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CELL DEATH AND REMOVAL IN THE CEREBRAL CORTEX DURING DEVELOPMENT

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1. INTRODUCTION

Naturally occurring cell death is a common phenomenon during normal development of the nervous system. The majority of observations have been made in birds, reptiles and amphibians (Cowan, 1973; Oppenheim, 1981, 1985; Cowan *et al.*, 1984; Bottjer and Sengelaub, 1989). Cell death also occurs in mammals during embryonic and postnatal life. In mammals, cell death has been observed in the spinal cord (Nurcombe *et al.*, 1981; Banker, 1982; Lance-Jones, 1982; Baulac and Meininger, 1983; Bennett *et al.*, 1983; Harris and McCaig, 1984; Comans *et al.*, 1987) and retina and its connections, including the lateral geniculate nucleus, optic tectum and colliculus (Arees and Astrom, 1977; Heuman and Rabinowicz, 1980, Sengelaub and Finlay, 1982; Cunningham *et al.*, 1982; Finlay *et al.*, 1982; Perry *et al.*, 1983; Dreher *et al.*, 1984; Sengelaub *et al.*, 1985; Penfold and Provis, 1986; Sengelaub *et al.*, 1986; Provis and Penfold, 1986; Finlay and Pallas, 1989), as well as in the parabigeminal nucleus of the rat (Linden and Piñón, 1987) and the cerebellum of the hamster (Janowsky and Finlay, 1983).

Naturally occurring cell death has also been found in the hippocampus of hamsters and rats (Janowsky and Finlay, 1983; Ferrer *et al.*, 1990b), pyriform cortex of mice (Friedman and Price, 1986) and neocortex of the hamster (Finlay and Slattery, 1983), mouse (Pearlman, 1985), cat (Ferrer *et al.*, 1989) and rat (Ferrer *et al.*, 1990a). Cell death in the cellular cortical layers during postnatal development in these studies correlates with other observations showing decreased numbers of cortical neurons in adults when compared with young animals (Heuman and Leuba, 1983; Ferrer *et al.*, 1988).

In addition, one of the most impressive structural transformations of the telencephalic mantle during

normal development comes up in the subplate zone, a transitory neuronal and nerve fiber organization located below the cortical plate which progressively disappears near birth. Most subplate neurons fade away by cell death (Kostovic and Rakic, 1980, 1990; Luskin and Shatz, 1985; Valverde and Facal-Valverde, 1987; Chun *et al.*, 1987; Shatz *et al.*, 1988; Chun and Shatz, 1989a; Ferrer *et al.*, 1990a). Some cells of the subplate do survive, nevertheless, as interstitial neurons of the white matter in adults (Kostovic and Rakic, 1980, 1990; Shatz *et al.*, 1988; Chun and Shatz, 1989b; Valverde and Facal-Valverde, 1988).

The present study is focused on naturally occurring postnatal cell death in the cerebral neocortex, cortical subplate and future subcortical white matter, and its relation to other progressive and regressive phenomena during normal development of the cerebral cortex.

Our knowledge about the mechanisms producing cell death is still fragmentary, and the reasons for cortical cell death remain elusive. Therefore, experiments have been conducted to gain more information in this field.

Finally, it has been shown that cellular debris is removed by neighboring cells and transitory exogenous phagocytes which penetrate the nervous tissue at early stages of development. These latter cells correspond to classical nascent or ameboid microglial cells, the function of which was, until recently, barely understood.

2. MORPHOLOGY OF DEAD CELLS

Dead cells are recognized in paraffin and semithin sections by their extremely shrunken and dark nucleus, which often fragments into several small and dense droplets. The cytoplasm may be preserved, or round and eosinophilic, or almost absent, depending on the stage (Fig. 1). Degenerating cells are also stained by silver methods (de Olmos and Ingram, 1971) during normal development and under different pathological conditions.

Characteristically, cell death in the cerebral cortex and future subcortical white matter affects scattered individual cells or cells in small clusters.

The term *pyknosis* designates the condensation of the cellular components, and the term *pyknotic cells* has been used to name degenerating or dying cells in the developing nervous tissue (Finlay *et al.*, 1982; Sengelaub and Finlay, 1982; Finlay and Slattery, 1983; Sengelaub *et al.*, 1985; Finlay and Pallas, 1989). Although pyknosis does not always imply irrecoverable cellular damage and cell loss (Cammermeyer, 1972), the appearance of the nucleus of degenerating cells, including fragmentation, is hardly consistent with the preservation of life. Pyknosis is also a nonspecific term since pyknotic cells may be found in two different types of cell death, necrosis and apoptosis, the characteristics of which will be discussed in the following paragraphs.

Electron microscopic morphology of naturally occurring dead cells during development of the nervous system has been described (Cantino and Sisto, 1972; Pannese, 1976; Pilar and Landemesser, 1976; Chu-Wang and Oppenheim, 1978; Sohal and Weidman, 1978; Giordano *et al.*, 1980; Cunningham, 1982; Warton and Jones, 1984; Trujillo *et al.*, 1987; Marrero *et al.*, 1987; Valverde and Facal-Valverde, 1987; Ferrer *et al.*, 1989, 1990a). There is the general agreement that most degenerating cells display complete chromatin condensation, polysomal disaggregation and increased numbers of cytoplasmic vesicles, together with cytoplasmic shrinkage. Other cells show fragmentation of the nucleus and vacuolated cytoplasm. Finally, some cells exhibit not only mild chromatin compaction, but also striking dilatations of the rough endoplasmic reticulum, Golgi complex and nuclear envelope. The first and third types correspond, respectively, to the so-called nuclear and cytoplasmic types of cell death (Pilar and Landmesser, 1976). The third type also coincides with type 3B of others (Clarke, 1990). Nuclear cell death corresponds to cell death type I described in embryonic and fetal rats (Scweichel and Merker, 1973), shrinkage necrosis (Kerr, 1971), and primary or precocious 'picnosis' (Beaulaton and Lockshin, 1982).

Our own studies on cell death in the cerebral cortex and sub cortical white matter of developing rats and cerebral cortex of kittens have shown that the most common type of cell death is characterized by primary nuclear damage and fragmentation which is followed by cytoplasmic abnormalities. Degenerating cells show chromatin condensation, often into coarse granular aggregates, dispersion of the nucleolus and fragmentation of chromatin masses. The cytoplasm shows, at first, only mild changes, and the rough endoplasmic reticulum, Golgi complex and mitochondria are well preserved, even in cells with an extremely shrunken nucleus. Later on, the cytoplasm is condensed and eventually fragments into small bodies which are engulfed by neighboring cells or by specialized phagocytes (Fig. 2).

The term apoptosis has been introduced to name a type of cell death with the morphological characteristics just described above. This type of cell death occurs in many other tissues in normal development, normal cellular turnover, and different pathological conditions including tumors (Kerr *et al.*, 1972, 1987; Wyllie *et al.*, 1980, 1984; Wyllie, 1981, 1987; Cotter *et al.*, 1990; Clarke, 1990).

In contrast to necrosis, apoptosis is a highly selective process which affects isolated cells or cells in small clusters, is usually triggered by physiologic stimuli, and massive inflammatory infiltrates (i.e. neutrophils or lymphocytes) are always absent (Wyllie *et al.*, 1980). In other cell systems, apoptosis does not depend on the external energy supply, but involves endonuclease activation (Wyllie *et al.*, 1980, 1984; Wyllie, 1987; Kondo, 1988; McConkey *et al.*, 1990). Nuclear cell death, morphologically analogous to apoptosis in other tissues is the most common type in the cerebral cortex and future subcortical white matter during normal development.

3. DISTRIBUTION OF DEAD CELLS IN THE TELENCEPHALIC MANTLE OF THE DEVELOPING RAT

As previously mentioned, cell death in the telencephalic mantle during normal development occurs in

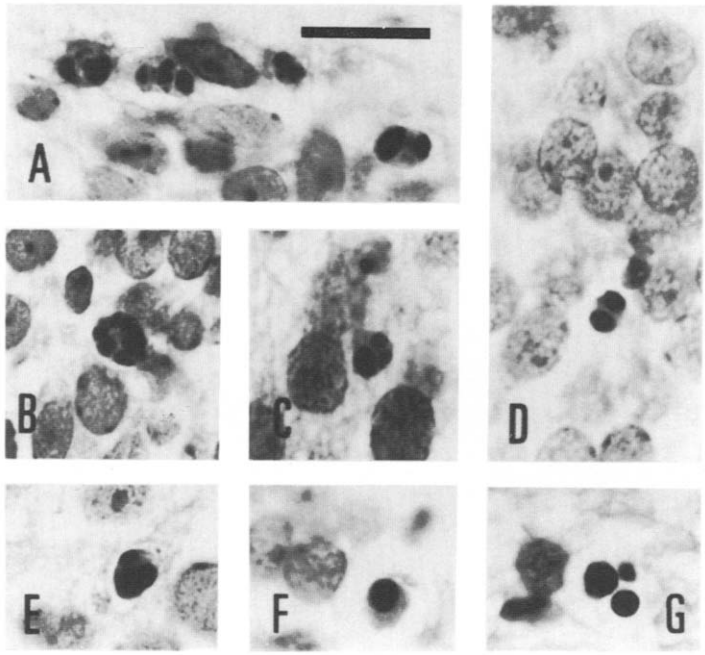

FIG. 1. Dead cells in the developing cerebral cortex and subcortical white matter of the rat. These cells are characterized by their extremely dark and shrunken, sometimes fragmented nuclei. The cytoplasm is pale or eosinophilic depending on the stage of cell death. A—Retrosplenial cortex, upper cortical layers; B—Somatosensory (SS) cortex, layers II–III; C—SS cortex, layer V; D—Occipital cortex, layers II–III; E—SS cortex, layer VIb; F and G—Subcortical white matter. A, C, D and F: Rats aged 5 days. B, E and G: Rats aged 7 days. Paraffin sections stained with hematoxylin and eosin. Bar = 20 microns.

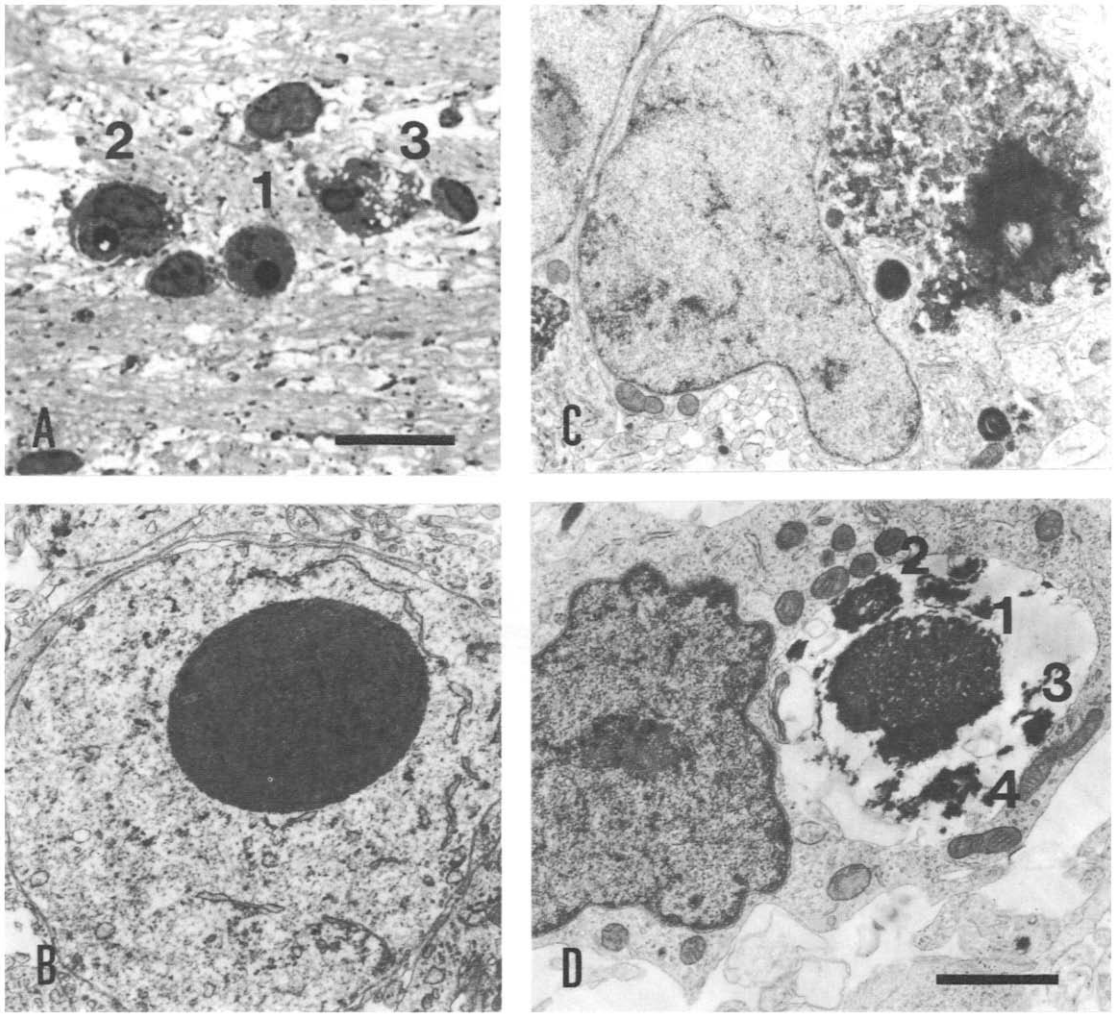

FIG. 2. Dead cells in the developing cortex of the rat. A—Semithin section of the subcortical white matter showing a dying cell with extremely dark and shrunken nucleus (1) a phagocyted dead cell (2) and one macrophage filled with clear cytoplasmic vacuoles (3) epon section stained with toluidine blue; B—Nuclear type of cell death in layers II–III of the cerebral cortex. The nucleus is extremely condensed but membranes and cytoplasmic organelles are preserved; C—Granular nuclear debris engulfed by a poorly-differentiated cell in the upper cortical layer; D—Fragmented nuclear debris (1–4) engulfed by a macrophage in the subcortical white matter. A and D: Rat aged 3 days. C and D: Rat 5 days old. A, bar = 20 microns. B, C and D, same magnification bar = 2 microns.

two different sites. One of them is the future subcortical white matter which is located between layer VIb and the periventricular germinal layer of the lateral ventricles. The other site is the cerebral cortex which includes the future layers I to VIb.

The distribution of dead cells in the cerebral cortex and subcortical white matter of the rat at representative postnatal ages is shown in Fig. 3.

The term 'cerebral cortex' is used here to name the cortical layers I to VIb.

Dead cells first appear, in small numbers, shortly before birth (embryonic days 21 and 22) in the subcortical white matter of the retrosplenial granular cortex and the border region between the retrosplenial cortex and subicular complex.

Dead cells increase in number in the subcortical white matter during the first week of postnatal life (postnatal days 0 to 7: P0 to P7) and decrease, thereafter, until about the end of the second week, at which time dead cells are rarely encountered in the

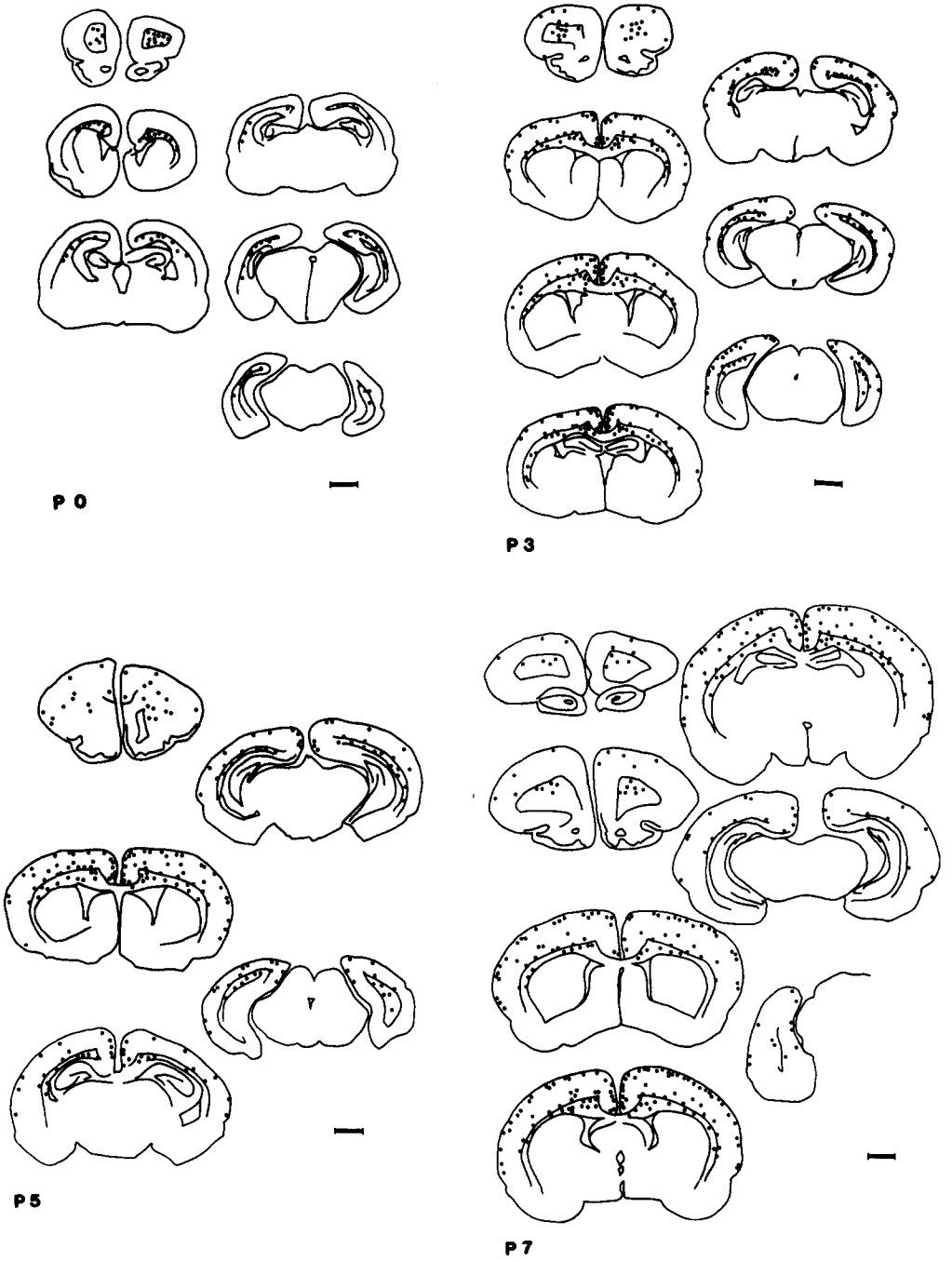

FIG. 3 (*caption overleaf*)

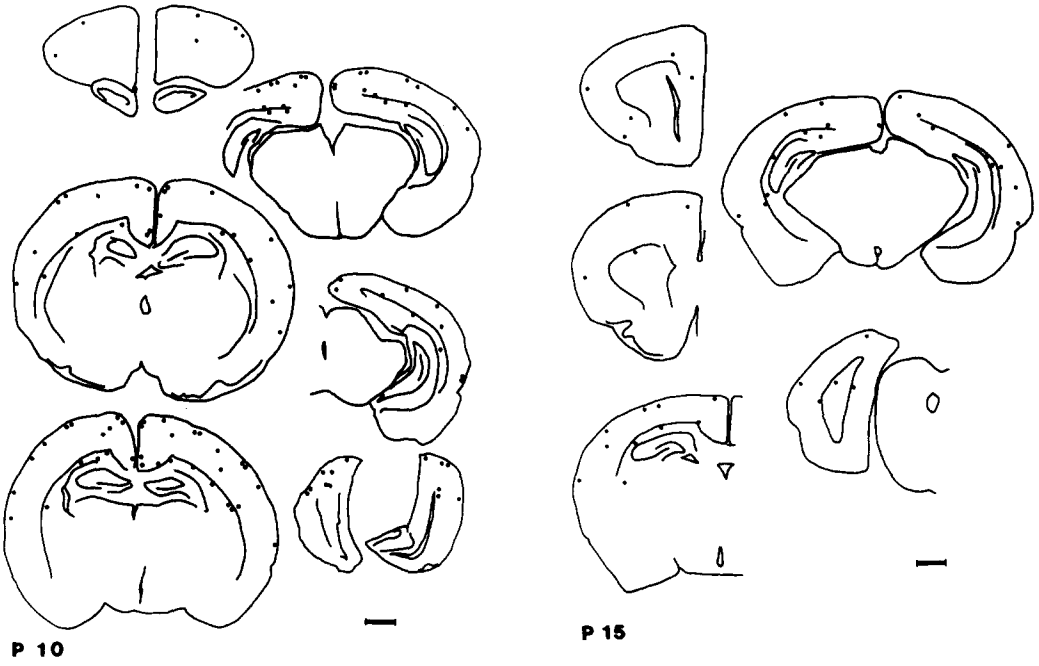

FIG. 3. Distribution of dead cells in the cerebral cortex and subcortical white matter of the developing rat at different postnatal days: 0 (newborn), 3, 5, 7, 10, 15 (P0, . . . , P15). Each dot represents one dead cell. Dead cells first appear in the subcortical white matter at P0. Later on, dead cells are found in the subcortical white matter and cerebral cortex as well. The number of dead cells increases from P0 to P7 and decreases thereafter. Cell death is almost absent in the rat aged 30 days (not shown). At every age, cortical dead cells predominate in the upper cortical layers (layers II–III) and in layer VIb. Whatever the cortical region, the number of dead cells is greater in the medial than in the lateral part of the cerebral hemisphere. At every age, the number of dead cells is greater in the parietal than in the frontal and occipital lobes. Camera lucida drawings of paraffin-embedded, hematoxylin and eosin-stained sections. Bar = 1 mm.

white matter. Dead cells in the future subcortical white matter of the postnatal rat predominate in the region underlying the cingulate and retrosplenial granular cortices, medially, and the white matter of the frontal (FR) region and parietal region 1 (Par 1) of the somatosensory cortex, laterally. Dead cells are lower in number, at every age, in the subcortical white matter of the lateral part of the somatosensory cortex (parietal 2: Par 2), occipital cortex and temporal region.

In the cerebral cortex, dead cells are seldom found at birth, but augment from P3 onwards to reach peak values at the end of the first week. Dead cells lessen during the second week and are no longer observed at the end of the first month. The majority of dead cells, at every age, are found in the upper cellular layers (layers II–III), which are recognized as separate layers at about P7–P8. This predominance is particularly marked at the end of the first week. Cell death also occurs in layers V and VIa, but is more common, mainly in relation to the total number of regional neurons, in layer VIb.

A few dead cells are also seen in the molecular layer during the first week of postnatal life.

Regional differences in the amount of dead cells also exist throughout development. Cell death predominates in the posterior cingulate cortex (Cg 2 and caudal part of Cg 1 regions), together with retrosplenial granular and agranular (RSA and RSG) cortices. It is followed by Par 1 and Par 2 of the somatosensory cortex, and the forelimb and hindlimb (FL and HL) regions of the motor cortex. Cell death is less frequent in the occipital (either in the Oc 1 or Oc 2 subdivision), frontal and temporal cortices.

In general terms, cell death in the neocortex follows a rough ascending gradient from the subcortical white matter to the upper cortical layers during the first week of postnatal life. Later on, from P10 onwards, dead cells are more disperse. This mimics the ascending (inside-out) gradient of neuroblast migration in the cerebral cortex of rodents (Angevine and Sidman, 1961; Berry and Rogers, 1965; Hicks and D'Amato, 1968; Smart and Smart, 1982).

4. QUANTITATIVE ASPECTS OF CORTICAL CELL DEATH IN THE RAT AND COMPARISON WITH CELL DEATH IN THE CEREBRAL CORTEX IN OTHER SPECIES

The magnitude of cortical cell death during normal development in the rat is illustrated in Tables 1 and 2. In these tables, the amount of cell death in the somatosensory and medial neighboring cortical areas, and the primary visual cortex (Oc 1 M) is expressed as the proportion of cortical dead cells per

1000 live neurons. Methods for the quantification of neurons and correcting factors used have been described in detail elsewhere (Ferrer *et al.*, 1990a). These data together with those provided in Fig. 3 permit us to draw some conclusions.

(1) Cell death is rather synchronous in the different cortical regions, and temporal gradients of cell death along the rostrocaudal and lateromedial axes are not seen in rats.
(2) Regional differences exist, nevertheless, in the amount of cell death. Thus, the proportion of dead cells in the medial part (Par 1) of the somatosensory cortex at P7 is 2.38 ± 0.37 dead cells per 1000 live neurons, whereas in the medial part of the primary visual cortex (Oc 1 M) the proportion of dead cells is 1.29 ± 0.48 at the same age.
(3) At every rostro-caudal level, cell death is more prominent in the medial than in the lateral parts of the cerebral cortex. As a paradigm, the greatest values of cell death are encountered in the retrosplenial cortex with 4.82 ± 1.10 and 3.91 ± 1.21 dead cells per 1000 live neurons at P5 and P7 respectively.

Cell death rates are slightly variable in mice and hamsters. Peak values of 4 dead cells per 1000 live neurons are found at P6, and values rapidly decrease at day 10 in the visual cortex of the mouse (Pearlman, 1985).

In the occipital cortex of the hamster, peak values at day 7 vary from less than 8 per 1000 live neurons in areas 17 and 18 b to 30 per 1000 in area 29 b (Finlay and Slattery, 1983). In contrast to the hamster, values of cortical cell death and differences in the amount of cell death among the subdivisions of the occipital cortex are almost negligible in the Wistar rat. For example, the following values were found in the occipital subdivisions of rats: Oc 2 M: 1.37 ± 0.38; Oc 2 L: 1.05 ± 0.27; Oc 1 M: 1.29 ± 0.48; Oc 1 L: 1.06 ± 0.19 dead cells per 1000 live neurons at P7 (mean values $\pm$ SD of 16 tissue sections from 8 animals).

TABLE 1

Days	Control	Enucleated
2	0.38 ± 0.11	0.29 ± 0.09
5	0.77 ± 0.41	0.89 ± 0.33
7	1.29 ± 0.48	1.27 ± 0.37
10	0.94 ± 0.29	1.08 ± 0.31
15	0.81 ± 0.36	0.92 ± 0.38
21	0.10 ± 0.28	0.25 ± 0.19

Number of dead cells (mean values $\pm$ SD) per 1000 live neurons in the primary visual cortex (Oc 1 M) of normal rats and rats bilaterally enucleated at birth, at different postnatal ages. Each value at every age corresponds to the examination of 9 or 10 animals (2 tissue sections per rat). Methods for the quantification of dead cells, neurons and correcting factors used are similar to those reported elsewhere (Ferrer *et al.*, 1990a). The nomenclature of cortical areas throughout the text follows Zilles (Zilles, 1985).

No differences are seen in the proportion of dead cells in both groups of rats. This suggests that elimination of main visual afferents at birth has no effect on naturally occurring cell death in the primary visual cortex.

TABLE 2

	Control		100 cGy/E18	
Days	Neurons	Dead cells	Neurons	Dead cells
2	11490 ± 1280	0.25	9610 ± 1430*	0.54
5	10560 ± 1493	1.11	8128 ± 1266*	1.01
7	9438 ± 1017	2.38	7155 ± 958*	0.97**
10	7380 ± 987	1.89	6023 ± 1038*	0.70**
15	5684 ± 735	0.55	4445 ± 820*	0.22
21	4815 ± 646	0.20	3288 ± 530*	0.11
mean accumulative cell death across time		6.38		3.55***

Number of neurons per section (mean values $\pm$ SD) and number of cortical dead cells (mean values) per 1000 live neurons in the cerebral cortex in control and X-irradiated (100 cGy) rats at embryonic day 18 (E18), examined at different postnatal ages. The quantitative study is focused on the cortical region between the callosal and rhinal fissures which included parietal regions 1 and 2 (Par 1 and Par 2) of the somatosensory cortex, and the fields HL (motor cortex, hindlimb), frontal regions FRl and FR2, and retrosplenial agranular and granular (RSA and RSG) cortices (see Zilles, 1985, for nomenclature of cortical areas).

Significant reductions in the number of cortical neurons were seen at every age in irradiated animals when compared to age-matched controls (Student's t test, $p < 0.05$). The proportion of dead cells per 1000 live neurons was significantly decreased in irradiated animals aged 7 and 10 days (**) (Student's t test, $p < 0.05$), As a result, the cumulative cell death across time was significantly decreased in the irradiated group (***) (Mann-Whitney U test, 2-tailed $p < 0.05$).

Whether these variations are related to species differences or are due to albinism is still not known.

In other animals, cortical cell death occurs shortly before birth. In insectivorous bats of the species *Myotis myotis*, massive cell death in the future subcortical white matter and cerebral cortex occurs in the preterm animal (Fig. 4); cortical cell death is almost absent in the newborn bat. It is noteworthy that the cerebral cortex is well developed at birth in these animals (Ferrer, 1989), and naturally occurring cell death at the preterm period could be an expression of the precocious cortical maturation in this species. Cortical cell death during normal development is a more complex and less understood phenomenon in gyrencephalic species. In the gyrus suprasylvius and adjoining sulci in the cat, mean values of cell death in animals aged 15 and 25 days vary from 5–10 per 1000 live neurons in the sulcal region to 2–4 per 1000 in the crown of the gyrus (Ferrer *et al.*, 1989). In addition, the amount of cell death progresses from the already formed suprasylvian sulcus to the formative sulcus lateralis (Ferrer *et al.*, 1988, 1989). Therefore, naturally occurring cell death in the cat proceeds following spatial or regional (i.e. more dead cells in sulcal regions than in gyri), and temporal (i.e. dead cells first appear in already formed sulci, and later in formative sulci) gradients. Based on the different percentages of cell death in the occipital cortical areas of the hamster, it has been suggested that the amount of cell loss is inversely related to numbers of neurons in adults in corresponding areas (Finlay and Slattery, 1983). Similar observations can be made in the cingular cortex of the rat and in the sulcal regions of

the cat, in which the number of neurons in adults is smaller than in the frontal and gyral zones, respectively. However, it must be stressed that cell death is not a major determining factor in the early formation of sulci in the cat (Ferrer *et al.*, 1989), nor in the gyrus cinguli in the rat (Ferrer *et al.*, 1990a), since these folds are already formed before dead cells appear in the cerebral cortex.

5. CELL DEATH IN THE CORTICAL SUBPLATE AND FUTURE SUBCORTICAL WHITE MATTER

5.1. General Aspects of the Cortical Subplate

As mentioned in previous sections, the cortical subplate contains fibers and neurons. Cortical afferents include thalamocortical and monoaminergic systems which wait for a certain period of time in this embryonic region before entering into the cortical plate (Molliver and van der Loos, 1970; Rakic, 1977; Lidov and Molliver, 1978; Kostovic and Rakic, 1980, 1990; Kordower and Rakic, 1986); thus the subplate can be considered a waiting compartment for afferent systems.

The second component of the subplate is formed by a diversified population of transitory neurons, collectively known as the subplate neurons, which are cogenerated with neurons of the marginal layer and are therefore some of the first postmitotic neurons of the telencephalic mantle (Marin-Padilla, 1970, 1971, 1978; Kostovic and Rakic, 1980, 1990; Luskin and Shatz, 1985; Chun *et al.*, 1987; Valverde and Facal-Valverde, 1987, 1988; Shatz *et al.*, 1988; Chun and Shatz, 1988a, 1988b, 1989a, 1989b; Huntley *et al.*, 1988; Friauf *et al.*, 1989; Antonini and Shatz, 1990). These neurons exhibit mature morphological features including well developed dendrites and synapses, cytoskeletal and calcium-binding proteins, neurotransmitters and neuropeptides, together with the capacity to evoke synaptic potentials and establish long axonal connections. During the 'waiting period' of thalamic afferents, subplate cells act as transient postsynaptic targets for these fibers (see Kostovic and Rakic, 1980, 1990). Recent studies have shown that the experimental ablation of subplate cells prevents the entry of thalamic fibers into the depleted region, thus suggesting that these cells guide thalamocortical fibers into the cortical plate (Friauf *et al.*, 1990; Ghosh *et al.*, 1990).

In addition, subplate cells also pioneer long projections to the thalamus and superior colliculus, as well as to the cortical plate (McConnell *et al.*, 1989; Antonini and Shatz, 1990). Efferent connections are probably mediated by neurotrophic interactions (Allendoerfer *et al.*, 1989). Later on, most subplate cells die, although some of them survive as interstitial neurons of the white matter.

Our knowledge of the composition and fate of subplate cells in the rodent brain has increased in recent years (Valverde *et al.*, 1989; Bayer and Altman, 1990; Cobas *et al.*, 1991), but is still fragmentary. For example, cell death in the cortical subplate of rodents is still controversial (Valverde *et al.*, 1989; Ferrer *et al.*, 1990a). We do not know whether transient expression of neurotransmitters in subplate cells eventually fades by cell death, as happens in kittens and monkeys.

Furthermore, studies of cortical development '*in vitro*' using organotypic slice cultures have not provided clear results. Whereas Bolz *et al.* (1990) suggested that subplate cells are not required for the settlement of corticothalamic and corticocortical connections, Blakemore and Molnar (1991) have suggested that the formation of similar connections at very early stages of development involves subplate cells.

For this reason we will examine the cellular and fibrillar composition of the cortical subplate, and intermediate zone–subventricular border (future subcortical white matter) in the brains of mice (and rats) before describing naturally occurring cell death in these regions.

5.2. Structural Organization of the Rodent Subplate and Future Subcortical White Matter

The cortical subplate in the mouse first appears at embryonic day 13 (E13), as a thin band of cells located just below the emerging cortical plate (Fig. 5A). Together with the marginal zone, the subplate derives from the primitive plexiform layer which surrounds the germinal ventricular zone at earlier embryonic stages. As the cortical plate grows by the addition of newly-generated postmitotic neurons, the subplate becomes a clearly individualized layer (Figs 5C and D), located above the intermediate and subventricular zones and the germinal ventricular zone.

Infragranular layers VIa and V first appear at E17, whereas layers IV to II appear a few days after birth. During the early postnatal period the subplate probably reorganizes to form the sublayer VIb, which is separated from layer VIa by a thin sparsely cellular band (Fig. 5E).

These observations agree with previous reports (Boulder Committee, 1970; Rickman *et al.*, 1977; Crandall and Caviness, 1984a and b; Valverde *et al.*, 1989; Bayer and Altman, 1991), and underline the fact that layer VIb is a particular cortical structure in rodents.

The neuronal composition of the subplate in the mouse (NMRI strain) was investigated by immunocytochemistry with antibodies against a variety of neuronal markers including microtubule-associated protein-2 (MAP2, Sternberger Meyer, used at a dilution 1:3000); neurotransmitters: gamma-aminobutyric acid (GABA, Immunotech, 1:2500) and glutamate (kindly supplied by Dr C. Matutes; also used in the brain of the rat); neuropeptides: neuropeptide Y (NPY, CRB, 1:2000), somatostatin (SRIF, Dakopats, 1:800), substance P (SP, Seralab, 1:1000) and cholecystokinin (CCK, Immunonuclear, 1:1500); and the calcium-binding protein calbindin (CaBP, kindly given by Dr M. R. Celio). Some results of the study have been reported elsewhere (Del Rio *et al.*, 1991a and b).

During the entire prenatal period examined (from E13 to E19), strong-labelled MAP2-immunoreactive cells heavily populated the subplate, and, more sparsely, the marginal (molecular) layer (Figs 5B, 6A). Large numbers of faintly-stained cells were

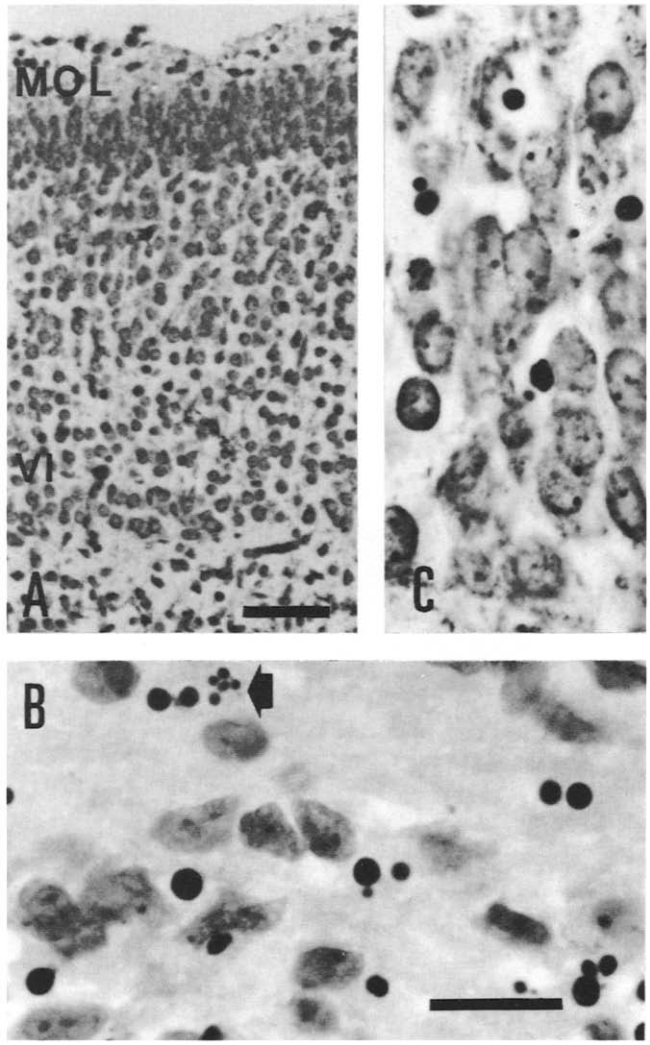

FIG. 4. Cell death in the cerebral cortex and subcortical white matter in the preterm bat *Myotis myotis*. A—General view of the cerebral cortex showing the molecular (MOL) and layer VI (VI); B—Dead cells in the subcortical white matter of the gyrus cinguli; C—Dead cells in the inner layers of the cerebral cortex. A: Hematoxylin and eosin; bar = 50 microns. B and C: Silver method; same magnification, bar = 20 microns.

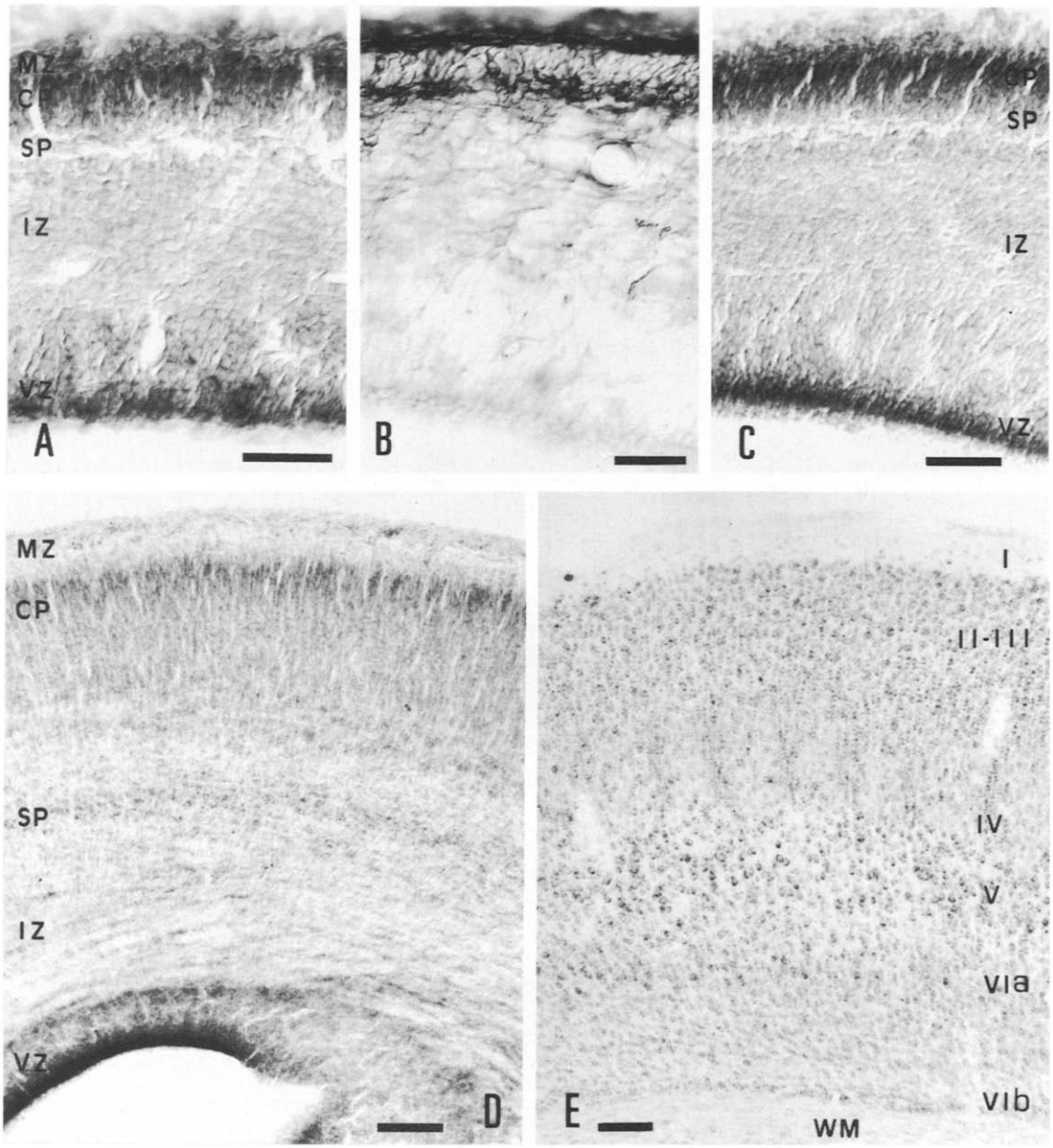

FIG. 5. Cytoarchitectonic layers of the telencephalic mantle of the mouse during development. A—Embryonic day 13 (E13); C—E14; D—E17; and E—Postnatal day 16 in Nissl preparations. B—Immunocytochemistry with antibodies against MAP2 at E13 of a brain region similar to that seen in B. MZ: marginal zone; CP: cortical plate; SP: cortical subplate; IZ: intermediate zone; VZ: ventricular zone. I–VIb: cortical layers I to VIb; WM: white matter. Note the marked development of the cortical subplate at embryonic day 17. A, B and C, bar = 50 microns; D and E, bar = 100 microns.

found in the lower region of the intermediate zone (intermediate zone-subventricular border population), and in the ventricular zone (Figs 6A, 7E). A few cells were also seen in the cortical plate and intermediate zone.

In the cortical subplate and intermediate zone, MAP2-ir neurons were large multipolar, bipolar or polymorphic cells, the dendrites of those located in the subplate zone being arranged tangentially or penetrating into the intermediate zone (Fig. 7A).

In contrast, MAP2-ir cells in the intermediate zone–subventricular border were smaller in size and displayed horizontally-oriented dendrites (Fig. 7E).

From E13 onwards in the mouse, GABAergic cells were found in the subplate, intermediate zone and intermediate zone–subventricular border (Figs 6B, 7B, 7C). A similar distribution of glutamatergic cells was observed in the cerebral cortex of the rat from E15 to E20 (Figs 6C, 7C, 7D). With both antibodies, neurons in the marginal zone were intensely labelled, whereas only a few cells were immunoreactive in the cortical plate. The morphology of GABA-ir and glutamate-positive neurons was similar to that observed with antibodies against MAP2. These populations of neurons became more conspicuous during the last days of the gestational period.

Subplate cells were also labelled with antibodies against calbindin during the prenatal period from E14 to E18 (Fig. 6E), although CaBP immunoreactivity decreased at the end of gestation, and reappeared in the cerebral cortex during postnatal development. Neurons in the ventricular zone were not labelled with this antibody. No positive staining for parvalbumin, another Ca^{2+} binding protein (Celio, 1990), was observed during the fetal period studied.

Subplate neurons were also the first neurons to be labelled with antibodies against NPY, CCK and SRIF from E17 onwards (Fig. 6D). Neuropeptide-immunoreactive cells in the subplate were particularly abundant during the perinatal period. In contrast with these observations, substance P-like positive cells have been found in the mouse cortical subplate only during the early postnatal period (Del Rio *et al.*, 1991a).

Our observations on the immunostaining characteristics of subplate cells agree with, and extend, previous studies in rodents regarding GABA (Chronwall and Wolff, 1980; Lauder *et al.*, 1986; van Eden *et al.*, 1989; Cobas *et al.*, 1991), MAP2 (Crandall *et al.*, 1986; Cobas *et al.*, 1991), calbindin (Enderlin *et al.*, 1987), and neuropeptides (McDonald *et al.*, 1982; Brené *et al.*, 1990; Hajós *et al.*, 1990).

Taken together these results demonstrate that subplate neurons in the rodent brain appear early during the prenatal period and exhibit a high degree of morphological maturity as shown by their developed dendritic arbors. Furthermore, subplate neurons constitute a very diversified and rich population as revealed by the different immunoreactive expressions for several neurotransmitters, neuropeptides and calbindin.

Although colocalization studies are needed to elucidate the degree of overlapping of the different immunocytochemically-labelled neuronal populations, it is feasible that, in addition to excitatory glutamatergic cells, inhibitory GABAergic neurons can be categorized into different subsets according to their different neuropeptide or CaBP contents, as happens in the cerebral cortex of adults (Jones and Hendry, 1986).

It is worth stressing that, in addition to subplate neurons, another neuronal population appears very early during the prenatal period in the rodent brain. These neurons (Fig. 7E) are located in the intermediate zone–subventricular border and are smaller than subplate neurons. These cells react with antibodies against MAP2, GABA and glutamate, and, as we will describe later, they are not related to thalamic axons (Figs 7E, F).

5.3. Relationships Between the Subplate and Intermediate Zone–Subventricular Border and the Development of Axonal Systems

One of the most typical features of the subplate in kittens and primates is the close relationship between subplate neurons and different afferent systems, particularly thalamocortical axons. This fact provides the morphological basis for the general agreement that subplate neurons may act as temporary recipients for these axonal systems.

To reinvestigate this possibility in the rodent brain small crystals of the lipophyllic dye DII (Molecular Probes) were placed into the dorsal thalamus at different prenatal ages. From E14 (where the first fibers are seen in the cortex) to E16, bundles of thalamic axons penetrated laterally into the telencephalic mantle through the internal capsule and populated exclusively the subplate. Thalamocortical fibers which went across the intermediate zone to the subplate progressed tangentially to the surface towards the cingulate region (Fig. 8A).

Axonal growth cones were easily seen in this system. The spatial links between thalamic fibers and subplate neurons were further illustrated by combined MAP2 immunocytochemistry and DII tracing (Figs 9C, D; 10D). From E17 onwards, thalamocortical axons started the invasion of the deep regions of the cortical plate, at the time when the intermediate zone began to be permeated by bundles of thalamic fibers (Fig. 8B). Massive trespass of thalamic fibers into the cerebral cortex occurred during the first week of postnatal life.

To investigate the course of corticothalamic fibers during the prenatal period, small crystals of DII were placed in the telencephalic mantle. Corticothalamic fibers were observed throughout the subplate following a similar course described for thalamocortical fibers (Fig. 9A).

In addition, GABAergic fibers from the striatal primordium and internal capsule penetrated into the cortical subplate from E14 onwards (Figs 8C, D). Corticofugal fibers were also immunolabelled with antibodies against glutamic acid (Fig. 8E), and although no definite conclusions can be drawn until double-labelling tracer studies are made, these findings suggest that thalamocortical, and corticothalamic fibers may contain GABA and glutamate from very early stages of development.

Subplate neurons, intermingled with fluorescent fibers, were consistently labelled after crystals were placed in the thalamus (Fig. 9E). A fluorescent

band of subplate neurons was also observed 2–3 mm away from the zone where DII was placed in the cerebral cortex (Fig. 9B). These findings indicate that subplate neurons send axons to the thalamus and to the cortical plate from E15 onwards.

The morphology of labelled cells was similar to that observed in immunocytochemical studies.

In both sets of experiments, scattered fluorescent neurons were also seen in the cortical plate and occasionally in the intermediate zone (Fig. 9B), but not in the intermediate zone–subventricular border.

In addition to the corticothalamic fiber system described above, a second system of efferent cortical fibers was observed from E18 onwards after the injection of DII in the cerebral cortex (Fig. 9A). This second system passed vertically across the intermediate zone and followed a periventricular course in close apposition with the intermediate zone–subventricular border (Figs 9A, 10A, B). From E18 to birth, fluorescent callosal axons stopped in the interhemispheric region or started to cross the contralateral hemisphere depending on the injection site in the cortex (Figs 10A, B, C). The relationships between these axons and cells is shown in Fig. 10D.

Early callosal axons were immunoreactive with antiglutamate antibodies, whereas a few GABAergic axons were seen in the corpus callosum during the prenatal period (Fig. 8E).

The present observations support recent findings in the rat cortex (Blakemore and Molnar, 1990; Catalano *et al.*, 1991), and confirm the early arrival of thalamic fibers, and the short duration of the waiting period for these fibers (only two days) in the rodent brain. These results also validate previous observations in kittens and primates regarding the close relationship between subplate neurons and thalamocortical and corticothalamic fibers.

In addition, we observed that a number of subplate neurons give rise to thalamic and intracortical projections. Similar findings have been found in the cat's cerebral cortex (McConnell *et al.*, 1989; Ghosh *et al.*, 1990; Antonini and Shatz, 1990).

Most important, the present studies with fluorescent tracers placed in different brain regions have permitted us to see that thalamic and callosal fibers in the rodent brain use different pathways during embryonic development. Thalamic fibers run along the cortical subplate, whereas callosal fibers are located in the intermediate zone–subventricular border. The exciting possibility that neurons in the intermediate zone–subventricular border may play a guiding role for callosal fibers, as subplate neurons do for thalamic axons during development, deserves further inquiry.

Finally, our immunocytochemical findings showing glutamate and GABA immunoreactivities in thalamic and callosal fibers are consistent with other studies (Lauder *et al.*, 1986; Antonini and Shatz, 1990) which suggest a role of neurotransmitters in the settlement of axonal connections.

5.4. Postnatal Loss of Subplate and Intermediate Zone–Subventricular Border Neurons

Immunocytochemical studies disclosed a dramatic reduction in the number of all immunolabelled neurons in the intermediate zone and intermediate zone–subventricular border between P0 and P10. Immunolabelled cells in the subplate also decreased in number during postnatal development (Figs 11 and 12).

Decreased numbers of immunostained neurons in these regions is coincidental with the presence of shrunken neuronal cell bodies, beaded and swollen dendrites and varicose cell processes (Fig. 11), which are currently interpreted as degenerating cells (Wahle and Meyer, 1987; Valverde and Facal-Valverde, 1987, 1988; Shatz *et al.*, 1988). Degenerating neurons were easily seen with all the different neuronal markers employed (MAP2, GABA, NPY, somatostatin, cholecystokinin, and calbindin), except glutamate since the strong immunoreaction of the neuropil hampered the identification of putative degenerating cells.

Shrunken neurons were found from P0 to P12, but were particularly numerous during the first week, at the time when cell death is very common in the subcortical white matter and cortical layer VIb (Figs 11 and 12).

These features suggest that the reduction in the number of the different neurotransmitter and neuropeptide immunoreactive neuronal populations in these regions is not due to shifting immunoreactive expression but, rather, to neuron degeneration and cell death.

To further uphold this notion, the following study was undertaken: pregnant mice received pulse injections of H^3-thymidine (Amersham), or the purine analogue 5′-bromodeoxyuridine (5′-BrdU, Sigma, 50 mg/Kg bw) at embryonic ages 10 or 11 (E10, E11) (Del Rio and Soriano, 1989; Soriano and Del Rio, 1991), when the first postmitotic cortical neurons are produced in the mouse (Angevine and Sidman, 1961; Caviness, 1982). Animals were killed at different prenatal and postnatal ages.

Most neurons in the subplate, and in the marginal layer (future molecular layer), were labelled in animals injected with 5′-BrdU at E10 and E11, and killed at E18. Large numbers of heavily-labelled cells were also seen in the intermediate zone–subventricular border, whereas only a few labelled cells were observed in the cortical plate (Figs 13A and 14). A marked decrease in the number of labelled cells occurred during postnatal development (Fig. 13B). As seen in Fig. 14 only a few cells were labelled in the subcortical white matter, and in the molecular layer (layer I) in the mouse aged 5 days. A progressive loss of labelled cells also occurred in layer VIb where only a few such cells were found in adulthood (Fig. 14).

This reduction in the number of labelled cells could be the result of successive neuronal generation cycles and dilution of the DNA markers, although it is a remote possibility for postmitotic neurons.

In contrast, numbers of BrdU-labelled cells had pyknotic, often fragmented, nuclei through the early postnatal period (Fig. 13C). The distribution of 5′-BrdU-immunoreactive and pyknotic nuclei in the cerebral cortex of mice aged 5 days, which received 5′-BrdU pulses at E10 is shown in Fig. 14. Degenerating cells born at E10 are very common in the layer VIb and subcortical white matter. This further reinforces the idea that most neurons in these regions

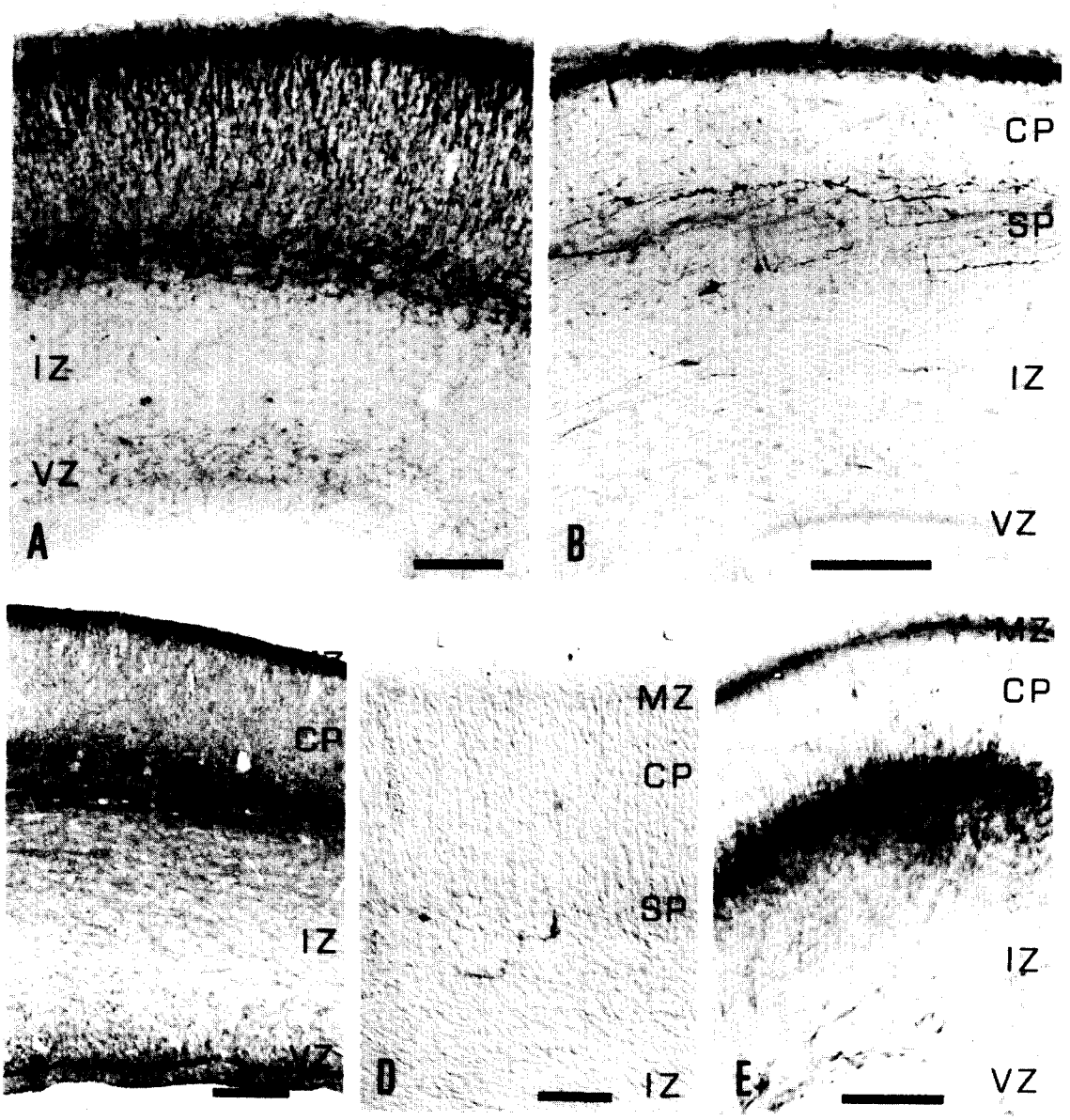

FIG. 6. Patterns of immunostaining for different neuronal antigens in the developing telencephalic mantle of the mouse. A—Immunolabelling for the cytoskeletal protein MAP2 at E16. Note the staining of the subplate and intermediate zone–subventricular border. B—GABA immunoreactivity at E15 is very marked in the marginal zone, cortical subplate and intermediate zone–subventricular border. C—Glutamic immunoreactivity in the telencephalic mantle of the rat at E15. Marked immunoreactivity is found in the subplate. D—The first appearance of NPY-immunoreactive cells in the mouse occurs in the cortical subplate at E17. E—Intense calbindin immunoreactivity is found in the cortical subplate of the mouse at E16. MZ: marginal zone; CP: cortical plate; SP: cortical subplate; IZ: intermediate zone; VZ: ventricular zone. The region named 'intermediate zone– subventricular border' is located between the intermediate and ventricular zones. A, B, C and E, bar = 100 microns; D, bar = 50 microns.

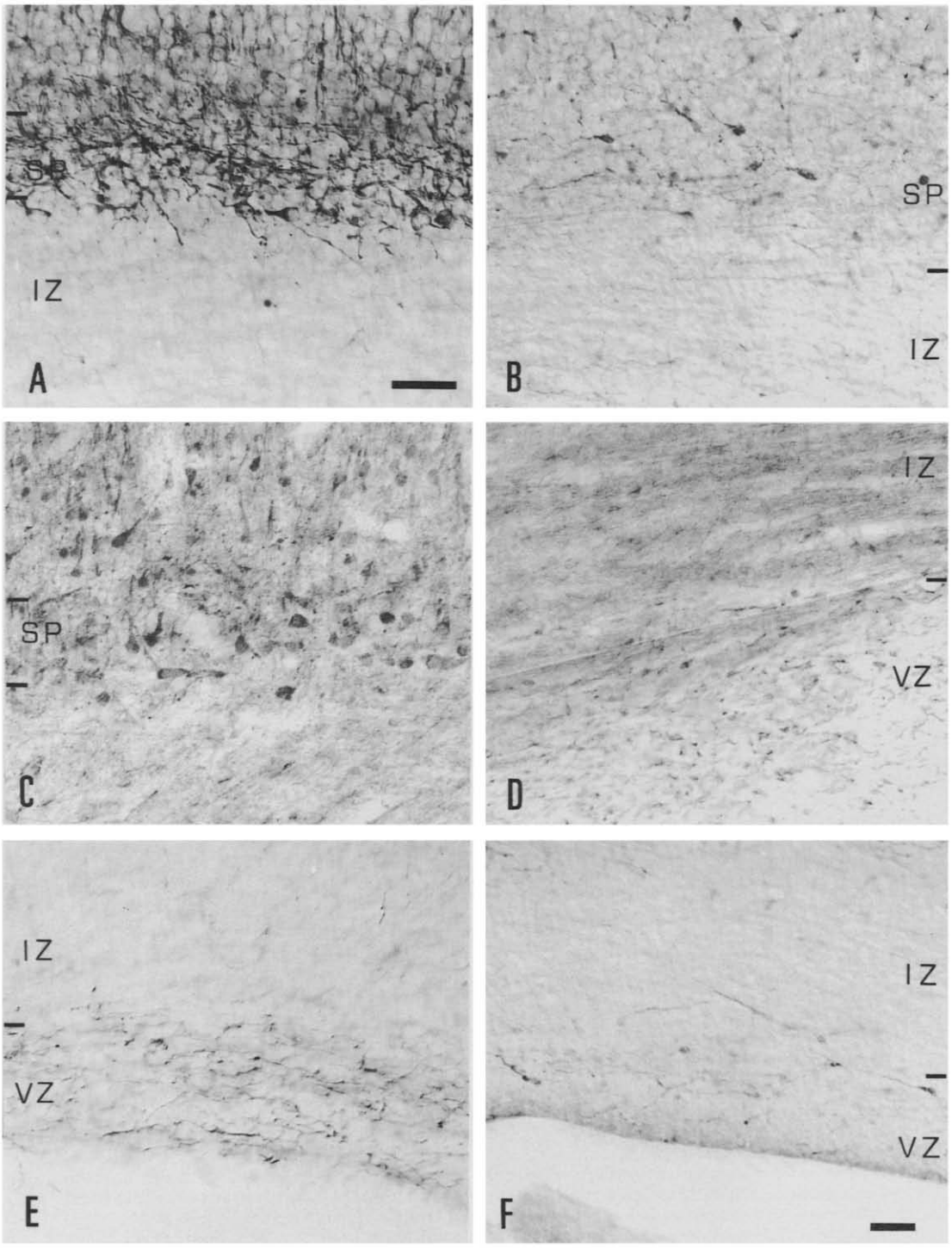

FIG. 7. Photomicrographs showing the morphology of neurons in the cortical subplate (A–C), and intermediate zone–subventricular border (D–F) during development as seen with different antibodies. A—MAP2 at E14; B—GABA at E18; and C—glutamate at birth (P0). D—glutamate at E19; E-MAP2 at E16; and F—GABA at E15. All photomicrographs are taken from the brain of mice, excepting C and D which are from rats. SP: cortical subplate; IZ: intermediate zone; VZ: ventricular zone. Note the presence of large numbers of glutamatergic fiber in the intermediate zone (D). Bar = 50 microns.

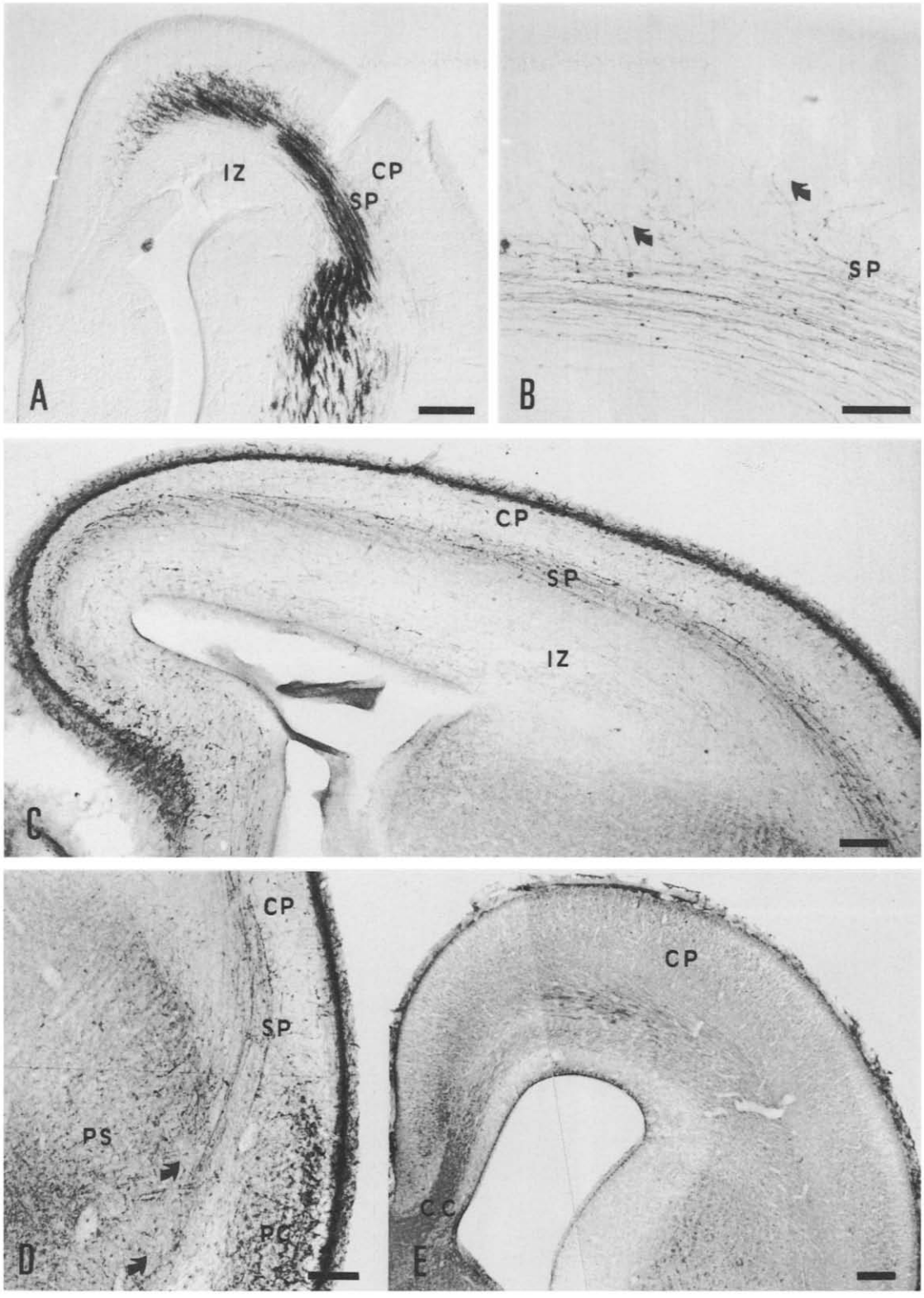

FIG. 8. A—Distribution of anterogradely-labelled thalamocortical fibers in the telencephalic mantle of the mouse at embryonic day 16 (E16) after placing a crystal of DII in the ipsilateral thalamus. Fibers are distributed in the cortical subplate. Section photoconverted with 0.2% diaminobenzydine tetrahydrochloride (DAB). B—DII-labelled thalamocortical fibers penetrating for the first time into the cortical plate at E17 (arrows). Section photoconverted with DAB. C—Coronal section of the cerebral vesicles of the mouse at E15 immunostained with antibodies against GABA. Note the presence of abundant GABAergic fibers in the cortical subplate. D—Bundles of GABAergic fibers (arrows) from the striatal primordium penetrate into the cortical subplate at the level of the pyriform cortex in the mouse at E15. E—Coronal section of the telencephalic vesicle of the rat at E19 immunostained with antibodies against glutamate. Immunoreactive fibers are found in the cortical subplate and lower intermediate zone. Some of these fibers are directed towards the corpus callosum. CP: cortical plate; SP: cortical subplate; IZ: intermediate zone; PS: striatal primordium; PC: pyriform cortex; CC: corpus callosum. A, bar = 100 microns; B, bar = 50 microns; C, D and E, bar = 200 microns.

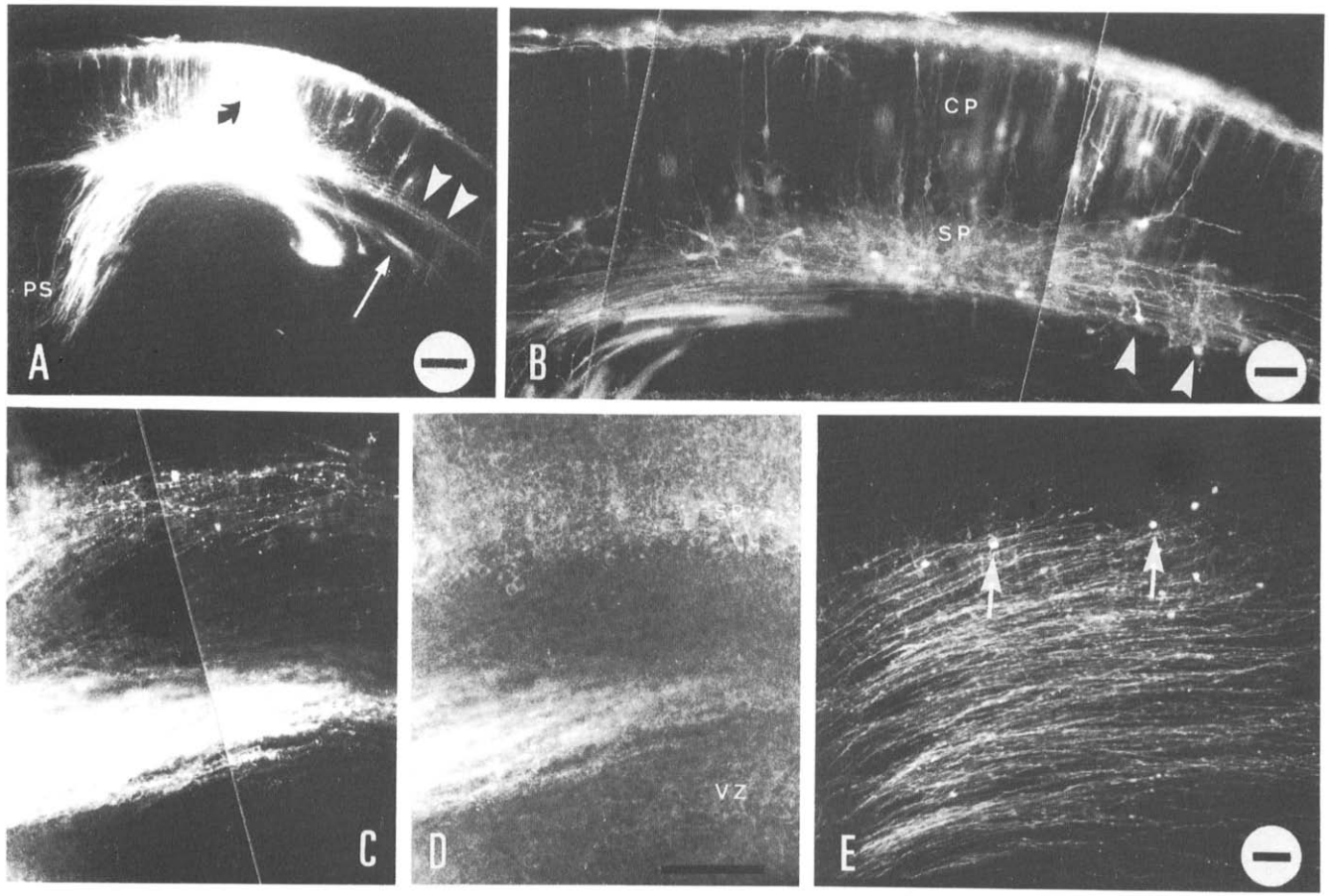

FIG. 9. A-DII-labelled fibers in the mouse at E18 after an intracortical placement of the tracer. The injection site is indicated by a curved arrow. Some fibers penetrate into the striatal primordium, whereas two bundles of fibers run, respectively, along the cortical subplate (arrowheads) and intermediate zone–subventricular border (arrow). B—The same mouse with a higher magnification showing DII-labelled cells 1 mm away from the injection site (arrowheads). C and D—Pair of photomicrographs illustrating the distribution of MAP2-immunoreactive cells in a DII labelled section, after injection of the tracer in the cerebral cortex. There is a close relationship between the upper bundle of DII fibers and the cortical subplate, and the inner bundle of fibers and the intermediate zone–subventricular border. E—DII-labelled cells (arrows) following an injection of the tracer in the ipsilateral thalamus. PS: striatal primordium; CP: cortical plate; SP: cortical subplate; VZ: ventricular zone. A, bar = 200 microns; B, bar = 25 microns; C and D, bar = 50 microns; E, bar = 25 microns.

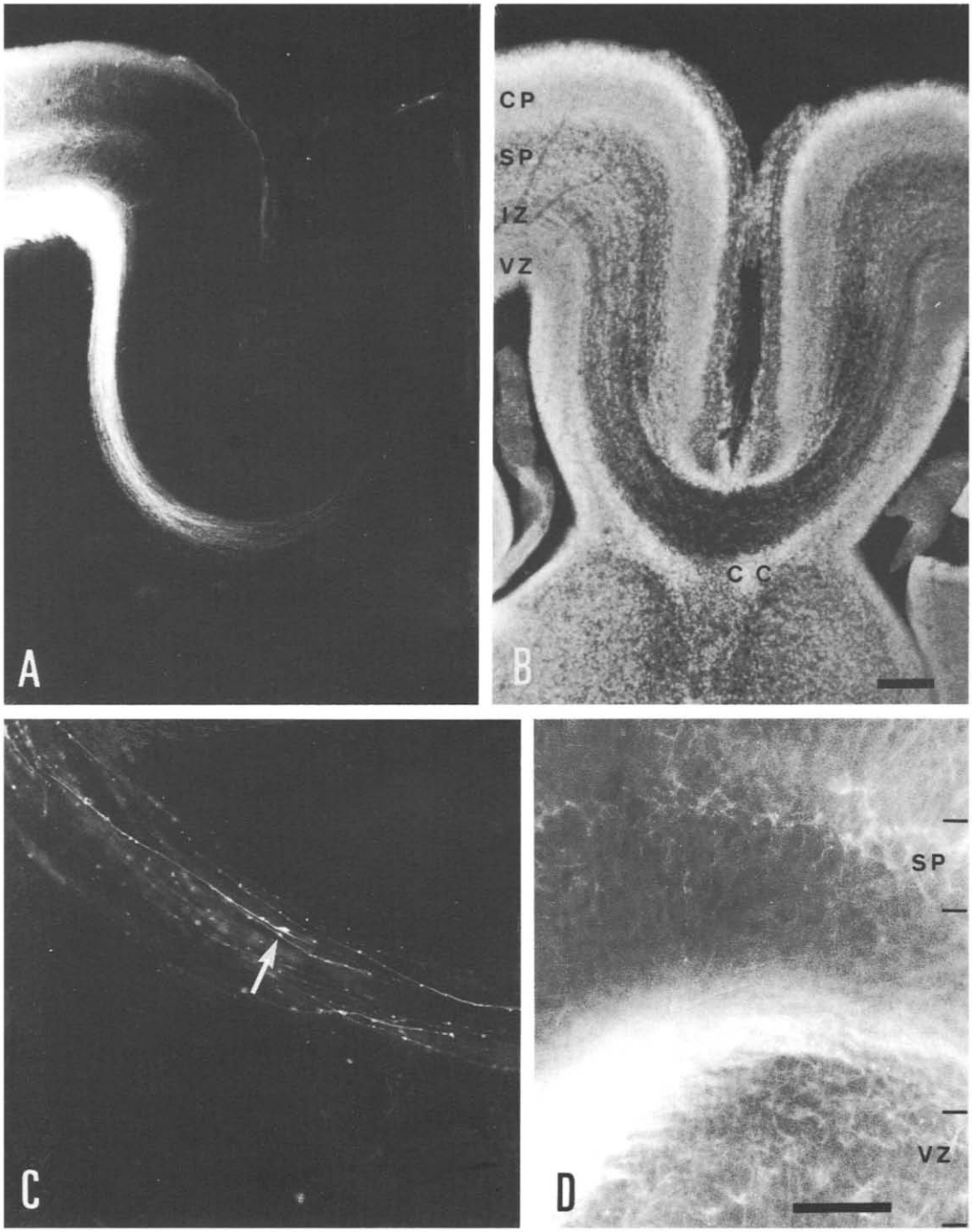

FIG. 10. A—Interhemispheric region of the mouse at E18 showing DII-labelled fibers after the injection of the tracer in the somatosensory cortex. B—Same field viewed with an UV filter net after counterstaining with a nuclear dye to show the cytoarchitectonic landmarks. Note the periventricular arrangement of the callosal fibers. C—Detail of early callosal fibers crossing the corpus callosum for the first time at E18. The arrow points to a growth cone. D—Distribution of callosal fibers in the intermediate zone–subventricular border (a region located just above the ventricular zone) in the mouse at E19, after intracortical injection of DII and immunostaining for the visualization of MAP2. CP: cortical plate; SP: cortical subplate; IZ: intermediate zone; VZ: ventricular zone; CC: corpus callosum. A and B, bar = 100 microns; C and D, bar = 50 microns.

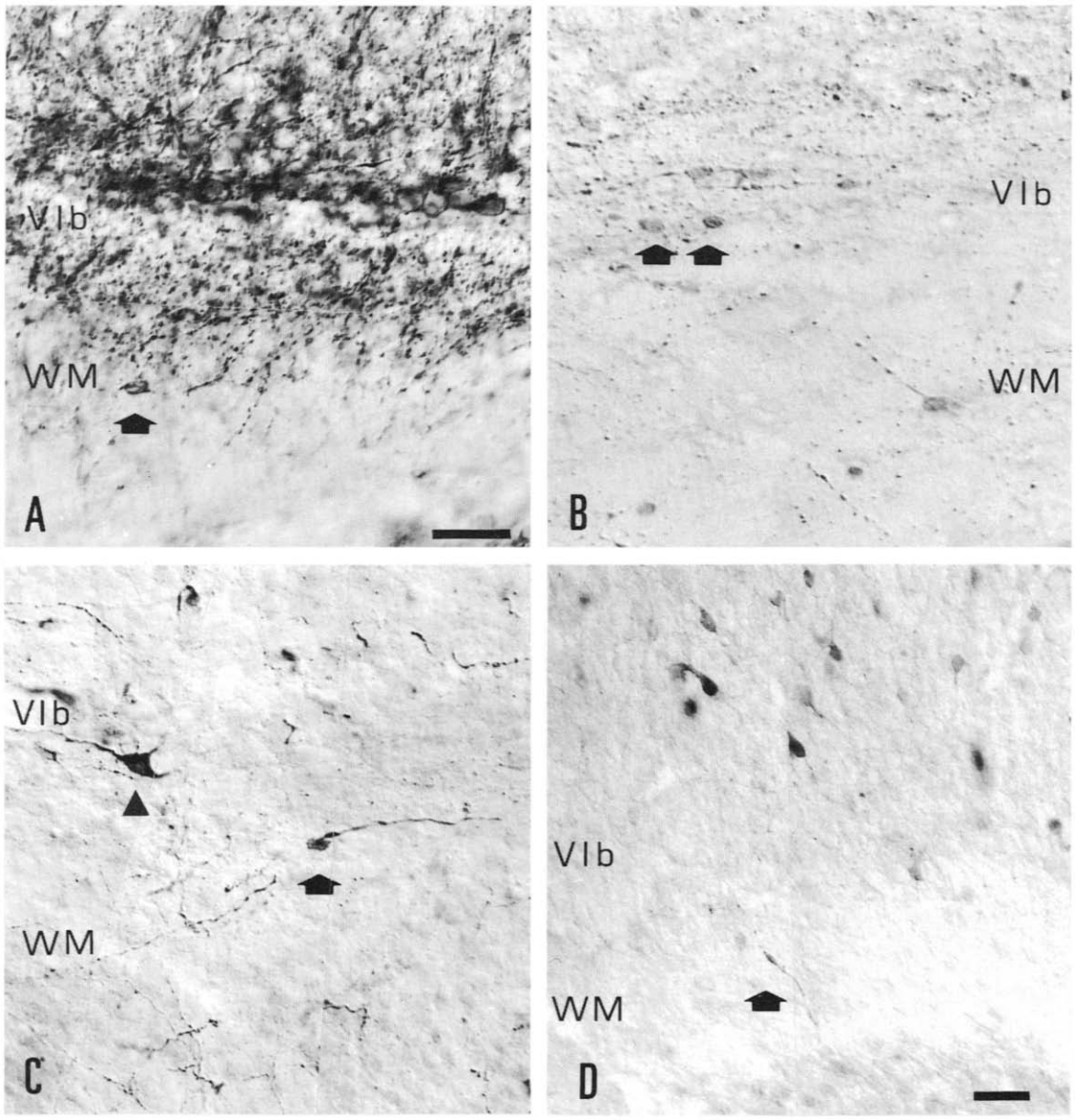

FIG. 11. Presumably degenerating neurons in layer VIb and developing white matter in the mouse at different postnatal ages (arrows). A—MAP2 immunostained section at postnatal day 5 (P5); B-Section stained with GABA antibodies at P5; C—NPY-immunoreactive cells at P2; D—Calbindin-immunoreactive cells at P5. VIb: layer VIb; WM: subcortical white matter. Bar = 50 microns.

die during the first week of postnatal life. As discussed later, similar conclusions can be applied to neurons in the molecular layer on the basis of the present studies with 5′-BrdU.

In the rodent brain the intermediate and subventricular zones progressively transform into the subcortical white matter, whereas at least part of the subplate evolves into layer VIb. However, preliminary studies suggest that the ontogenesis of this layer is not as simple as could be expected. In another set of experiments geared to detect the birth dates of cortical nonpyramidal neurons, we used double-immunolabelling methods with 5′-BrdU injections at several prenatal ages which were combined with antibodies against different neuropeptides at various postnatal ages (Soriano and Del Rio, 1991).

Injection of 5′-BrdU at E14-E15, when neurons of layers IV and upper cortical layers become postmitotic (Angevine and Sidman, 1961; Caviness, 1982), resulted in the consistent labelling of cells in layer VIb and white matter in young adults. Double-labelling studies revealed that some 5′-BrdU positive cells were actually neurons. This is shown in Fig. 15 which illustrates the distribution of 5′-BrdU-positive NPY-immunoreactive neurons in a mouse which received a 5′-BrdU pulse at E15 and was killed at P45.

Double-labelled neurons were observed in different cortical layers including layer VIb, and in the white matter. These findings demonstrate that, although the large number of neurons in layer VIb is generated at very early embryonic ages, there is also a continuous recruitment of neurons to this layer through the prenatal period.

Our results, in summary, suggest that in the rodent brain two different subcortical transitory structures can be identified.

One of them is the cortical subplate which is composed of different neuronal types which probably serve as transitory targets for thalamic projections. These neurons also send corticocortical and corticothalamic axons. Compared with subplate neurons in kittens and primates, subplate cells in rodents have similar morphology and establish similar connections. Likewise subplate neurons in cats and monkeys and most subplate cells in rodents die shortly after birth; however, remaining neurons in rodents probably evolve into layer VIb.

Some neurons in the subplate are, however, generated at later embryonic ages. These findings support the diffuse mode of deposition proposed for certain types of interneurons in the rat's cerebral cortex (Cavanagh and Parnavelas, 1988, 1989). The second early subcortical transitory structure is found in the intermediate zone–subventricular border, which is composed of neurons which are smaller in size. Since early callosal fibers run along this zone, it may be postulated that this second group of neurons guides callosal fibers to the contralateral hemisphere. Most neurons in this zone degenerate and die during the first week of postnatal life, and this transitory region transforms into subcortical white matter.

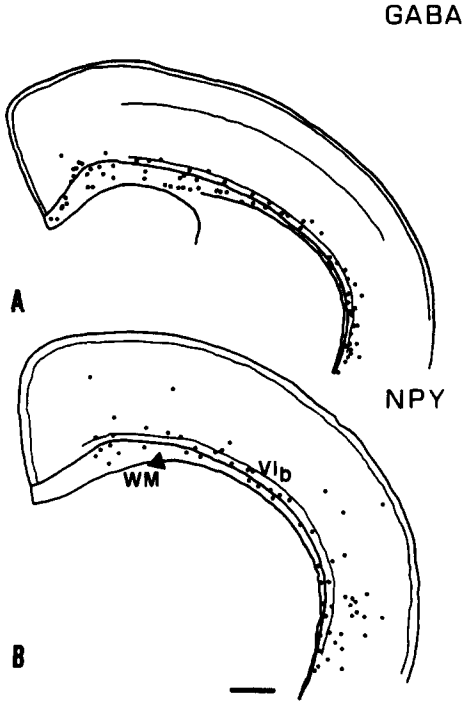

FIG. 12. Camera lucida drawings illustrating the distribution of presumably degenerating neurons at postnatal day 5 (P5) in the mouse. A—Distribution of degenerating GABAergic cells in one vibratome section 50 micron thick. B-Distribution of degenerating NPY-immunoreactive cells in 2 sections 50 micron thick. Degenerating neurons predominate in layer VIb and subcortical white matter. VIb: layer VIb; WM: white matter. Bar = 0.4 mm.

6. CORTICAL CELL DEATH AND OTHER PROGRESSIVE AND REGRESSIVE PHENOMENA IN THE CEREBRAL CORTEX

The mechanisms that trigger cell death in the cerebral cortex during normal development are not well understood. In other regions of the nervous system, naturally occurring cell death has been found to compensate variations in neuron–target ratios, and target-dependent and afferent dependent cell death have been separately found in different neuronal populations (Oppenheim, 1981, 1985, 1989, 1991; Purves, 1988). For naturally occurring cell death in the cerebral cortex the following sections will be discussed:

—elimination of exuberant cortical efferents;
—cortical cell death and innervation of targets;
—cortical afferents and cell death;
—local-circuit neurons and cortical cell death;
—regulation of cortical cell numbers.

6.1. ARE DEAD CELLS IN THE CEREBRAL CORTEX REALLY NEURONS?

In contrast to transitory cells in the cortical subplate and subcortical white matter which are neurons (Luskin and Shatz, 1985; Chun *et al.*, 1987; Valverde and Facal-Valverde, 1987, 1988; Wahle and Meyer, 1987; Shatz *et al.*, 1988; Chun and Shatz, 1989a; Kostovic and Rakic, 1990; present results), the nature of dying cells in the cerebral cortex has not been

rightly assessed. This is because most studies have been focused on the presence of pyknotic cells, and this method does not permit a distinction between glial cells and neurons among dead cells.

It is worth noting that cortical cell death is coincidental with the major development of glial cells in the cerebral cortex, and glial cells can also die during development (Knapp *et al.*, 1986).

At present, the assumption that most dying cells in the cerebral cortex are really neurons is inferred from the observation that the total number of cortical neurons is reduced during the time in which naturally occurring cell death is found.

In the cerebral cortex of the mouse a decrease of about 25% in the number of layer VI neurons during the first ten days of postnatal life has been observed (Heumann and Rabinowiz, 1980; Heumann and Leuba, 1983). In the gyrus suprasylvius and adjoining sulci of the cat there is a decrease in the number of neurons of about 15% from day 15 to day 25, and a cumulative loss of about 20% in the cat aged six months (Ferrer *et al.*, 1988).

6.2. Transitory Cortical Efferents

Exuberant cortical efferents have been found in the corticospinal, corticotectal, corticocerebellar and corticocortical ipsilateral and callosal pathways, as well as in intracortical circuits during normal development in different species (Innocenti *et al.*, 1977; Wise *et al.*, 1977; Distel and Hollander, 1980; Innocenti and Caminiti, 1980; Innocenti, 1981; Chow *et al.*, 1981; Reh and Kalil, 1982; Feng and Brugge, 1983; Innocenti and Clarke, 1984; Panneton and Tolbert, 1984; Tolbert and Panneton, 1984; Luhmann *et al.*, 1986; O'Leary and Stanfield, 1986; Pittman and Tolbert, 1988; Killackey *et al.*, 1989).

In the cerebral cortex of rats, corticospinal fibers are more extensively distributed during development than in the adult (Stanfield *et al.*, 1982; Bates and Killackey, 1984; Schreyer and Jones, 1988a and b). Neurons in the visual cortex transitorily project to the spinal cord during the first week of postnatal life, but these projections are restricted to middle levels of the pons during the second and third weeks (Adams *et al.*, 1983; Stanfield *et al.*, 1982; Stanfield and O'Leary, 1985; O'Leary and Stanfield, 1985; O'Leary and Terashima, 1988).

Corticotectal projections extend from the visual cortex to the ipsilateral optical layer, intermediate layers, and inner and deep gray layers of the superior colliculus during postnatal days 5 to 14 in the rat; the adult pattern, with projections restricted to the upper layers, is found at the end of the third week (Thong and Dreher, 1986, 1987). Transitory fibers from the motor cortex to the intermediate layer of the ipsilateral colliculus are also eliminated at the end of the second week (Thong and Dreher, 1987).

Callosal projections in rats are mainly produced during the first week of postnatal life; most exuberant connections are, however, eliminated at the end of the second week (Ivy *et al.*, 1979; Ivy and Killackey, 1981; Olavarria and van Sluyters, 1984, 1985).

Studies based on the injection of anterograde and retrograde tracers have demonstrated that elimination of transitory cortical projections is due to the reduction of axonic collaterals and not to the elimination of nerve cells (Ivy and Killackey, 1981; O'Leary *et al.*, 1981; Innocenti *et al.*, 1986; Pittman and Tolbert, 1988; Chalupa and Killackey 1989; LaMantia and Rakic, 1990).

It must be also stressed that cortical cell death in the rat mainly occurs during the first week of postnatal life, and therefore, elimination of transitory fibers is not coincidental in time with the occurrence of cortical cell death. This feature further supports the concept that elimination of exuberant projections is not the result of neuron loss.

6.3. Cortical Cell Death and Innervation of Targets

Target-dependent cell death during development is well known in some cell populations (Oppenheim, 1981, 1991).

A classical example is motorneuron cell death during innervation of muscle fibers, and the modification in the number of spinal motorneurons after experimental hazards of the limb muscles (Hamburger and Levi-Montalcini, 1949; Cowan, 1973; Oppenheim, 1981, 1991; Hamburger and Oppenheim, 1982; Lamb, 1982; Clarke, 1985; Betz, 1987). In this system, elimination of targets produces marked neuron loss of innervating cells, whereas increasing the availability of targets, naturally occurring cell death is reduced.

Since dead cells in the cerebral cortex appear while exuberant cortical projections permeate large regions of the brain, the extent to which cortical cell death is dependent on the innervation of specific targets has been investigated. Elegant studies carried out in hamsters have shown, however, that the number of dead cells in the cerebral cortex is not significantly increased after destruction of the targets of specific neurons. For example, preserved neuron density and normal cell death rates are found in the visual cortex after early tectal ablation (Pallas *et al.*, 1988). Similarly, the number of neurons in different cortical regions is not modified after sectioning the corpus callosum in neonatal hamsters (Windrem *et al.*, 1988).

Likewise, the number of layer V pyramidal neurons is not altered following early pyramidal tract sections (Ramirez and Kalil, 1985).

These results suggest that elimination of one of the many targets (including the 'main' putative targets) has no effect on the survival of cortical neurons. Nerve cells can continue living because alternative contacts are established through axonic collaterals.

6.4. Cortical Afferents and Cell Death

Reduction of afferent inputs to neurons shortly before or during the period of cell death results in a significant increase in the number of dead cells in some neuronal populations (Oppenheim, 1991).

Ablation of the visual cortex during development produces marked elimination of nerve cells in the lateral geniculate nucleus (Raabe *et al.*, 1986), whereas hemispherectomy causes loss of neurons in the extraocular motor nuclei (Gonzalo and Torremadé, 1988).

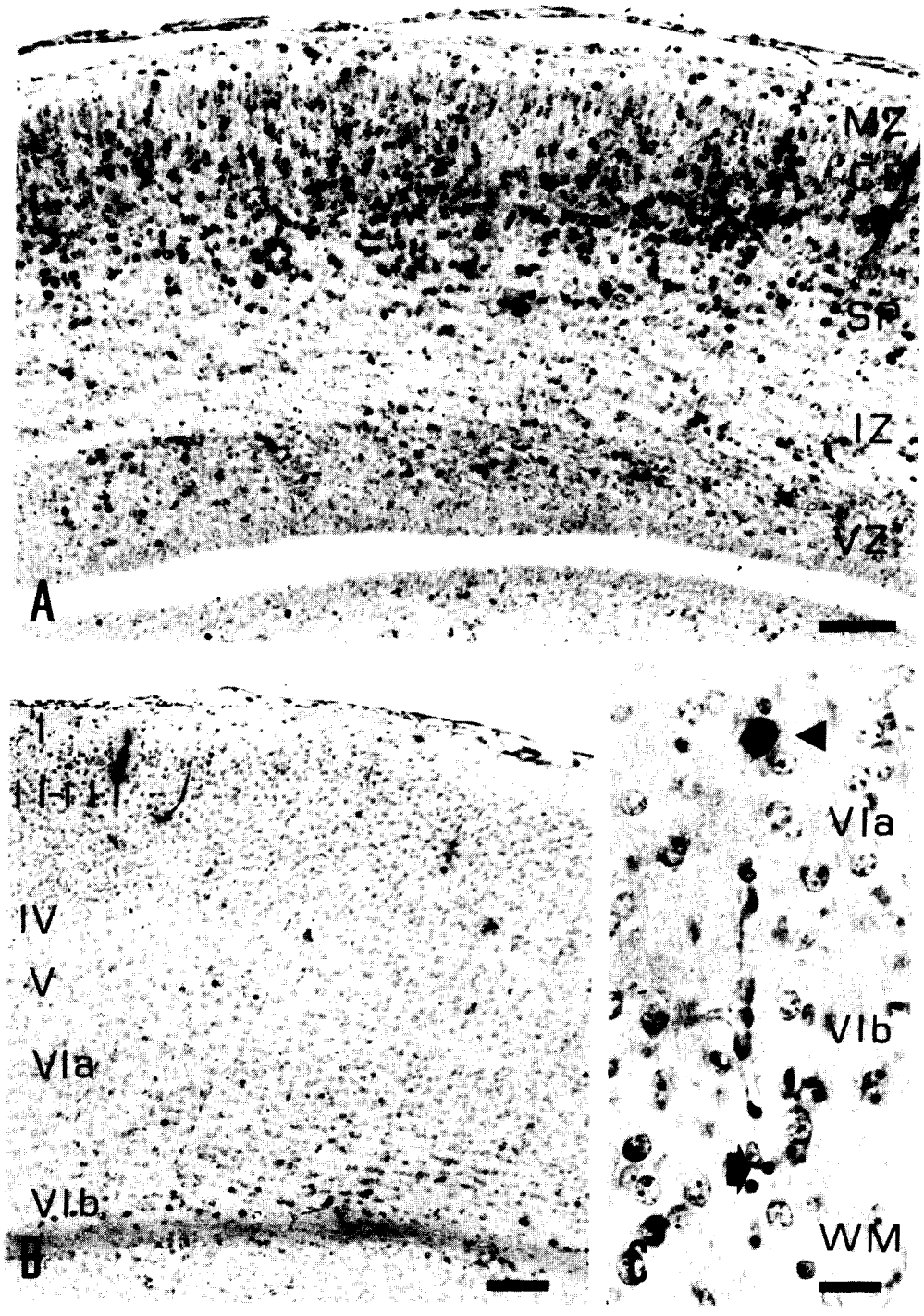

FIG. 13. A—Photomicrograph showing the distribution of 5′-BrdU-immunolabelled cells in the cerebral cortex of the mouse at embryonic day 18 (E18), following a BrdU pulse at E10. Most labelled cells are found in the cortical subplate and intermediate zone–subventricular border. B—5′-BrdU positive cells in the cerebral cortex of the mouse at postnatal day 5 (P5), following a pulse of BrdU at E10. Only a few cells are labelled when compared with E18. C—Cingulate cortex of a mouse at P5 showing a normal BrdU-labelled cell (arrowhead), and a pyknotic BrdU-labelled nucleus (arrow). A and B, bar = 100 microns; C, bar = 25 microns.

These data suggest that the integrity of the lateral geniculate nucleus and extraocular motor nuclei depends on the integrity of the cortical afferent projections to these nuclei. Conversely, increased cortical cell death after reductions of afferent inputs to the cerebral cortex would suggest that cell death in the cerebral cortex is afferent dependent.

In rats, cell death in the upper cortical layers is coincidental with the arrival and settlement of thalamocortical and corticocortical connections (Wise and Jones, 1976, 1978; Lund and Mustari, 1977; Killackey and Belford, 1979). Notwithstanding, the effects on cortical cell death after destruction of cortical afferents are confounding.

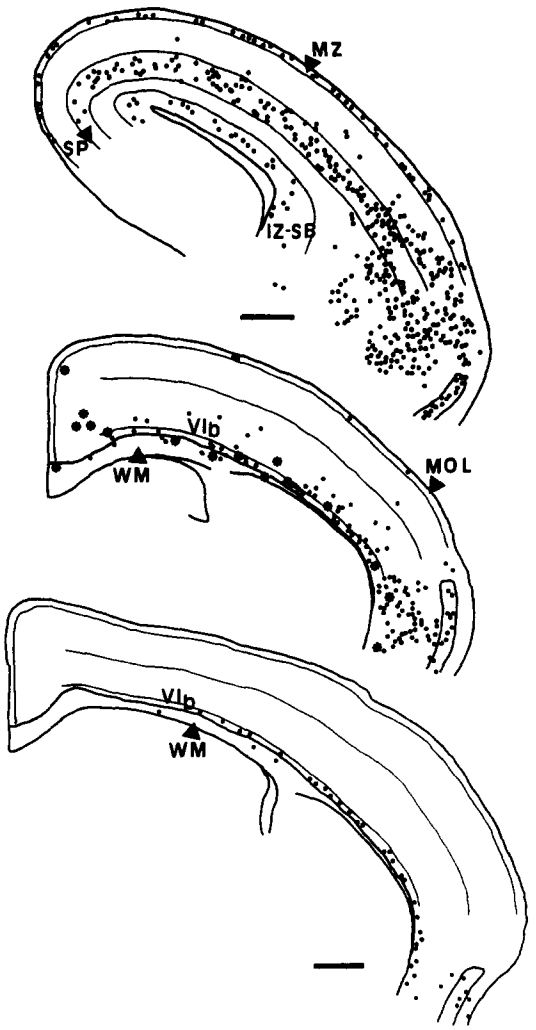

FIG. 14. Camera lucida drawings illustrating the distribution of BrdU-labelled cells (dots) at embryonic day 18 (E18) (upper), postnatal day 5 (P5) (middle), and postnatal day 30 (P30) (lower), following pulses of 5′-BrdU at E10. The number of labelled cells decreases during postnatal development in layer VIb and subcortical white matter. BrdU positive cells in the marginal zone (molecular layer) at E18 decrease in number at P5 and are no longer present at P30. Each dot represents one labelled cell. Asterisks in P5 (middle) are BrdU pyknotic cells. MZ: marginal zone; CP: cortical plate; SP: subcortical plate; IZ-SB: intermediate zone–subventricular border; MOL: molecular layer; VIb: layer VIb; WM: white matter. A, bar = 0.2 mm.; B and C, bar = 0.4 mm.

The section of the corpus callosum has no effect on the total number of dead cells (Windrem *et al.*, 1988), but early thalamic lesions produce a marked increase in the number of dead cells in the cerebral cortex (Windrem and Finlay, 1985; Windrem *et al.*, 1986).

We have examined the rate of naturally occurring cell death in the primary visual area (Oc 1 M) in rats bilaterally enucleated at birth. As seen in Table 1, we did not find significant differences in the amount of cell death between normal and enucleated animals at birth when examined at different postnatal ages.

These findings suggest that cortical cell death does not depend on the elimination of one of the many afferents in a given cortical area, but cell death can be increased after massive removal of afferents plus efferents.

Olfactory bulb removal at birth produces increased cell death in the deep part of layer IIb and in layer III of the pyriform cortex (Friedman and Price, 1986), but not in layer IIa, which is the only one that receives olfactory projections but does not send fibers to the olfactory bulb. As stated by the same investigators (Friedman and Price, 1986), 'since most of the dying cells are located in the deeper layers of the olfactory cortex, any alteration due to olfactory bulb removal', before day 15 of postnatal life, 'may be due to retrograde as well as transneuronal effects'.

More delicate strategies are probably needed to elucidate possible relationships between cortical cell death and afferent projections. The developing cerebral cortex has enormous plastic capacities, and reduction of one of the many afferents can be rapidly replaced by other fibers (Lund *et al.*, 1973; Innocenti and Frost, 1979, 1980; Innocenti, 1981; Lund *et al.*, 1984; Innocenti *et al.*, 1985, 1986; Olavarria and van Sluyters, 1984; Olavarria *et al.*, 1988). Alternative cortical afferents produced at very appropriate stages of development may protect cortical nerve cells from dying.

6.5. TRANSITORY EXPRESSION OF NEUROTRANSMITTERS AND NEUROPEPTIDES IN CORTICAL NEURONS (LOCAL-CIRCUIT NEURONS) AND CORTICAL CELL DEATH

The prospect that cell death is confined to local-circuit neurons must be examined in detail because many studies have shown a decrease in the number of several peptidergic cells in the cerebral cortex during the late stages of development. It is known that peptidergic neurons are GABAergic nonpyramidal cells with functions of local-circuit neurons (Jones *et al.*, 1987).

For example, in the cerebral cortex of kittens, some populations containing neuropetide Y (NPY) decrease in number during the third and fourth weeks of postnatal life (Wahle and Meyer, 1987). Similarly, in rabbits and monkeys (Ramón y Cajal-Agueras *et al.*, 1986; Hayashi and Oshima, 1986), vasointestinal polypeptide-immunoreactive (VIP-ir) neurons decrease in number following peak values a few days after birth. A similar pattern occurs for substance P-(SP-) (Hayashi and Oshima, 1986; Yamashita *et al.*,

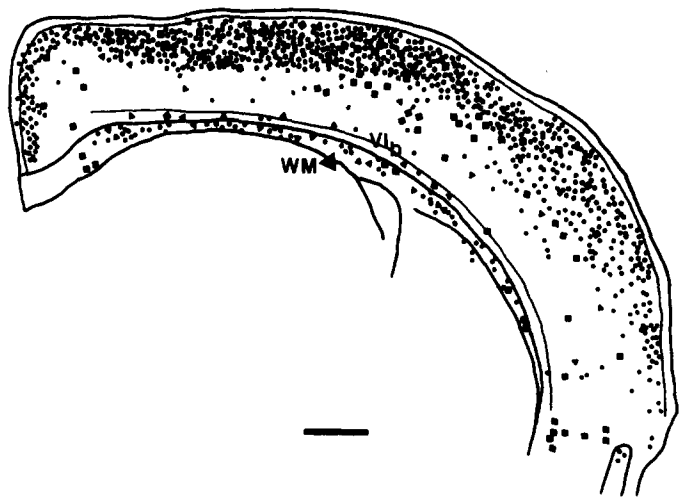

FIG. 15. Camera lucida drawing showing the distribution of BrdU-positive cells (dots), NPY-immunoreactive (-ir) cells (squares) and double-labelled BrdU-positive-NPY-ir cells (triangles) in the cerebral cortex of a mouse aged 45 days, after an injection of 5'-BrdU at embryonic day 15. One dot represents 2 cells. Many double-labelled cells are found in layer VIb and subcortical white matter. VIb: layer VIb; WM: white matter. Bar = 0.4 mm.

1990) and somatostatin- (SRIF-) (Yamashita *et al.*, 1989) immunoreactive neurons in the cerebral cortex of monkeys.

In the cerebral cortex of rats, there is a decrease in the number of SRIF- (Cavanagh and Parnavelas, 1988), VIP- (Cavanagh and Parnavelas, 1989) and SP- (McGregor *et al.*, 1982) immunoreactive neurons, and VIP-ir fibers (Hajós *et al.*, 1990) during the second and third weeks of postnatal life. In agreement with these findings, a decrease in SRIF-RNA expression during development has been reported in rats and monkeys (Sekitani *et al.*, 1990; Hayashi *et al.*, 1990).

We investigated the developmental expression of SP in the cerebral cortex of the mouse (Del Rio *et al.*, 1991a). SP-like-immunoreactive cells were abundant from P2 to P5 in layer VIb and in the subjacent future white matter. Although some cells were also present in layer V, positive cells were not found in the upper cortical layers. The number of SP-like-immunoreactive cells gradually decreased, being almost absent by P12, but from P16 onwards the adult pattern of SP-like immunoreactivity emerged with a few immunopositive cells scattered throughout the cortical layers. These results were consistent with the interpretation that transitory immunoreactive expressions in layer VIb and subcortical white matter were due to cell death.

However, the developmental pattern of most neuropeptide and neuronal protein immunoreactivities is different, and involves neurons in the upper cortical layers.

We have studied the development of calbindin- (CaBP) and SRIF-immunoreactive cells in the somatosensory cortex of rats, and compared these results with the rates of naturally occurring cell death in the same cortical region. Sections 60 microns thick of paraformaldehyde-fixed brains were obtained with a freezing microtome and processed free-floating according to the avidin and biotin (ABC, Vector Labs) method. Calbindin antibody (Sigma, clone no CL-300, Mouse IgG1) was used at 1/800–1/1000. Somatostatin antibody (raised in rabbit, Biogenex Labs) was used at a dilution 1/800.

Calbindin is a calcium-binding protein which, in the cerebral cortex, is found in many GABAergic (local-circuit) neurons (Celio, 1990). Pyramidal neurons in layers II and III are also weakly stained in the cerebral cortex of rodents (Celio, 1990). Somatostatin immunoreactivity is found in a widely distributed subpopulation of nonpyramidal cells during development (McDonald *et al.*, 1982).

CaBP-immunoreactive neurons in the cerebral cortex of the rat increase in number during the first eleven days of postnatal life. After this age, there is a decrease, which is rapid until day 15 and slower thereafer, in the number of immunoreactive cells to border on adult values at the end of the third week. This decrease is greater in layers V and VI than in the upper cortical layers. In addition, we have observed modifications in the morphology of CaBP-ir cells throughout development. During the first eleven days (in the SS cortex) heavily labelled cells are polymorphous neurons with variegated dendritic arborizations, bitufted cells and bipolar neurons distributed in all cortical layers. In the second half of the first month, bitufted and bipolar cells are encountered in the upper cortical layers, and only small numbers of multipolar neurons are found in layers V and VI (Fig. 16).

Besides nonpyramidal neurons, pyramidal cells in layers II and III exhibit the typical homogeneous labelling of adults (Celio, 1990) from the age of 15 days onwards.

The number of SRIF-ir cells increases during the first two weeks of postnatal life, and decreases thereafter with a second peak at P30. This pattern differs from that found in CaBP-ir cells.

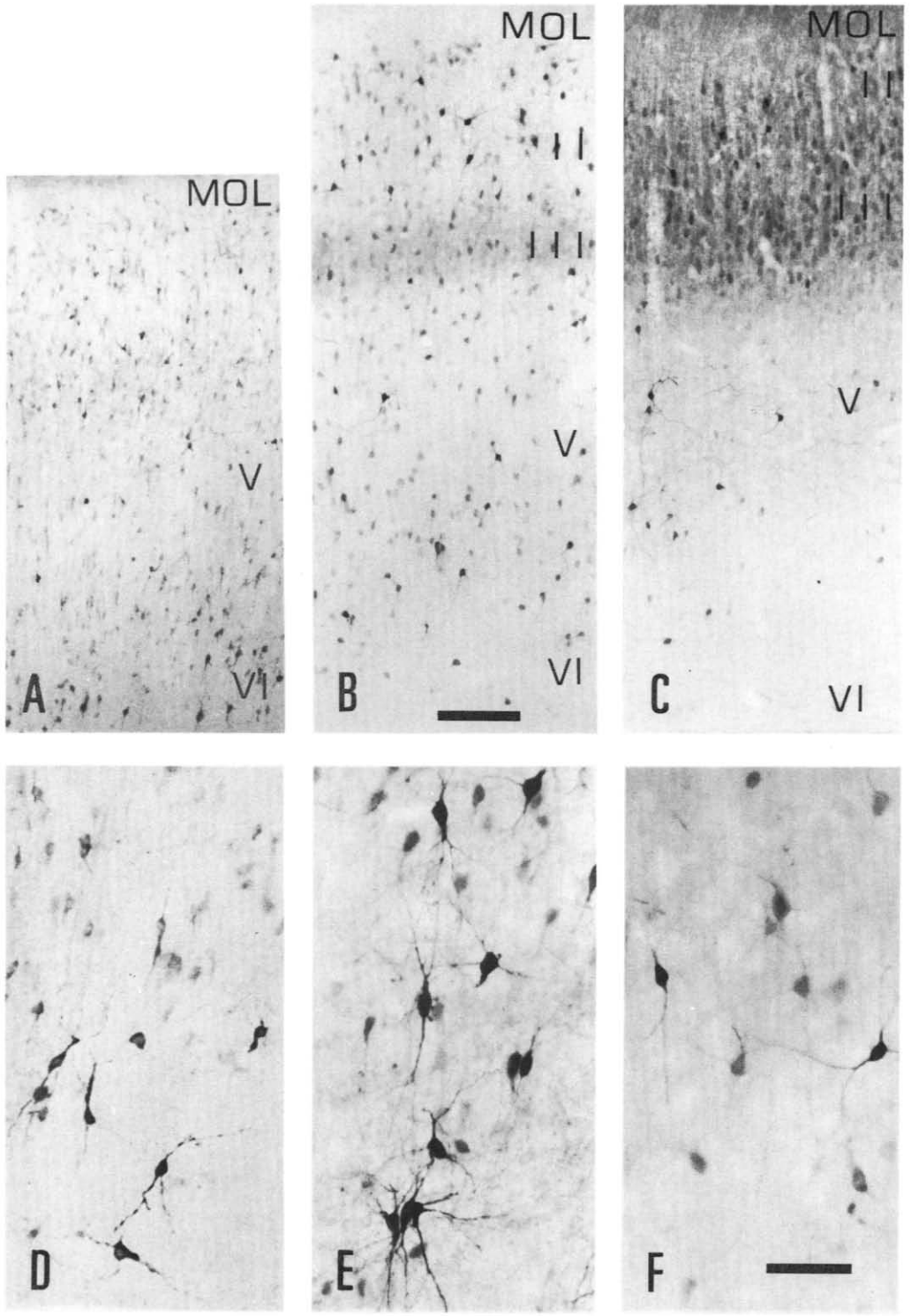

FIG. 16. Development of calbindin (CaBP) immunoreactive cells in the cerebral cortex of the rat. A and D: 6 days; D and E: 11 days; C and F: 21 days. All pictures are from the somatosensory cortex Par 1. A to C: The number of CaBP-ir cells increases during the first two weeks of postnatal life, and decrease thereafter. This decrease is particularly noticeable in layers V and VI. In rats aged 15 days and older (i.e. 21 days) pyramidal neurons in layers II and III are homogeneously stained. D to F: The morphology of CaBP-ir cells also varies in the course of development. In addition to bipolar and bitufted cells, multipolar neurons are very common during the first fortnight, but much rarer in animals older than 15 days. A, B and C, same magnification, bar = 100 microns. D, E and F, same magnification, bar = 75 microns.

The progressive changes in the number of CaBP- and SRIF-ir neurons in the somatosensory cortex of developing rats is shown in Fig. 17. These quantitative data can be compared with the rates of cell death (as expressed by the proportion of dead cells per 1000 live neurons) in the same cortical region during the same period.

These figures indicate that naturally occurring cell death and decreased CaBP and SRIF expressions in the cerebral cortex of the developing rat are not coincidental. Hence, it may be suggested that cell death is not primarily involved in the regulation of the total number of cortical local-circuit neurons, at least once these cells start to express determinate proteins or peptides. Furthermore, cell death predominates in the upper cortical layers, while the main changes in the morphology and number of CaBP- and SRIF-ir neurons take place in layers V and VI.

Along these lines, we can also postulate that the decrease in the number of peptidergic and CaBP-ir neurons during normal development is the result of reduced immunoreactive expressions rather than the

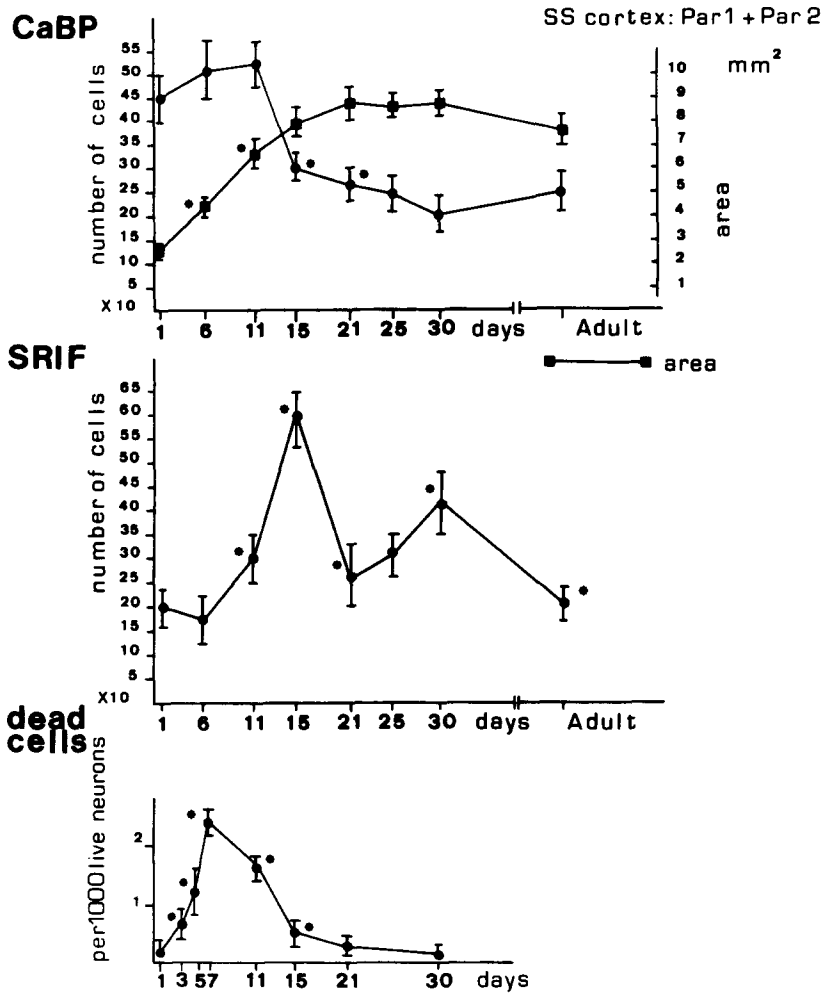

FIG. 17. Number of calbindin- (CaBP-) and somatostatin- (SRIF-) immunoreactive neurons in the somatosensory cortex (Par 1 + Par 2) of the rat during normal development, compared with the number of dead cells (as expressed by the number of dead cells per 1000 live neurons) in the same cortical region. CaBP-ir neurons increase in number from birth to day 11 in this cortical region, and decrease abruptly at day 15 and more slowly thereafter to reach adult values at the end of the third week. SRIF-ir neurons increase in number from birth to day 15 and decrease afterwards. A second peak is, however, found at the end of the first month which is followed by a gentle decrease to adult values in the next months. The number of dead cells increases from birth to day 7 and rapidly decreases during the second week, and more slowly until postnatal day 30. Asterisks in every case mark significant differences (Mann-Whitney U test, $p < 0.05$) with immediately anterior counts. Curves representing the number of CaBP- and SRIF-immunoreactive neurons are not synchronous, thus suggesting that immunoreactive expressions are independent for the neuropeptide and the calcium-binding protein. These curves are not coincidental with the one representing the number of dead cells, a feature which does not support the possibility of reduced immunoreactive expressions being due to cell death. Finally, modifications in the number of CaBP- and SRIF-ir neurons are not the result of dilution of the same number of neurons in the growing brain, as can be inferred from the observation of the areal increase of the SS cortex during the period studied.

effect of cell death, and that shifting immunoreactive expressions during development of the cerebral cortex are particular for each neuropeptide and neuronal protein.

These features are in contrast, as we have previously seen, with observations in the cortical subplate, in which lessening neuropeptide immunoreactivity is accompanied by cell death and neuron loss.

Specific thalamic afferents may play a role in the modelling of the expression of certain neuropeptides in cortical populations. Somatostatin-immunoreactive cells increase in number in the visual cortex of adult rats enucleated at birth (Jeffery and Parnavelas, 1987; Parnavelas and Cavanagh, 1988), or subjected to early light deprivation (Papadopoulos *et al.*, 1988). Likewise, early lesion of mysticial vibrissae in rats results in an increase of SRIF-labelled cells in the somatosensory cortex (Parnavelas *et al.*, 1990).

6.6. Cortical Cell Death is Related to the Total Number of Cortical Neurons

The possibility that cell death may be related to the control of cell numbers has been postulated in other parts of the nervous tissue (Purves, 1980, 1988; Oppenheim, 1981, 1991). We have observed that naturally occurring cell death in the cerebral cortex decreases in micrencephalic rats produced after prenatal X-irradiation (Ferrer *et al.*, 1991a). This suggests that postnatal cell death may be related to the total number of cortical neurons.

In the following experiment, Wistar rats were treated with 100 cGy X-rays at embryonic day 18 (E18) in an attempt to kill neurons which should populate the upper cortical layers (Berry and Rogers, 1965; Hicks and D'Amato, 1968; Raedler and Raedler, 1978). Irradiated animals studied at different postnatal ages had lower brain weight and decreased cortical thickness when compared to age-matched controls; furthermore, although the inner cortical layers were preserved, layers II and III were reduced one half ($n = 8$ at every age; Mann-Whitney U test, $p < 0.05$). These findings agree with reduced thickness of layers II and III and decreased corpus callosum in rats irradiated at later stages of gestation (Hicks and D'Amato, 1966; Jensen and Altman, 1982; Ferrer *et al.*, 1984).

The morphology and distribution of dead cells in the cerebral cortex and subcortical white matter were similar in controls and irradiated animals, but the number of dead cells in the cerebral cortex was dramatically reduced in micrencephalic rats (Table 2). For example, the proportion of dead cells in the somatosensory cortex of irradiated rats was 0.97 ± 0.38 dead cells per 1000 live neurons at P7, which was in striking contrast to 2.38 ± 0.37 dead cells per 1000 live neurons in controls ($n = 16$, Student t test, $p < 0.05$).

Reduction of dead cells was not homogeneous. As shown in Fig. 18, the number of dead cells in the future subcortical white matter was preserved throughout development in irradiated rats, whereas cell death decline was restricted to the upper and inner cortical layers.

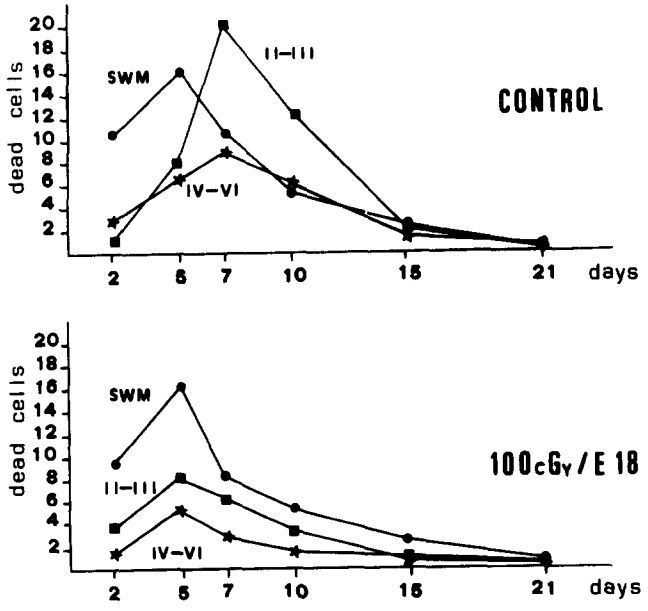

FIG. 18. Number of dead cells in the subcortical white matter (SWM) and cortical layers II and III and IV to VI in control and X-irradiated rats (100 cGy, embryonic day 18: E18), at different postnatal days. The region examined corresponds to the cortical region between the callosal and rhinal fissures of the left cerebral hemisphere, which includes the somatosensory cortex, hindlimb region of the motor cortex, frontal regions FR1 and FR2, and retrosplenial agranular and granular cortices. Numbers correspond to mean values of dead cells per tissue section from 9–10 animals at every age. The number of dead cells is markedly reduced in the cortical layers of irradiated animals when examined at the ages of 7 and 10 days. The number of dead cells is not affected in the subcortical white matter.

These results harmonize with the observation that the number of neurons per unit column is quite constant in different brain regions in different species (Rockel *et al.*, 1980). These data also point to the likelihood that cell death in the cerebral cortex adjust cell numbers accordingly to the total number of cortical neurons.

7. CELL DEATH IN THE MOLECULAR LAYER

The presence of a few dying cells in the molecular layer during the first week of postnatal life renews interest in the fate of Cajal-Retzius (CR) cells.

Several hypotheses have been proposed to explain the decrease in the number of CR cells during late stages of development (Fox and Inman, 1966; Barón and Gallego, 1971; Bradford *et al.*, 1977; Edmunds and Parnavelas, 1982; Marín-Padilla and Marín-Padilla, 1982; Parnavelas and Edmunds, 1983; Marín-Padilla, 1984). These include, (1) preservation of original cell numbers and resulting dilution into a growing neuropil; (2) transformation into other non-pyramidal neurons of layer I; and (3) degeneration and neuron loss.

Recent autoradiographic studies have shown that neurons, labelled with tritiated thymidine early in fetal life, in the molecular layer of the visual cortex of the cat progressively decrease as maturation spreads (Luskin and Shatz, 1985). As reported in Section 5.4 and Fig. 14, similar observations were made in the cerebral cortex of the mouse after the injection of 5′-BrdU at E10 and Ell when the animals were examined at different prenatal and postnatal ages.

Large numbers of labelled neurons were found at E18, but only a very few cells were labelled at P5. No labelled cells were present in the molecular layer in mice aged 45 days. Electronmicroscopic studies in the mouse brain have demonstrated degenerating nerve cells, as well as dead cells engulfed by probable microglial cells in the molecular layer (Derer and Derer, 1990).

Finally, CR cells in the cerebral cortex of the monkey are calbindin immunoreactive throughout the fetal period, and parvalbumin (another calcium-binding protein) immunoreactive between embryonic days 130 and 150. However, CR cells are no longer immunostained from few days after birth onwards (Huntley and Jones, 1990).

We have used anti-Hu antibodies to elucidate the development of Hu-antigen expression in nerve cells during fetal and early postnatal periods in the rat, and demonstrated that anti-Hu is a good marker of developing and adult nerve cells (Graus and Ferrer, 1990).

Anti-Hu antibodies are produced in patients with lung cancer and paraneoplastic encephalomyelitis (Graus *et al.*, 1986; Anderson *et al.*, 1988). By immunohistochemistry, anti-Hu stains neurons and small cell lung cancer, but not other normal tissues or other tumors (Graus *et al.*, 1986; Budde-Steffen *et al.*, 1988).

We observed (Graus and Ferrer, 1990) that horizontal neurons in the molecular layer, immunolabelled with the antibody anti-Hu, progressively vanish, at the time that globular cells appear in the molecular layer. As a result, cells with characteristics of CR neurons are no longer found after the first week of postnatal life in the rat (Fig. 19).

Whether these cells die or transform into other cellular types is still debatable, but taken together these features, including our birthdating studies with 5′-BrdU in mice, would suggest that CR cells ultimately disappear in adulthood. However, in another study we have found calbindin-immunoreactive horizontal cells in the molecular layer in the adult human temporal cortex (Ferrer *et al.*, 1991b), but not in the frontal cortex. Therefore, these features indicate that the fate of CR cells is, at least in the human brain, subjected to regional variations.

8. TRANSITORY PHAGOCYTES DURING NORMAL DEVELOPMENT

Rio Hortega first described microglial cells as non-neuroectodermal elements with phagocytic functions in normal development and pathological conditions (Rio Hortega, 1919, 1920, 1921).

In the rat, nascent or ameboid microglial cells appear during the late fetal and early postnatal periods. These cells have condensed chromatin, vacuolated cytoplasm and plasma membranes with finger-like projections, and exhibit nonspecific esterase, peroxidase, thyamine pyrophosphatase and acid phosphatase activities (Ling and Tan, 1974; Ling, 1976, 1977, 1980; Das, 1976a and b; Boya *et al.*, 1979; Ferrer and Sarmiento, 1980a; Imamoto, 1981; Ling *et al.*, 1982; Murabe and Sano, 1982; Kaur *et al.*, 1982). Nascent microglial cells react with anti-macrophage and anti-granulomonocyte antiserums and anti-MAC-1 surface antigen (Valentino and Jones, 1981; Murabe and Sano, 1983; Miyake *et al.*, 1984; Matsumoto and Ikuta, 1985), and anti-leucocyte common antigen. They also exhibit other cell surface antigens including F4/80, Fc, CR3, CD4, which are important in the immune response (Perry and Gordon, 1988).

The cytoplasmic membrane of ameboid microglial cells can be labelled with lectins (Boya *et al.*, 1991). Also, lectins are bound to specific membrane receptors and later internalized in living microglial cells (Kaur *et al.*, 1990). This indicates that the plasma membrane of nascent microglial cells contains carbohydrates which may play a role in the process of cell recognition and phagocytosis (Duvall *et al.*, 1985; Springer, 1990).

Ameboid microglial cells isolated from developing rat brains contain nonspecific esterase activity, the macrophage surface antigens MAC-1 and MAC-2, and acetylated low-density lipoprotein receptors (Giulian and Baker, 1986). Ameboid microglial cells are labelled by injecting carbon particles or horseradish peroxidase (Ling, 1979; Ling *et al.*, 1980; Kaur *et al.*, 1986). Furthermore, the injection of mononuclear blood cells labelled with tritiated thymidine from a donor to developing animals permits the observation of labelled cells at the sites where nascent microglial cells are usually found (Ferrer and Sarmiento, 1980a).

Taken together, these features indicate that nascent or ameboid microglial cells belong to the mononuclear phagocyte system, and penetrate the nervous system attending operative stimuli.

In the developing rat, nascent microglial cells are found in large numbers in the subcortical white matter from shortly before birth until about the second week of postnatal life. Lower numbers of microglial cells are encountered below the cortical surface from P0 to P10. In addition to vacuoles filled with neutral lipids, ameboid microglial cells have polymorphous inclusions and engulfed cytoplasmic and nuclear debris (Fig. 20).

These features point to the likelihood that removal of degenerating cellular processes and dead cells are major functions of nascent microglial cells (Perry and Gordon, 1988; Ferrer *et al.*, 1990a; Ashwell, 1991). Similar conclusions have been proposed regarding transitory phagocytes in the subcortical white matter of the kitten, which engulf the remnants of decayed transitory corticocortical projections (Innocenti *et al.*, 1983a and b), and in the cerebellum of the developing mouse regarding transitory microglial cells which scavenge dead cells (Ashwell, 1990).

9. CELL DEATH AND THE FORMATION OF CEREBRAL CONVOLUTIONS

9.1. Formation of Normal Convolutions

The formation and growth of the cerebral gyri and sulci has been recently analyzed in great detail. Major coincidental features during the process of gyrogenesis in normal development are maturation of nerve cells and dendrites, synaptogenesis, arrival of afferents (mainly thalamic and corticocortical), laminar aggregation and segregation, orientation of nerve cells, glial proliferation and rearrangement of cells and fibers (Welker, 1990).

All these features harmoniously assemble following precise areal and temporal patterns, most probably, commanded by the orderly arrival and maturation of cortical afferents (Welker and Campos, 1963; Welker and Johnson, 1965; Goldman-Rakic and Galkin, 1978; Goldman-Rakic, 1980; Goldman-Rakic and Rakic, 1984; Johnson, 1980; Smart and McSherry, 1986a and b; Ferrer *et al.*, 1988; Rakic, 1988). This assessment is based on the following observations: (1) Convolutions are formed following differences in growth between the gyral and sulcal regions, and between neighboring folding units (Smart and McSherry, 1986a and b; Ferrer *et al.*, 1988); (2) Some convolutions are anatomically and functionally defined conforming to their thalamic afferents (Welker and Campos, 1963; Welker and Johnson, 1965; Johnson, 1980); (3) Abnormal gyri and sulci are produced after removal of specific nuclei or connections early in development while the cerebral surface is still smooth (Goldman-Rakic and Galkin, 1978; Goldman-Rakic, 1980; Goldman-Rakic and Rakic, 1984; Rakic, 1988; Welker, 1990).

In line with these findings, it has been shown that the size of the subplate zone anticipates regional differences related to the late configuration of the cerebral surface because the subplate zone in the gyral regions is larger than in the sulcal zones (Kostovic and Rakic, 1990). For this reason, it has been proposed that the subplate zone may play a role not only in the parcelation of cytoarchitectonic areas, but in the formation of cerebral convolutions (Rakic, 1988; Kostovic and Rakic, 1990).

It has been suggested that neurons in the subplate zone may die as a consequence of deafferentation once afferent fibers waiting in the future subcortical white matter penetrate the cerebral cortex. If we accept this hypothesis, naturally occurring cell death in the subplate zone does not have a pivotal role in the formation of cerebral convolutions. However, if the alternative hypothesis is considered, in which cortical afferents move towards the cerebral cortex because transitory neurons in the cortical subplate die, then cell death in the subplate eventually promotes the formation of cerebral convolutions by permitting the arrival to the cerebral cortex of large amounts of fibers which stimulate and gear the maturation of cortical neurons.

Although it seems premature to accept (or reject) a role of dead cells in the cortical subplate in the formation of the cerebral convolutions, it has been clearly shown that naturally occurring cell death in the cerebral cortex, at least in the cat, does not have a significant function in the process of cortical folding. This is simply because cell death in the cerebral cortex of kittens takes place just after gyri and sulci are formed (Ferrer *et al.*, 1989).

Even though it does not shape the external configuration of the brain, cell death in the cerebral cortex of the cat probably models the final cellular organization in the gyral and sulcal zones. This is supported by the fact that the sum of dead cells is larger in the sulcus than in the crown of the gyrus, a feature which is in accordance with the small cortical thickness of the sulci when compared to the gyri in adulthood (Ferrer *et al.*, 1986, 1987, 1988, 1990).

9.2. Formation of Pathological Gyri

In contrast to normal development, in which cortical folding is independent of naturally occurring cell death in the cerebral cortex, cortical necrosis at appropriate times of development may promote the formation of abnormal convolutions. Observations in spontaneous cortical abnormalities in humans and in experimentally-induced cortical malformations in rodents support these hypotheses.

Human polymicrogyria is characterized by multiple, irregular and small gyri, often not exteriorized by sulci (Bielchowsky, 1916; Crome, 1952; Larroche, 1984; Barth, 1987; Friede, 1989). In some cases, polymicrogyria is the result of laminar cortical necrosis produced as a consequence of an hypoxic accident occurring after neuronal migration to the cerebral cortex is almost completed and before secondary and tertiary sulci have developed (Richman *et al.*, 1974; Williams *et al.*, 1976). In other cases, polymicrogyria is, probably, secondary to circulatory failure in the radial and unbranched cortical arteries penetrating from the meningeal surface, and which vascularize the cerebral cortex at mid-gestation, before the end of the period of neuroblast migration to the cortical plate (Ferrer and Catalá, 1991). The particular

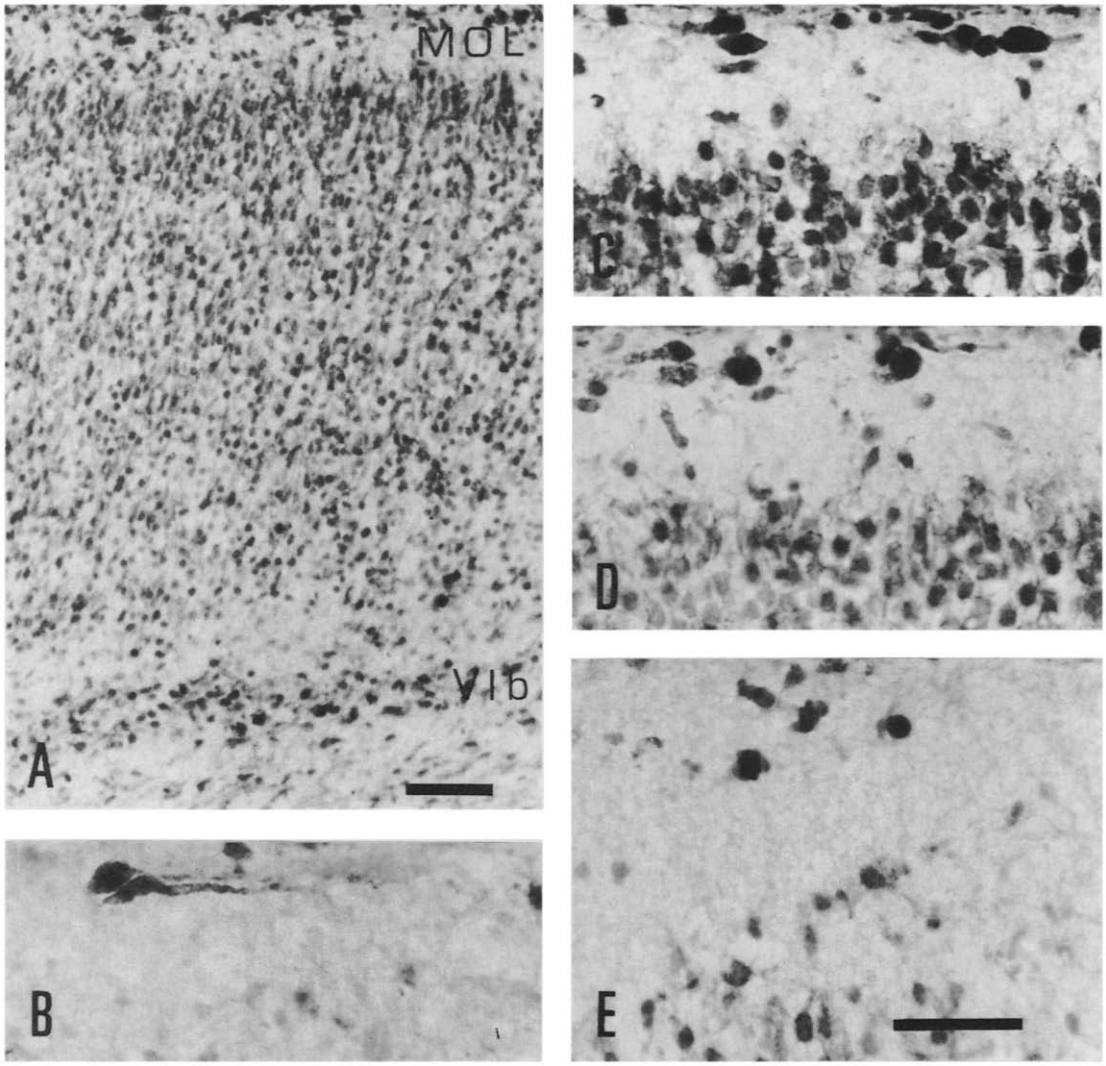

FIG. 19. Neurons in the cerebral cortex of the developing rat immunolabelled with the anti-Hu antibody (see Graus and Ferrer, 1990). A—Newborn rat showing MOL: molecular layer and layer VIb (VIb), separate this latter from the other cortical layers by a thin band almost devoid of nerve cells; B to E—Neurons in the molecular layer in newborn (B), 3 day-old (C), 5 day-old (D) and 10 day-old (E) rat. Horizontal neurons with morphological characteristics of Cajal-Retzius cells progressively vanish in the molecular layer, while globular neurons appear instead. Original serum diluted at 1/1000. Avidin and biotin method. A, bar = 100 microns. B to E, same magnification, bar = 50 microns.

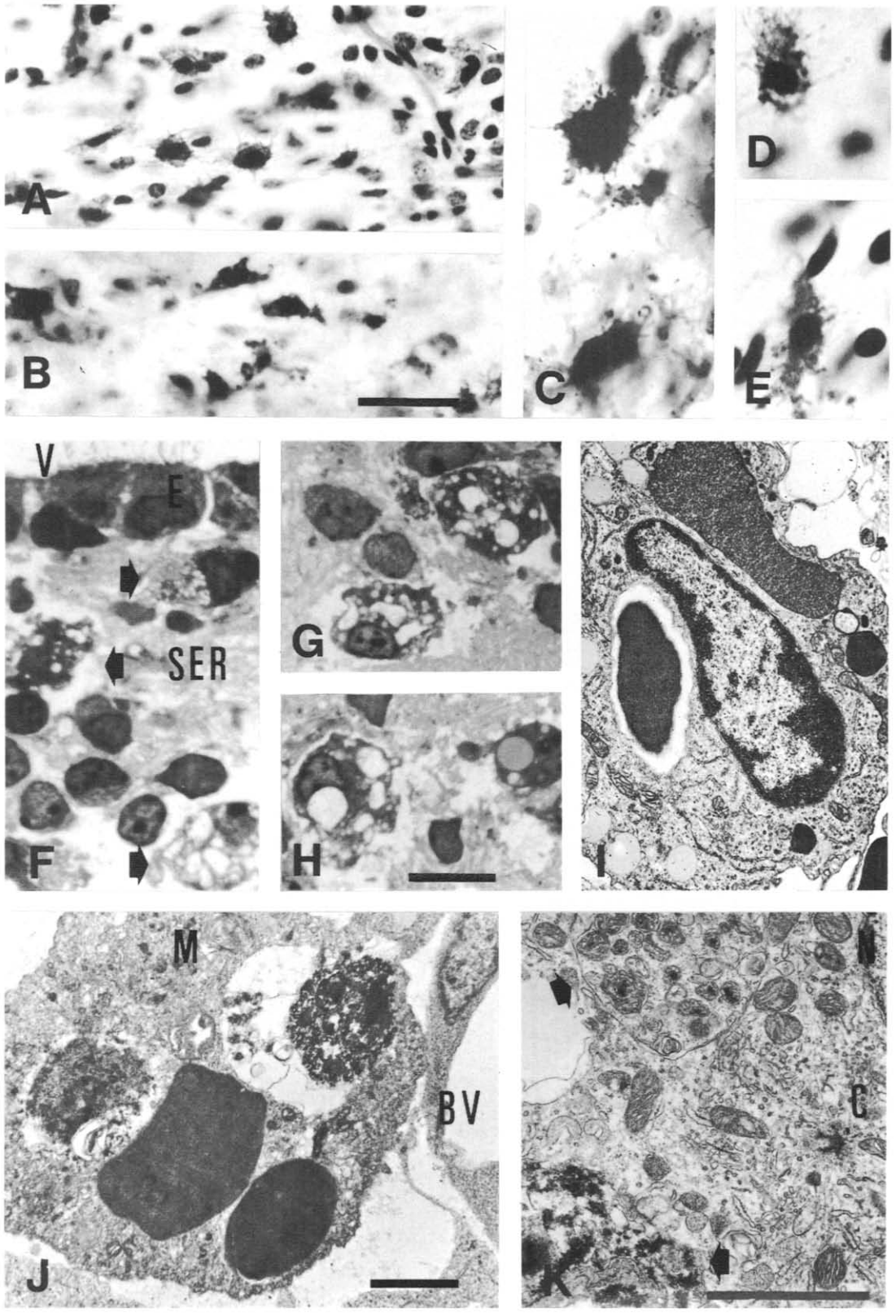

FIG. 20. Microglial cells in the developing cortex and subcortical white matter of the rat. A to E—Classical ameboid microglial cells stained with the Rio Hortega's method for microglia. A: Subcortical white matter of the 3 day-old rat; B: Subcortical white matter in the rat aged 7 days; C: Ameboid microglial in the subcortical white matter of the rat aged 5 days; D and E: Ameboid and ramified, respectively, microglial cells in the upper cortical layers (layers II–III) in rats aged 7 days. F to H—Epon-embedded, semithin sections stained with toluidine blue showing ameboid cells (arrows) in the rat aged 5 days. F: Subependimal region (SER) (V): ventricle; (E): ependima. G and H: Subcortical white matter. I and J—Microglial cells engulfing nuclear debris (M): microglia; (BV): blood vessel K—Microglial cell engulfing cellular debris (arrows); (C): centriole. A and B, bar = 100 microns. C to H, same magnification, bar = 20 microns. I and J, bar = 2 microns. K, bar = 1 micron.

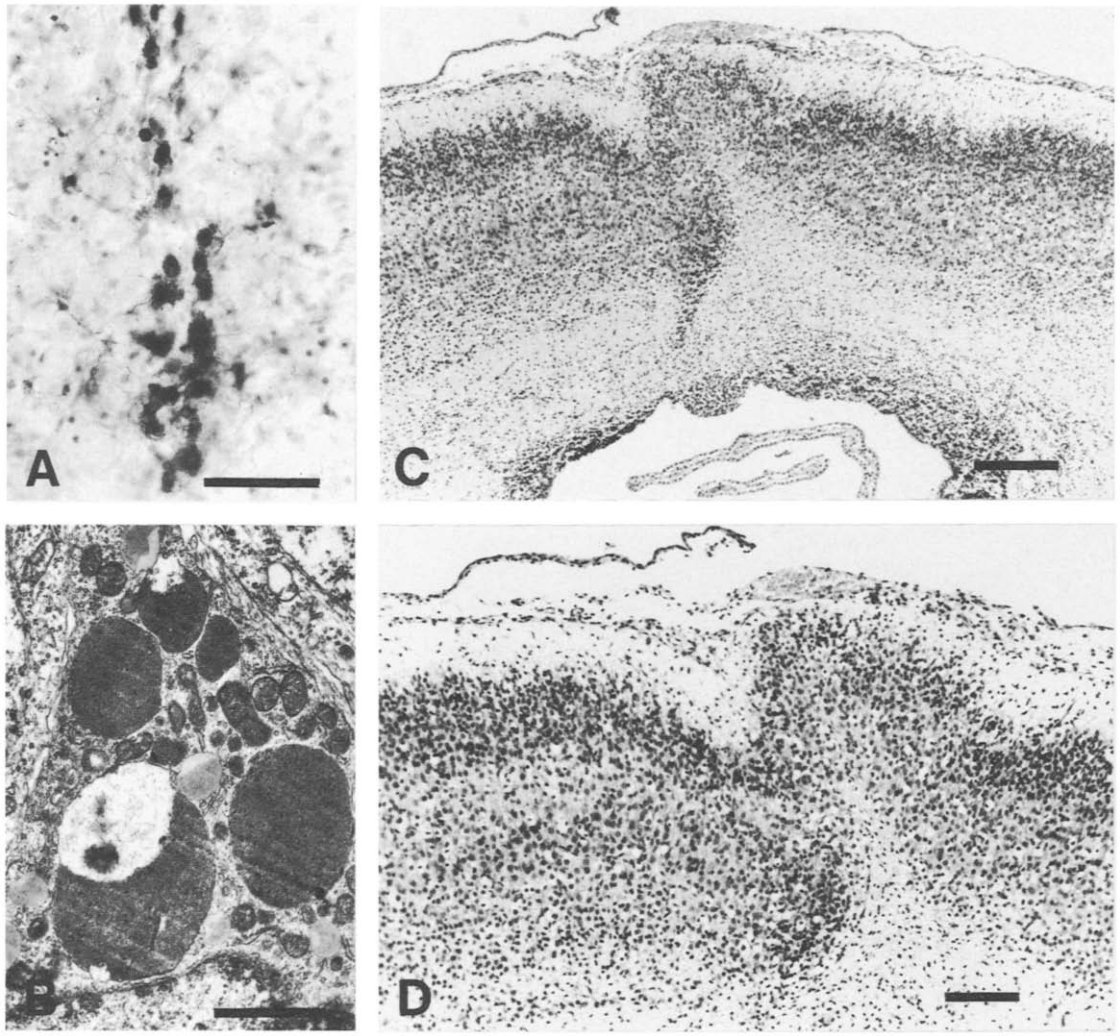

FIG. 21. Development of an abnormal gyrus in the rat following gentle rubbing of the cortical meninge at birth radial necrosis of the cerebral cortex is accomplished, probably, due to destruction of radial unbranched blood vessels which penetrate from the meninge to vascularize the immature cerebral cortex. A—Reactive microglial cells, similar in morphology and function to ameboid microglial cells, appear after 24 hr, and phagocyte cellular debris in the necrotic radial territories. Rio Hortega's silver method for microglia. B—Reactive microglial cell engulfing large numbers of cellular debris. C and D—Formation of abnormal gyri with 'fused' molecular layers in a rat aged 7 days. Cortical lamination is lost at the site of the cortical abnormality. A, bar = 100; B, bar = 200; C, bar = 100; D, bar = 2 microns.

morphology of this cortical abnormality finally depends on the imbalance in the tangential growth of adjoining cortical areas variably destroyed by tissue necrosis (Ferrer, 1984).

Microgyria has been experimentally produced in rats by freezing the cortical surface in newborn animals, just at the time when the last neuroblasts migrate to their definitive emplacements in the cerebral cortex (Dvorák and Feit, 1977; Dvorák *et al.*, 1978; Humphreys *et al.*, 1991). In these experiments tissue necrosis was caused by applying a freezing probe to the skull overlying the cerebral hemispheres. We have obtained similar results after gentle rubbing of the meninge with small blunt needles after covering their ends with cotton.

Microgyria was often observed in animals treated at birth or in rats 1 day old, but microgyria is never produced in animals older than 2 days. Sequential studies showed focal areas of necrosis of the cerebral cortex which followed oblique or perpendicular trajectories to the surface. With electron microscopy, necrotic cells exhibited shrunken cytoplasm with dilated endoplasmic reticulum, perinuclear membranes and Golgi complex, together with condensation of the mitochondrial matrix. The nucleus was dark and the nucleolus condensed. These characteristics differed from those found in normal postnatal cell death, but were typical of the so-called cytoplasmic type of cell death (Pilar and Landmesser, 1976).

After 24 hr, areas of necrosis were populated by large numbers of macrophages, as previously described in rats subjected to cortical lesions at birth (Ferrer and Sarmiento, 1980b).

Reorganization of radial cortical necrosis led to the formation of abnormal cortical folds not exteriorized by sulci in animals older than two weeks (Fig. 21). These results give further support to the notion that cortical folding ultimately builds upon the unbalanced distribution of intracortical neurons.

In summary, cortical cell death during normal development is a late phenomenon probably contributing to the final stabilization of cell numbers in the sulcal and gyral regions (Ferrer *et al.*, 1989). On the contrary, pathological cell death at very precise stages of development may induce the formation of abnormal cortical folds.

10. FINAL COMMENTS

Many mechanisms have been proposed to explain why some neurons die during normal development.

The nerve growth factor theory (Hamburger and Oppenheim, 1982; Oppenheim, 1985, 1989, 1991; Purves, 1988) derives from the observation that nerve growth factor is necessary for the survival of certain neuronal populations (Hamburger and Levi-Montalcini, 1949; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1966; Hamburger, 1980; Levi-Montalcini and Aloe, 1981; Hamburger *et al.*, 1981), and states that neurons compete for trophic factors produced by their targets (Hamburger and Oppenheim, 1982; Purves, 1988; Davies, 1988; Barde, 1989; Oppenheim, 1989, 1991). Since these are produced in small amounts, it is postulated that only nerve cells with appropriate levels of growth factors can survive.

Other populations of neurons depend on hormones. Neuronal death in amphibian metamorphosis is related to thyroxine levels (Kollros, 1981). Death of abdominal neurons in the moth *Manduca sexta* is associated with the decline in ecdysteroids (20 HE) (Truman and Schwartz, 1980), and treatment with 20 HE at physiological concentrations prevents neuronal death (Truman and Schwartz, 1984). Sexually dimorphic nuclei in the spinal cord and other brain regions of rodents are androgen dependent (Nordeen *et al.*, 1985; Wright and Smolen, 1983, 1987; Sengelaub and Arnold, 1989). Similarly, the survival of forebrain neurons which control the song of male songbirds (e.g. zebrafinch) is steroid dependent (Nordeen *et al.*, 1987).

Programmed neuron death in the nematode *C. elegans* is a result of the activation of different genes which encode different proteins involved in cell killing and engulfment (Horvitz *et al.*, 1982; Chalfie and Wolinsky, 1990; Ellis *et al.*, 1991).

This short overview reveals that naturally occurring cell death may be triggered by many different factors and we do not yet know which can act in the telencephalic mantle during normal development. Deprivation of distinct trophic factors may play a role in cortical cell death, but much work must be done before this theory is proved. On the other hand, the proposal that the expression of certain proteins is a putative marker for cell death during development (Al-Ghoul and Miller, 1989; Naegele *et al.*, 1991), cannot be justified in some cases (Valverde *et al.*, 1990).

Naturally occurring cell death in the cerebral cortex, cortical subplate and future subcortical white matter resembles apoptosis, which in other systems is characterized by 'nuclear condensation associated with the activation of a specific endonuclease which cleaves the cell's DNA into microsome-like fragments' (Wyllie, 1981, 1987). Ca^{2+}/Mg^{2+} endonuclease activation has been demonstrated in apoptotic lymphocytes and thymic cells (Wyllie *et al.*, 1980, 1984; Wyllie, 1981, 1987; Duke *et al.*, 1983; Cohen and Duke, 1984; Cotter *et al.*, 1990; McConkey *et al.*, 1990).

Recent studies have proposed that a similar active process may occur in some nerve cell populations *in vitro* (Martin *et al.*, 1988; Scott and Davies, 1990) and *in vivo* (Fahrbach and Truman, 1987; Oppenheim *et al.*, 1990), as deduced from the observation that actinomycin-D and cycloheximide, which are inhibitors of protein and RNA synthesis, may reduce the amount of cell death in these systems.

In addition, flunarazine, a Ca^{2+} channel antagonist which probably blocks intracellular entry of Ca^{2+}, protects neurons from death after axotomy or nerve growth factor deprivation (Rich and Hollowell, 1990).

Cycloheximide reduces ischemic delayed cell death in the rat hippocampus (Goto *et al.*, 1990), while irradiation-induced apoptosis in the developing rodent cerebellum (Inouye and Kameyama, 1983; Harmon and Allan, 1988) is also inhibited by cycloheximide (Inouye *et al.*, 1991).

However, further studies are needed to elucidate whether novel RNA and protein syntheses are required in relation to cortical cell death during normal development.

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