on ED 15 (ventricular layer ca. 15 cell rows, 155 μm, primordial plexiform layer 90 μm), with the first cells of the cortical plate within the primordial plexiform layer.

The lateral regions of the cortex can now be described as follows: The ventricular layer with radially arranged oval to fusiform nuclei and small internuclear spaces consists of about 12–14 rows of cells (120 μm). Above can be recognized the formation of the subventricular layer (105 μm), which is characterized by the loss of the radial arrangement of its cells and an enlargement of the internuclear spaces partly due to increasing intercellular spaces, partly due to growing numbers of fibre bundles between the pericarya. The cells of this layer show polymorphous nuclei and an undifferentiated cytoplasm. While the subventricular layer is very prominent in this lateral part of the neocortex on ED 17, it becomes very much thinner medially and almost disappears in the most medial part of the neural wall.

Within the primordial plexiform layer there has now formed a cortical plate with about 5 cell rows (45 μm) of perpendicularly oriented, very densely packed (cells/area index 11) cells with relatively large and light vertical oval nuclei. Electron microscopically, the cytoplasmic equipment of these cells resembles that of the ventricular cells. In the pial part of the pericaryon, one or more Golgi complexes (dictyosomes), few profiles of rough ER and numerous neurofilaments can be detected. Regarding the amount of cytoplasm and cell organelles, the deepest preneurons within the cortical plate are considered to be the most developmentally advanced. Because of the bipolar appearance of its cells, this layer is hence-forward called 'bipolar cortical plate'. Below the bipolar cortical plate there can now be recognized the lower part of the former primordial plexiform layer, mainly consisting of horizontal fibres, and the cells with large light nuclei described above. Ultrastructurally, these cells display a rather advanced developmental stage insofar as concerns nuclear and cytoplasmatic differentiation. The cytoplasm is markedly enlarged compared to former stages and concentrated at one pole of the pericaryon. It contains accumulations of mitochondria, several Golgi complexes and numerous neurofilaments and ribosomes. The nucleus often shows an indentation. These cells occupy mostly the basal part of the cortical plate. In our opinion, the cells correspond to the cells of Layer VII of Marin-Padilla (1978).

Beneath these cells another new layer is observable, the intermediate layer. It is characterized by relatively few cells (cells/area index 8) and wide intercellular spaces which are traversed by numerous fibres. Most of the cells display polymorphous, not oriented nuclei and ultrastructurally resemble the ventricular resp. subventricular cells. Some of them possess a bipolar shaped, but not necessarily radially oriented pericaryon. The border between the subventricular and intermediate layer on the one hand, and between the intermediate layer and the cortical plate on the other hand is not sharply demarcated. Above the cortical plate lies Layer I (previously the upper part of the primordial plexiform layer), about

30 μm thick. It contains mainly two types of cells, the Cajal-Retzius cells with round to oval nucleus, abundant cytoplasm and long slender processes, and cells with small, dark, polymorphous nuclei and little cytoplasm. Ultrastructurally, the Cajal-Retzius cells have developed considerably compared to ED 15. Their pericaryal cytoplasm has increased further and contains, apart from several well developed Golgi complexes, numerous mitochondria and some dense bodies, a marked accumulation of granular endoplasmic reticulum which is already beginning to form a parallel array.

In the lateral part of the neocortex of animals injected with ^{3}H-thymidine on ED 9 and ED 11, no radioactivity is exhibited at all, while application on ED 12 results in a labelling of the Cajal-Retzius cells of Layer I almost exclusively (surface index: 4) (Figs. 4a, b). In the basal part of the cortical plate only few cells exhibit a considerable number of grains. Animals injected on ED 13 and perfused on ED 17 show, in the lateral parts of the neural wall, no labelling throughout the ventricular and subventricular layers, sparse labelling of numerous cells in the cortical plate without a preference for upper or lower part and heavy labelling of cells above and below the bipolar cortical plate (Figs. 4c, d), i.e. the horizontal cells of Layer I (surface index: 21) and the preneurons of the prospective Layer VII of Marin-Padilla (surface index: 18). In addition, some cells with small, dark polymorphous nuclei in Layer I and few bipolar preneurons in the cortical plate are heavily radioactive.

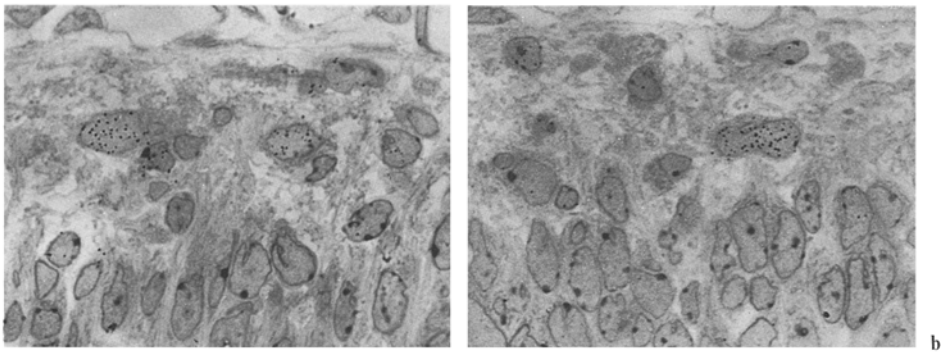
Injection of ^{3}H-thymidine two days later (ED 15) resulted in a heavy radioactivity of numerous cells in the bipolar cortical plate (Fig. 5a). A few heavily labelled, immature appearing cells were found in the subventricular layer, intermediate layer and Layer I. Several predominantly undifferentiated cells in ventricular, subventricular and intermediate layer displayed a moderate degree of radioactivity. Estimating the percentage of future horizontal cells out of the totality of labelled cells in Layer I on ED 17 by analysing the different labelled cell types of Layer I on ED 21, it appears that only 15 percent of labelled cells on ED 17 develop into Cajal-Retzius cells. This would correspond to a surface index of 2 on ED 17. On the other hand, we were unable to estimate the surface index of prospective Layer VII neurons because of absence of typical LM and EM characteristics apart from those in stages of advanced maturation.

If injected 4 resp. $1^1/_2$ h before sacrifice, the foetuses showed heavy labelling of most cells, predominantly in the upper half of the ventricular layer (Figs. 5b, c). Many heavily radioactive cells could be seen in the subventricular layer, too. In the medial part of the neocortex, application of ^{3}H-thymidine on ED 9, 11 and 12

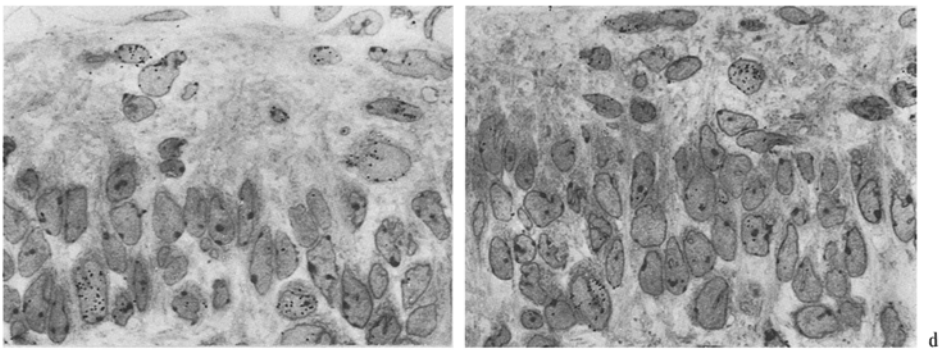
Fig. 4a and b. Rat neocortex after ^{3}H-thymidine application on ED 12, sacrifice on ED 17. Labelled Cajal-Retzius cells in Layer I. ×825

Fig. 4c and d. Rat neocortex after ^{3}H-thymidine application on ED 13, sacrifice on ED 17. Heavily labelled nuclei above (Cajal-Retzius cells) and below (presumptive neurons of Layer VII) the bipolar cortical plate. Sparse labelling of cells in the cortical plate. ×675

Fig. 5. a Rat neocortex after ^{3}H-thymidine application on ED15, sacrifice on ED17. Labelling of numerous nuclei in the bipolar cortical plate. ×530. **b** Rat neocortex after application of a high range dose of ^{3}H-thymidine on ED 17, sacrifice 4 h later. Labelling of nuclei in the upper half of the ventricular layer (survey). 135. **c** Enlarged section of Figure 5b. ×530



4 a b



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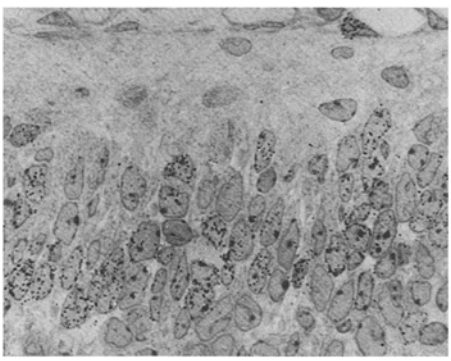
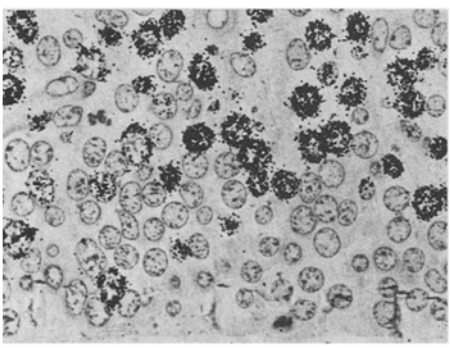


5a



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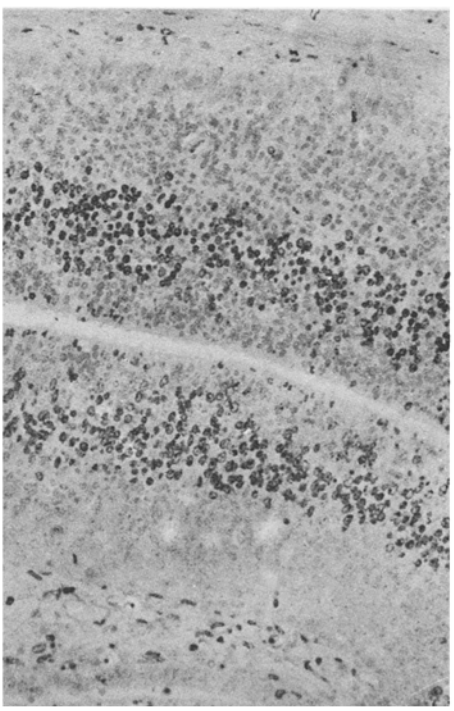

b

resulted in hardly any labelling at all. Injection of radioactive thymidine on ED 13 was followed by labelling of the more differentiated cells above and below the first cells of the bipolar cortical cells, i.e. the Cajal-Retzius cells and the cells of the lower part of the former primordial plexiform layer.

Animals injected on ED 15 show heavily labelled cells, especially in the cortical plate and Layer I.

ED 21

At this day of gestation there are, histologically, no significant differences to be observed between the medial and the lateral parts of the neural wall. The ventricular layer has thinned considerably (50–55 μm) and now contains about 2–4 rows of cells with radially arranged, oval nuclei. The subventricular layer above is about 105 μm wide and shows cells with small, dark polymorphous nuclei without any recognizable orientation of cell axes. Between these cells are numerous horizontal fibre bundles. The border between the subventricular and the intermediate layer is hard to define. Like the subventricular layer, the intermediate layer now consists mainly of cells with dark polymorphous nuclei, ultrastructurally undifferentiated cytoplasm and abundant fibre bundles. Both intermediate and subventricular layer therefore have the appearance of white matter.

The cortical plate has thickened to a width of about 260 μm. The upper part of the cortical plate (bipolar cortical plate) still shows the radial arrangement of cells with large, oval, light nuclei, very densely packed (cells/area index 10). Ultrastructurally, these cells still show an immature appearance with sparse cell organelles and numerous free ribosomes, although the nucleus is less electron dense and the pericaryal cytoplasm seems to have increased. In the lower part of the cortical plate the cells also possess very large, roundish, light nuclei without definite layering. In this part, the internuclear space is larger. Electron microscopically these cells display an abundant pericaryal cytoplasm giving rise to several cell processes, and an accumulation of granular ER which spreads to the basal part of the main dendrite. Although different stages of cytological maturation can be observed in the entire cortical plate – whereby the maturer cells occupy the lower levels – no different types of preneurons can be distinguished. Besides the immature and the more advanced preneurons of the cortical plate, a great number of undifferentiated cells with dark polymorphous nuclei can be observed, predominantly in the lower part. These cells resemble that of the subventricular layer. Layer I has even a greater cell paucity than before. The cell types present are the Cajal-Retzius cells, which display a lighter nucleus than previously, and a still bipolar shaped pericaryon which contains an extensive Nissl body in the vicinity of the nucleus and emits two long, slender, horizontally oriented processes. These cells are still the most mature of the neocortex. The second cell type are the cells with a dark polymorphous nucleus described above. The third cell type is very sparse and resembles the undifferentiated preneurons of the bipolar cortical plate. Ultrastructural examination of the pial basement membrane reveals triangular end-feet containing numerous ribosomes, similar to the pial cell processes observed on ED 13. Rarely can these cell processes be found to originate from the cells with dark polymorphous nuclei of Layer I.

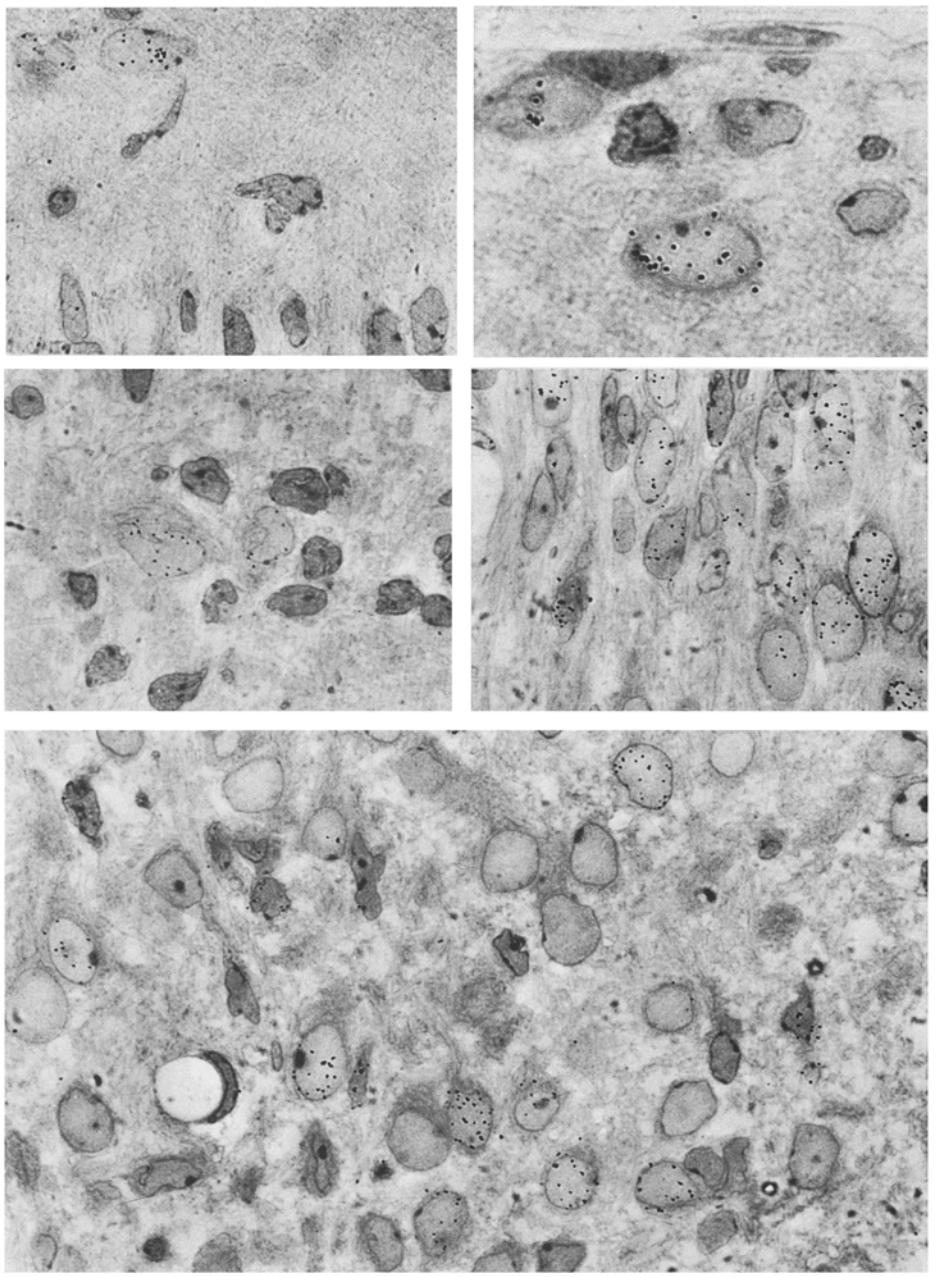

Fig. 6. a and b Rat neocortex after ^{3}H-thymidine application on ED 12, sacrifice on ED 21. Labelled nuclei of Cajal-Retzius cells in Layer I. **a** ×900; **b** ×1440. **c** Rat neocortex after ^{3}H-thymidine application on ED 13, sacrifice on ED 21. Labelled nuclei in the lowest part of the cortical plate (presumptive Layer VII). ×900. **d and e** Rat neocortex after ^{3}H-thymidine application on ED 17, sacrifice on ED 21. Labelled nuclei of cells of the upper (**d**) and lower part (**e**) of the cortical plate. ×900

Injection of ^{3}H-thymidine on ED 9 and ED 11 results in no labelling of nuclei, while animals injected on ED 12 and 13 show labelling of the nuclei of Cajal-Retzius cells (Figs. 6a, b) and the cells in the lowest part of the cortical plate in the lateral part of the neocortex (Fig. 6c), although labelled cells are generally very rare (only 1–3 cells per section). Injection of ^{3}H-thymidine on ED 15 resulted in hardly any labelling of the ventricular, subventricular and intermediate layers. On the other hand, numerous cells of the cortical plate, chiefly those in the lower part, were heavily radioactive. A few of the cells in Layer I, too, showed strong labelling. Most of them are obviously not Cajal-Retzius cells.

The animals which had received ^{3}H-thymidine on ED 17 showed sparse labelling of numerous cells in the ventricular, subventricular and intermediate layer. Heavy labelling is to be seen in the cortical plate. Nearly all nuclei in this layer demonstrated grains. In Layer I hardly any cells were radioactive.

In the medial part of the neocortex, the first labelled cells could, after injection of ^{3}H-thymidine on ED 13, be detected in Layer I (Cajal-Retzius cells) and in the lower part of the cortical plate. Application of radioactive thymidine on ED 15 resulted in a labelling of cells of Layer I as well as of the cortical plate, mainly in its lower part, while animals injected on ED 17 showed grains over the nuclei of cells of the whole cortical plate (Figs. 6d, e).

Discussion

The Ventricular Layer

The ventricular layer is common to all parts of the developing CNS and represents a proliferation zone which gives rise to almost all cells of the central nervous tissue, neurons as well as glial cells. It is generally accepted that neurogenesis precedes gliogenesis (Berry and Rogers, 1967; Fujita, 1967). The ventricular layer passes through certain phases (Kahle, 1951; Fujita, 1967; Smart, 1973) during development of the CNS. Stage I is characterized by a proliferation which results in an increasing number of new stem cells without differentiation into more mature cell types. This means that both daughter cells will remain ventricular cells and take part in further proliferation. The increasing number of ventricular cells causes an enlargement of the surface of the neural wall, but only a slight increase in thickness (Smart, 1973). In stage I of development of the neocortex the ventricular layer consists only of a few rows of radially oriented cells. These cells are ultrastructurally characterized by a fusiform nucleus with dark chromatin clumps, polyribosomes, very little granular ER, some mitochondria and a Golgi complex in a bipolar pericaryon.

The basal cell process of the ventricular cell reaches the cerebral ventricle where it is attached by desmosomes to the neighbouring cell processes. The apical cell process extends to the outer circumference of the neural wall, where it assumes a triangular shape and is attached to the pial basement membrane. Neighbouring cell processes are connected with less pronounced junctions in comparison to the basal ones. These apical end-feet, as upper cell processes of the ventricular cells, can be observed in the EM up to ED 13 in the lateral part of the neocortex and even longer in the medial part.

When the first horizontal axons reach the lateral part of the neocortex beneath the pial membrane, as revealed by Golgi preparations (Marin-Padilla, 1971) and EM, the first ventricular cell nuclei lose their perpendicular orientation and the first preneurons are to be found forming a second distinguishable cell layer. At this time the second phase of development, which will last until the end of the proliferative activity of the ventricular layer, starts (stage II of Kahle, 1951; Fujita, 1967; Smart, 1973) and is characterized by a synchronous proliferation and differentiation, i.e. the mitosis of at least a part of the ventricular cells, produces one daughter cell which migrates towards the pial membrane and begins to differentiate. Our results suggest that the ventricular layer of the lateral part of the neocortex enters stage II between ED 11 and ED 13, while the medial part remains in stage I and so enlarges the surface of the cerebral vesicle (see also Smart, 1973). Interestingly, at this time pial blood vessels start to invade the neural wall (Bär, 1972), possibly due to the fact that the differentiating cells have lost both a pial and a basal cell process. Short-time labelling experiments ($1^1/_2$ resp. 4 h) show that ventricular cells in the S-zone (Fujita, 1967) present a heavy labelling a short time after administration of tritiated thymidine. But ventricular cells of the I-zone are labelled, too, though to a lesser degree and number, indicating that either DNA-synthesis begins after the postmitotic cells have left the M-zone to migrate upwards or persists, when the ventricular cells depart from the S-zone and migrate through the I-zone. The question of whether a ventricular cell in the I-zone is premitotic or postmitotic is not easy to resolve because, contrary to Fujita (1967), we were unable by means of EM-autoradiography to find reliable ultrastructural differences (see also Lyser, 1964). This applies to ED 13 as well as to the later days analysed, while on ED 11 this labelling pattern is less pronounced, a distinction between a M-S-and I-zone being more difficult; all ventricular cells are labelled heavily, possibly due to a shorter generation time (Sidman et al., 1959). Moreover, the ultrastructure of ventricular cells of ED 11 as well as their arrangement are almost the same as on ED 21, as revealed by the cells/area index. There are certain hints that the apical cell process of ventricular cells mentioned above continues to exist until the presumed end of neurogenesis (Berry and Rogers, 1967; Morest, 1970). Ultrastructurally pial end-feet, quite similar to those observed on ED 13, could in fact be detected up to the end of gestation. A persistence of a connection between pial end-feet and ventricular cells beyond ED 13 is confirmed by SEM analysis of prenatal neocortex (Meller and Tetzlaff, 1975) and Golgi preparations (Morest, 1970). These cell processes could conduct the migrating nuclei up to the pial membrane, where in the end the division of the cytoplasm may occur.

The Primordial Plexiform Layer resp. Layer I

Whereas on ED 11 the neural wall consists only of ventricular cells which with their apical and basal cell processes are extended between the pial basement membrane and the ventricular border (Morest, 1970), on ED 13 horizontally oriented axons, presumably thalamocortical fibres (Marin-Padilla, 1971), are detectable ultrastructurally beneath the pial membrane in almost the whole circumference of the neural wall. In the lateral part of the neocortex, a distinct layer is observable even light

microscopically which consists mainly of horizontal fibres and a few cell elements. This layer is named primordial plexiform layer (Marin-Padilla, 1971) according to a comparable layer in adult amphibian cortex or, synonymously, pallial anlage (Rickmann, 1977). As will be explained later, this primordial plexiform layer is not identical with the marginal zone (synonyms: Layer I, molecular layer) of the Boulder Committee (1970). Light and electron microscopically, the cells of the primordial plexiform layer are quite different from ventricular cells. One type (the horizontal cells of Cajal-Retzius (König et al., 1975, 1977; Raedler and Sievers, 1976)), is situated in the upper third of the primordial plexiform layer and shows on ED 13 a horizontally oriented nucleus with a chromatin distribution similar to that of the ventricular cells. The enlarged pericaryon of these cells contains numerous mitochondria, Golgi complexes, microtubules and several profiles of granular ER, and appears to be of bipolar, horizontally oriented, shape. The Cajal-Retzius cells continue to differentiate in the following EDs and maintain their advanced stage of maturation compared with the other neurons of the neocortex. On ED 17 they display a considerable accumulation of granular ER which already begins to take on a parallel array, and on ED 21 a more or less extensive Nissl body is formed.

The typical bipolar shape (Marin-Padilla, 1971; Bradford et al., 1977) of the fetal Cajal-Retzius cells suggests a rather archaic neuronal cell type which, in neocortex, is only comparable with immature preneurons, while all other neocortical neurons differentiate into multipolar cells (Meller and Tetzlaff, 1975).

While administration of ^{3}H-thymidine on ED 9 failed to label any preneurons of the primordial plexiform layer, the Cajal-Retzius cells became heavily labelled when radioactive thymidine was administered on ED 12 and ED 13. Some horizontal cells seem to undergo their last mitosis as early as ED 11, but could be traced only up to ED 13, possibly due to their percentually lower number on later days of gestation. Analysing the day of origin of Cajal-Retzius cells, using a surface index of labelled cells after application of ^{3}H-thymidine on different EDs, we found that most probably the horizontal cells begin to originate on ED 11 and attain their peak on ED 13. The number of newly formed Cajal-Retzius cells decreases considerably towards ED 15 (surface index: 5) and no Cajal-Retzius cells could be detected originating on ED 17. Therefore these cells are regarded as the first preneurons of the neocortex (Duckett and Pearse, 1968; Marin-Padilla, 1971; Raedler and Sievers, 1975, 1976; König et al., 1975, 1977).

On ED 13 a second cell type in the primordial plexiform layer is characterized by an undifferentiated appearance with polymorphous, dark nuclei containing coarse chromatin clumps, and numerous ribosomes, few mitochondria and little rough ER. However, two days later (ED 15), in the lower half of the primordial plexiform layer preneurons are to be found with a lighter round to oval nucleus and an enlarged pericaryon. At the end of gestation, when most of the preneurons of the cortical plate are still rather undifferentiated, these cells display a dispersed nuclear chromatin with a prominent nucleolus, numerous pericaryal cell organelles, and particularly large amounts of rough ER inside an abundant cytoplasm which emits several cell processes.

Labelling experiments revealed that the very first of these preneurons, which from ED 15 to 17 onwards will be situated below the bipolar cortical plate, are born on ED 12. On ED 13 a much greater number of these cells originates. Later on, the

rate of birth of these cells is somewhat difficult to estimate because, ultrastructurally, they hardly differ from the preneurons of the cortical plate. Next to the horizontal Cajal-Retzius cells in the upper part of the primordial plexiform layer, these cells seem to be the most mature ones judged on the basis of the ultrastructural criteria of neuronal differentiation (Marin-Padilla, 1971; Raedler and Sievers, 1975). This would indicate an outside-in gradient within the primordial plexiform layer, as is common to other areas of the CNS (Rakic, 1977). Regarding localisation, stage of maturation and labelling pattern, the preneurons in the lower part of the primordial plexiform layer seem to correspond to the neurons of the prospective Layer VII of Marin-Padilla (1978). By the formation of the bipolar cortical plate on ED 15 to 17 within the primordial plexiform layer (Marin-Padilla, 1971; König et al., 1975; Rickmann, 1977) the latter is divided into an upper part, hence Layer I, and a lower part, the prospective Layer VII (VIb), and the intermediate layer.

In Layer I, few non-Cajal-Retzius preneurons (Bradford, 1977; Rickmann, 1977), in ultrastructural appearance similar to the preneurons of the cortical plate, could be detected from ED15 onwards. We consider these cells to be the non-horizontal cells of Layer I described by Bradford (1977).

Analysing the whole circumference of the neural wall on ED 13 it is obvious that the first components of the developing primordial plexiform layer are horizontal fibres beneath the pial basement membrane. In Golgi studies these cell processes are seen to be of thalamic origin (Marin-Padilla, 1971). Labelling experiments revealed that the first definite postmitotic cells appear shortly after the arrival of these axons. This observation probably further supports the suggestion that these thalamic fibres are responsible for the onset of differentiation of the neocortical preneurons (Morest, 1969; Marin-Padilla, 1971). The multiple contacts between the horizontal fibres and undifferentiated cells could be considered as the substrate of this developmental influence (see also Derer et al., 1977).

The Cortical Plate

The bipolar part of the cortical plate originates on ED 15 in the very lateral aspect of the neocortex and is responsible for the division of the primordial plexiform layer into a superficial Layer I and a basal part, Layer VII and intermediate layer. On ED 17 the formation of the bipolar cortical plate is nearly complete along the whole circumference of the neural wall with the exception of the most medial part of the neocortex. The impression of the cortical plate as a distinct layer is caused by its small internuclear/intercellular spaces and the uniform appearance of its cells. Because of their radial orientation with a basal and an apical cell process originating from a bipolar shaped pericaryon containing only few cell organelles, they resemble the ventricular cells save for a more oval and less electron dense nucleus. Labelling experiments revealed that the vast majority of the preneurons forming the bipolar cortical plate does not originate before ED 15. Numerous cells born on this day in the lateral part of the neocortex do not need more than 48 h to reach the cortical plate. This coincides with the finding of Hicks and D'Amato

(1968) that this time interval seems to be necessary for the migration of preneurons from the ventricular layer to the cortical plate, at least in early neurogenesis.

The fact that cells labelled on ED 15 appear at the end of gestation predominantly in the lower part of the cortical plate justifies the postulation of an inside-out gradient (Angevine and Sidman, 1962; Berry and Rogers, 1965; Langman and Welch, 1967; Hicks and D'Amato, 1968; Shimada and Langman, 1970) which was disputed recently (Bisconte and Marty, 1975; Lund et al., 1977; Smart and Smart, 1977). Postulating an inside-out gradient in the cortical plate, a migration of young preneurons through the already formed part of this layer must be expected (Angevine and Sidman, 1962; Marin-Padilla, 1971; Rickmann, 1977). Considering the small intercellular spaces in the cortical plate, this seems not very probable. The narrow intercellular space, on the other hand, may be the result of migration of younger preneurons into the upper part of this layer.

Although in our experiments an inside-out gradient regarding the date of origin of the preneurons is not very pronounced, there seems to be a strong gradient regarding the onset of maturation. This is possibly due to the presence of large numbers of ingrowing corticopetal fibre bundles (Hicks and D'Amato, 1968; Marin-Padilla, 1971; Rickmann, 1977) below the cortical plate, perhaps triggering first the beginning of differentiation of the neighbouring preneurons (Morest, 1969; Marin-Padilla, 1971).

Intermediate Layer/Subventricular Layer

The intermediate layer originates as the lowest part of the primordial plexiform layer after the formation of the bipolar cortical plate, and is characterized by its large internuclear and in the beginning large intercellular spaces and increasing number of fibre bundles. Besides the migrating bipolar shaped preneurons, the predominating cell type is an undifferentiated cell with a dark polymorphous nucleus. This kind of cell can be observed in Layer I and in the subventricular layer, as well as in the cortical plate, at the end of gestation. Ultrastructurally, these cells resemble the ventricular cells except for the radial orientation of the pericaryon and the fusiform shape of the nucleus.

The failure to detect heavy labelling of their nuclei after longer time intervals (more then two days) after administration of ^{3}H-thymidine, may be due either to a differentiation into the preneuronal cell types described above or to a constant mitotic activity which would define them as glioblasts (Berry and Rogers, 1967; Fujita, 1967). In contrast to Rickmann (1977), we found no reliable ultrastructural features which defined the undifferentiated cells during this part of corticogenesis as future glial cells.

References

Angevine, J.B., Sidman, R.L.: Autoradiographic study of histogenesis in the cerebral cortex of the mouse. Anat. Rec. **142,** 210 (1962)

Bär, T., Wolff, J.R.: The formation of capillary basement membranes during internal vascularisation of the rat's cerebral cortex. Z. Zellforsch. **133,** 231–248 (1972)

Berry, M., Rogers, A.W.: The migration of neuroblasts in the developing cerebral cortex. J. Anat. **99,** 691–709 (1965)

Berry, M., Rogers, A.W.: Histogenesis of mammalian neocortex. In: Evolution of the forebrain, (Hassler, R. and Stephan, H., eds.) pp. 197–205. New York: Plenum Press 1967

Bisconte, J.-C., Marty, R.: Analyse chronoarchitectonique du cerveau de rat par radioautographie. I. Histogenèse du télencéphale. J. Hirnforsch. **16,** 55–74 (1975)

Boulder Committee: Embryonic vertebrate central nervous system: revised terminology. Anat. Rec. **166,** 257–261 (1970)

Bradford, R., Parnavelas, J.G., Liebermann, A.R.: Neurons in Layer I of the developing occipital cortex of the rat. J. Comp. Neur. **176,** 121–132 (1977)

Brückner, G., Mareš, V., Biesold, D.: Neurogenesis in the visual system of the rat. An autoradiographic investigation. J. Comp. Neur. **166,** 245–256 (1976)

Derer,P., Caviness, V.S., Sidman, R.L.: Early cortical histogenesis in the primary olfactory cortex of the mouse. Brain Res. **123,** 27–40 (1977)

Duckett, S., Pearse, A.G.E.: The cells of Cajal-Retzius in the developing human brain. J. Anat. **102,** 183–187 (1968)

Fischer, H.A.: Autoradiography of specimens for electron microscopy-presentation of a method with special remarks on the use of samples from the nervous system. Brain Res. **85,** 237–240 (1975)

Fujita, S.: Applications of light and electron microscopy, autoradiography to the study of cytogenesis of the forebrain. In: Evolution of the forebrain, (Hassler, R. and Stephan, H., eds.), pp. 180–196. New York: Plenum Press 1967

Hicks, S.P., D'Amato, C.J.: Cell migration to the isocortex in the rat. Anat. Rec. **160,** 619–634 (1968)

Kahle, W.: Studien über die Matrixphasen und die örtlichen Reifungsunterschiede im embryonalen menschlichen Gehirn. Dt. Zschr. Nervenheilk. **166,** 273–302 (1951)

Kennedy, L.A., Persaud, T.V.N.: Pathogenesis of developmental defects induced in the rat by amniotic sac puncture. Acta Anat. **97,** 23–35 (1977)

König, N., Roche, G., Marty, R.: The onset of synaptogenesis in rat temporal cortex. Anat. Embryol. **148,** 73–87 (1975)

König, N., Valat, J., Fulcrand, J., Marty, R.: The time of origin of Cajal-Retzius cells in the rat temporal cortex. An autoradiographic study. Neurosc. Lett. **4,** 21–26 (1977)

Langman, J., Welch, G.W.: Excess vitamin A and development of the cerebral cortex. J. Comp. Neur. **131,** 15–25 (1967)

Luft, J.H.: Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9,** 409–414 (1961)

Lund, J.S., Boothe, R.G., Lund, R.D.: Development of neurons in the visual cortex (area 17) of the monkey (macaca nemestrina): A Golgi study from fetal day 127 to postnatal maturity. J. Comp. Neur. **176,** 149–188 (1977)

Lyser, K.J.: Early differentiation of motor neuroblasts in the chick embryo as studied by electron microscopy. I. General aspects. Developm. Biol. **10,** 433–466 (1964)

Marin-Padilla, M.: Early prenatal ontogenesis of the cerebral cortex (neocortex) of the cat (felis domestica). A Golgi study. I. The primordial neocortical organisation. Z. Anat. Entwickl.-Gesch. **134,** 117–145 (1971)

Marin-Padilla, M.: Prenatal ontogenetic history of the principal neurons of the neocortex of the cat (felis domestica). A Golgi study. II. Developmental differences and their significances. Z. Anat. Entwickl.-Gesch. **136,** 125–142 (1972)

Marin-Padilla, M.: Dual origin of the mammalian neocortex and evolution of the cortical plate. Anat. Embryol. **152,** 109–126 (1978)

Meller, K., Tetzlaff, W.: Neuronal migration during the early development of the cerebral cortex. A scanning electron microscopic study. Cell Tiss. Res. **163,** 313–325 (1975)

Morest, D.K.: The growth of dendrites in the mammalian brain. Z. Anat. Entwickl.-Gesch. **128,** 290–317 (1969)

Morest, D.K.: A study of neurogenesis in the forebrain of opossum pouch young. Z. Anat. Entwickl.-Gesch. **131,** 45–67 (1970)

Raedler, A., Sievers, J.: The development of the visual system of the albino rat. Adv. Anat. Embryol. Cell Biol. **50,** Fasc. 3 (1975)

Raedler, A., Sievers, J.: Light and electron microscopical studies on specific cells of the marginal zone in the developing rat cerebral cortex. Anat. Embryol. **149,** 173–181 (1976)

Rakic, P.: Genesis of the dorsal lateral geniculate nucleus in the Rhesus monkey: Site and time of origin, kinetics of proliferation, routes of migration and pattern of distribution of neurons. J. Comp. Neur. **176,** 23–52 (1977)

Rickmann, M., Chronwall, B.M., Wolff, J.R.: On the development of nonpyramidal neurons and axons outside the cortical plate: The early marginal zone as a pallial anlage. Anat. Embryol. **151,** 285–307 (1977)

Schultze, B., Maurer, W., Hagenbusch, H.: A two emulsion autoradiographic technique and the discrimination of the three different types of labelling after double labelling with ^{3}H- and ^{14}C-thymidine. Cell Tissue Kinet. **9,** 245–255 (1976)

Shimada, M., Langman, J.: Cell proliferation, migration and differentiation in the cerebral cortex of the golden hamster. J. Comp. Neur. **139,** 227–244 (1970)

Sidman, R.L., Miale, I.L., Feder, N.: Cell proliferation and migration in the primitive ependymal zone: An autoradiographic study of histogenesis in the nervous system. Exper. Neurol. **1,** 322–333 (1959)

Smart, I.H.M.: Proliferative characteristics of the ependymal layer during the early development of the mouse neocortex: a pilot study based on recording the number, location and plane of cleavage of mitotic figures. J. Anat. **116,** 67–91 (1973)

Smart, I.H.M., Smart, M.: The location of nuclei of different labelling intensity in autoradiographs of the anterior forebrain of postnatal mice injected with ^{3}H-thymidine on the eleventh and twelfth days post-conception. J. Anat. **123,** 515–525 (1977)

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