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CHAPTER 5

The Development of the Primordial Plexiform Layer and Its Subsequent Partitioning into Layer I and the Subplate (Layer VII)

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Marin-Padilla (1971, 1978, 1983) proposed that the mammalian cerebral cortex develops in two stages. The first stage is the formation of the primordial plexiform layer consisting of cells of the future layer I (marginal layer) and layer VII (subplate). The primordial plexiform layer is hypothesized to represent the phylogenetically old reptilian cortex. The second stage is the formation of the mammalian cortical plate (the future layers VI–II) that partitions the primordial plexiform layer into superficial and deep parts. In this chapter, we present [^{3}H]thymidine autoradiographic observations that support and extend Marin-Padilla's hypothesis.

The embryonic observations were based on the sequential order of neurogenesis between the Cajal-Retzius and subplate neurons (Chapter 3). First, appropriate [^{3}H]thymidine injection groups were selected from the sequential-survival series of autoradiograms that would differentiate Cajal-Retzius neurons from subplate neurons. Since close to 64% of the Cajal-Retzius neurons are generated on E14 (Fig. 3–2A), most of them should be heavily labeled in embryos following an E13 [^{3}H]thymidine injection but not in embryos following an E15 injection. On the other hand, 45% of the subplate cells are generated on E15 (Fig. 3–2A), and about half of them should be heavily labeled in embryos following an E15 injection. Consequently, we examined the neocortex after an E13 injection to track movements of the Cajal-Retzius neurons and, after an E15 injection, to track movements of the subplate neurons. (See Appendix 2 for the details of sequential-survival autoradiographic methods.)

In Chapter 2 we briefly described two sets of extracellular channels that appear sequentially in the embryonic neocortex, channel 1 on E14 and channel 2 on E17 (Fig. 2–12A, D). Such channels have been described in the incipient optic tract (Silver and Sidman, 1980), the optic tectum (Krayanek, 1980), the retina (Krayanek and Goldberg, 1981), the corpus callosum (Silver et al., 1982), and the dorsal column of the spinal cord (Altman and Bayer, 1984). In this chapter, we will show that the partitioning of the primordial plexiform layer is related to these two channels. The Cajal-Retzius neurons rapidly move to the surface of the cortex and settle in channel 1. In contrast, the polymorph neurons first assemble beneath channel 1 in a zone where the cortical plate will form later. As a new extracellular matrix, channel 2, develops beneath the cortical plate, the polymorph neurons descend into channel 2 and form a morphologically identifiable subplate.

5.1 THE FORMATION OF CHANNEL 1 AND THE SETTLING OF THE CAJAL-RETZIUS CELLS

The examination of the developing neocortex in normal methacrylate-embedded embryonic brains presented in Chapter 2 shows the successive formation, and then the gradual dissolution, of two systems of

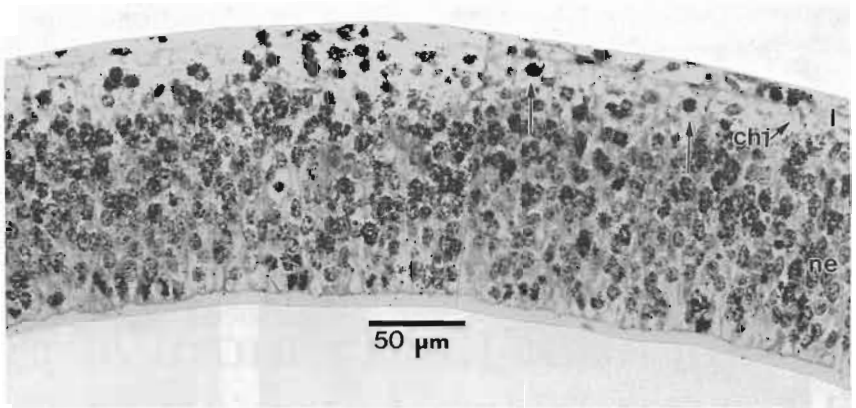

FIG. 5–1. The dorsal neocortical wall in a rat embryo on E14 that was exposed to a single injection of [^{3}H]thymidine on E13 and survived for 24 hours. Heavily labeled cells (*arrows*, presumed birthdays on E13) are already outside of the neuroepithelium among the spaces in the superficial extracellular channel system (ch1). (3 μm methacrylate coronal section, hematoxylin stain.)

extracellular channels, channel 1 and channel 2. On days E12 (Fig. 2–3) and E13 (Fig. 2–4), the primordium of the neocortex consists only of radially oriented cells in the neuroepithelium, and an organized extracellular network is not apparent at the light microscopic level. By day E14, channel 1 appears just beneath the pia (ch1, Figs. 2–12A and 5–1), consisting of large membrane-bound extracellular spaces with a few round or horizontally oriented cells embedded in them. Derer and Nakanishi (1983) have also described a subpial channel network in the embryonic mouse neocortex. Channel 1 remains prominent on E15 (ch1, Fig. 2–12B) but is not obvious by E16 and E17 (Fig. 2–12C, D), possibly due to the ingrowth of neuronal processes.

In rats that received [^{3}H]thymidine on day E13 and were killed on day E14, heavily labeled cells begin to accumulate outside of the neuroepithelium beneath the pia in channel 1 (Fig. 5–1). In rats injected on day E13 and killed on day E15 (Fig. 5–2), a row of large heavily labeled cells clearly delineates the outermost part of

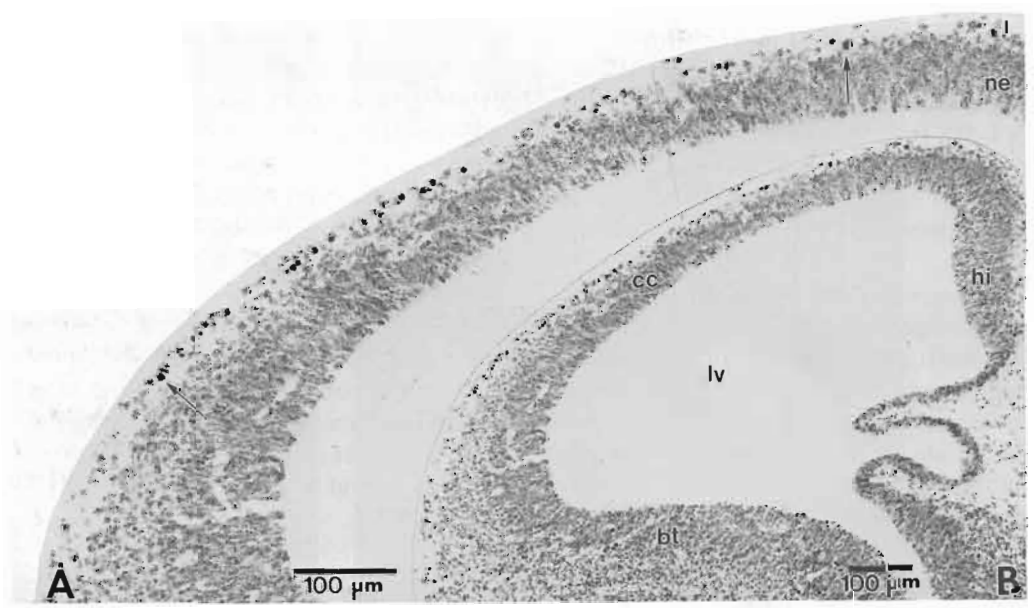

FIG. 5–2. Low magnification **(B)** and high magnification **(A)** views of the coronally sectioned embryonic neocortex on E15, 2 days after a single [^{3}H]thymidine injection on E13. Many heavily labeled large cells (*arrows*, presumed birthdays on E13 and E14) are accumulating just beneath the pial membrane (cut edge of the brain). The heavily labeled cells stop as the pallium curves downward to form the medial wall, where components of the hippocampal region (hi) will develop. (6 μm paraffin section, hematoxylin stain.)

the primordial plexiform layer. These are presumed to be the Cajal-Retzius neurons since it is unlikely that subplate neurons would be heavily labeled by an E13 injection. The Cajal-Retzius neurons appear concurrently in the ventrolateral and dorsal parts of the cerebral cortex (the lateral limbic and neocortical primordia), in accordance with the absence of a lateral-to-medial neurogenetic gradient (Chapter 3). However, the large heavily labeled cells stop abruptly in the medial cortical wall where components of the hippocampal region will develop (hi, Fig. 5–2B). In animals that survived for 3 or more days after a [^{3}H]thymidine injection on E13, the heavily labeled large cells beneath the pial membrane remain a consistent feature of the developing neocortex, except that they become more sparse as the neocortex expands.

A remarkable feature of the Cajal-Retzius neurons is that they settle in their permanent positions within 1 day after their generation. That characteristic sets the Cajal-Retzius neurons apart from both the subplate neurons and the layer VI–II neurons. In the next section, we show that it will be several days before the subplate neurons reach their permanent position. In Chapter 7 we show that all of the layer VI–II neurons that accumulate in the cortical plate sojourn in specific bands in the transitional field for 1 to 2 days after their generation. The rapid settling of the Cajal-Retzius neurons beneath the pial membrane (Fig. 5–2B) may be related to the early ingrowth of cortical afferents into channel 1. It is well known that the first cortical afferents are distributed superficially (Cajal, 1911; Stensaas, 1967d; Marin-Padilla, 1972), just where the channel 1 system is located. Several studies indicate that these fibers come from brainstem monoamine nuclei (Seiger and Olson, 1973; Molliver and Kristt, 1975; Schlumpf et al., 1980; Caviness and Korde, 1981; Lidov and Molliver, 1982; Crandall and Caviness, 1984; Fujimiya et al., 1986; Mulligan and Törk, 1987; Kalsbeek et al., 1988). Correlating with the early ingrowth of these fibers, the first synapses appear superficially on the dendrites of Cajal-Retzius neurons (Stensaas, 1967c; Meller et al., 1968; Molliver and van der Loos, 1970; Molliver et al., 1973; König et al., 1975; Kristt and Molliver, 1976; Raedler and Sievers, 1976; Rickmann et al., 1977; Zheng et al., 1990). Early synapse formation may be responsible for the fact that Cajal-Retzius neurons remain in a superficial position throughout the rest of neocortical development (Cajal, 1911; Marin-Padilla, 1971, 1978).

The three-dimensional reconstructions of the E15 and E16 embryonic neocortex (Chapter 2) show that the primordial plexiform layer and the ventricular zone are coextensive in both the mediolateral and anteroposterior axes on E15 (Color Fig. 1). As early as E16 (Color Fig. 2), the ventricular zone begins to shrink dorsomedially but the primitive cortical plate first appears ventrolaterally beneath the most ventrolateral extent of the channel 1 system outlined by the settling Cajal-Retzius neurons. Possibly the original extent of the Cajal-Retzius neuron distribution sets the linear limits for the cortical plate.

5.2 THE FORMATION OF CHANNEL 2 AND THE SETTLING OF NEURONS IN THE SUBPLATE

The second extracellular channel system emerges on day E16 far ventrolaterally underneath the cortical plate and spreads laterally and dorsomedially by day E17 (ch2 and CP, Fig. 2–12D). By day E18, a horizontal band of cells begins to accumulate within channel 2 (ch2 and SP, Fig. 2–12E), which, according to the [^{3}H]thymidine autoradiographic evidence, are subplate neurons. Chun and Shatz (1988) and Kostovic and Rakic (1990) described a similar channel system among the subplate neurons in the embryonic cat neocortex. Channel 2, which has become wider by E18, is split into a superficial and a deep component by the subplate neurons. This pattern persists through days E19 to E22 (Figs. 2–13, 2–14, and 2–15), and the subplate is still recognizable as a separate layer on P5 (Fig. 3–1A). Considering that most subplate neurons are generated earlier than cortical plate neurons (Chapter 3), it seems paradoxical that the subplate becomes morphologically distinct after the cortical plate.

We tracked the positions of subplate neurons in [^{3}H]thymidine autoradiograms of embryos that survived 1–5 days after their dams received a single injection on day E15 (E16 to E20, Figs. 5–3 and 5–4). The sagittally sectioned dorsomedial neocortex is illustrated, where development lags behind the more mature ventrolateral cortex. In this part of the cortex, E15 is the peak day of neurogenesis in the subplate (50%; MO, Fig. 3–2B), while neurogenesis in layer VI has just begun (slightly over 10%; FR, Fig. 3–8). Consequently, most of the heavily labeled cells in the differentiating layers outside of the neuroepithelium are presumptive subplate neurons.

In rats labeled on day E15 and killed on day E16 (Fig. 5–3A), heavily labeled cells are found in two locations. First, a high proportion of them gather in irregular clusters in the upper part of the neuroepithelium (*vertical arrows,* Fig. 5–3A); these may be some early postmitotic neurons sequestered in the superficial part of the neuroepithelium or slowly cycling mitotic cells (Chapter 4). Second, there is a smaller concentration of heavily labeled cells in a horizontal band outside of the neuroepithelium (*brackets*), just beneath the unlabeled Cajal-Retzius neurons (*horizontal arrows*). That band is uniformly distributed throughout

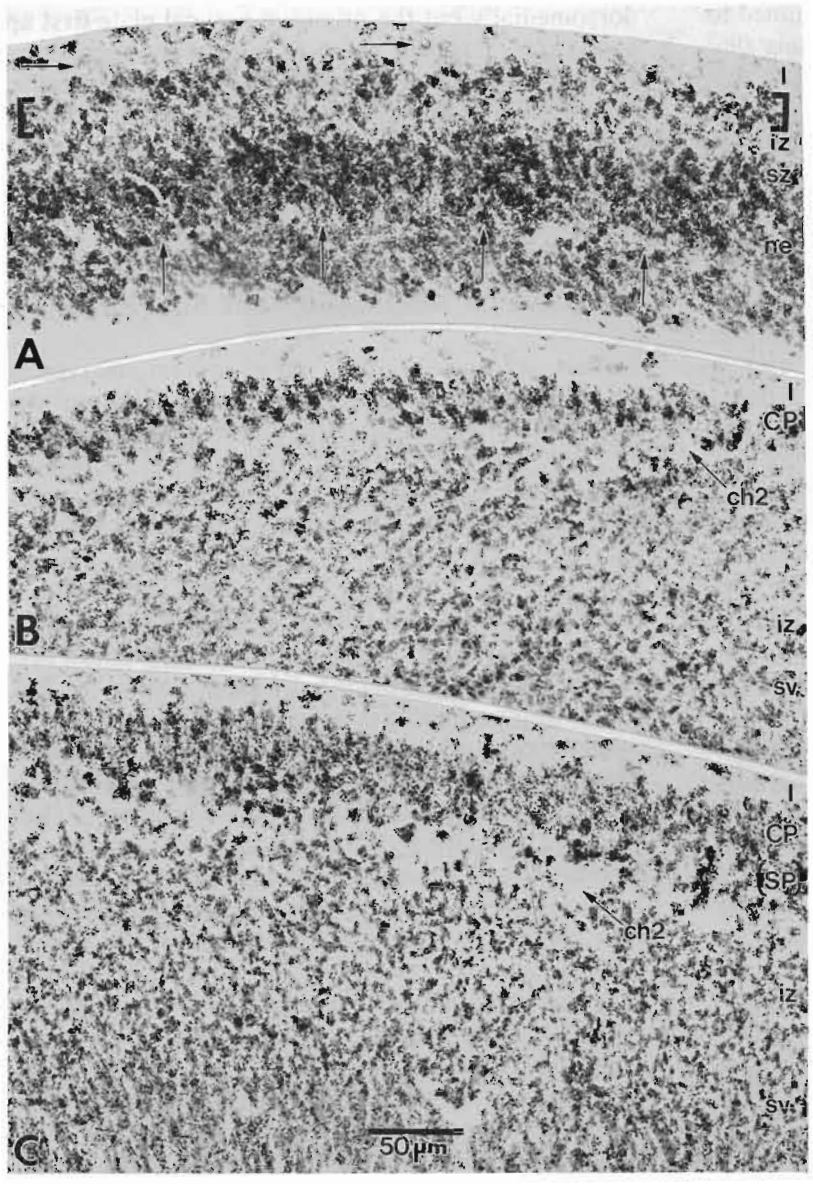

FIG. 5–3. A series of autoradiograms showing the variable positions of cells heavily labeled by a single [^{3}H]thymidine injection on E15 in the sagittally sectioned dorsomedial neocortex (6 μm paraffin section, hematoxylin stain). These cells are presumed to have birthdays on E15 and many will settle permanently in the subplate. In **A** (E16), 1 day after the injection, many heavily labeled cells are in a band (*brackets*) outside of the neuroepithelium (ne). *Vertical arrows* indicate clusters of heavily labeled cells that stay in the neuroepithelium; *horizontal arrows* indicate unlabeled cells above the band of heavily labeled cells, presumably the Cajal-Retzius neurons with birthdays on E14. In **B** (E17), 2 days after injection, heavily labeled cells are scattered throughout the cortical plate (CP). In **C** (E18), three days after injection, heavily labeled cells are at the base of the cortical plate and are beginning to accumulate in the subplate (SP).

the developing cortex in contrast to the bands that appear in the intermediate zone (described in Chapter 7)[1] and presumably contains subplate neurons that have variable (mostly horizontal) cell orientations.[2] In this stage of neocortical development, there is an unpartitioned primordial plexiform layer with the earlier generated Cajal-Retzius neurons superficial (mainly E14 birthdays), later-generated subplate neurons deep (many E15 birthdays).

From E17 through E20, the primordial plexiform layer is partitioned by the developing cortical plate into a superficial layer I and a deep subplate. In rats labeled on day E15 and killed on day E17 (Fig. 5–3B), heavily labeled cells are virtually limited to one location, the cortical plate (CP), which occupies exactly the same position as the horizontal band of heavily labeled cells on E16. The only difference is that the cells in the cortical plate are almost all radially oriented (Chapter 8). By E18 (Fig. 5–3C), 2 days after injection, most of the heavily labeled cells are at the base of the cortical plate (CP), and some are descending into the cell-sparse zone (ch2) to form the subplate (SP in brackets, Fig. 5–3C). By E19 (Fig. 5–4A), 3 days after injection, the cortical plate (CP) now contains only later-generated cells that are lightly labeled. But many heavily labeled cells are in the subplate (SP, Fig. 5–4A). By

[1] The superior bands in the intermediate zone (sb1 and sb2) have ventrolateral-to-dorsomedial gradients of morphogenesis. Since the band we are describing here has a nearly uniform appearance, another group of neurons must be accumulating within it.

[2] It is important to note that the variably oriented cells in the dorsomedial cortex are continuous with the more radially aligned cells that accumulate in the primordial cortical plate farther ventrolaterally on E16.

E20 (Fig. 5–4B), 4 days after injection, most of the heavily labeled subplate neurons are situated within the extracellular spaces of channel 2. A few heavily labeled cells are also seen at the base of the cortical plate; these may be the early-generated layer VI neurons.

The sequential events during partitioning of the primordial plexiform layer are diagrammed in Figure 5–5. Unlike the Cajal-Retzius neurons (striped ovals, Fig. 5–5) that settle immediately in their final locations in the extracellular matrix of channel 1, the subplate cells (*dotted ovals,* Fig. 5–5) occupy two temporary positions before settling in the morphological subplate that is surrounded by the later developing channel 2 system. Subplate neurons are generated on E14 and E15 (Fig. 3–2), rapidly move out from the ventricular zone by E16, and first accumulate beneath the Cajal-Retzius neurons (Fig. 5–3A). During all of E17 and early E18, subplate neurons assume mainly a radial orientation and are the first to form the cortical plate. The extracellular matrix of channel 2 appears on E17 (Figs. 2–12D and 5–5), and on E18 and E19 subplate neurons leave the cortical plate and permanently settle there (Figs. 5–3 to 5–5), suggesting that the subplate neurons have an affinity for the fibers that are growing into channel 2.

The ingrowth of a second afferent system below the cortical plate has been described in several previous studies (Cajal, 1911; Stensaas, 1967d; Marin-Padilla, 1972; Rickmann et al., 1977; Crandall and Caviness, 1984; Kalsbeek et al., 1988). Some of the fibers arborizing in the subplate contain monoamines (Crandall and Caviness, 1984), specifically dopamine (Kalsbeek et al., 1988) and serotonin (Fujimiya et al., 1986), while other fibers are known to come from thalamic relay nuclei (Rakic, 1983; Crandall and Caviness, 1984;

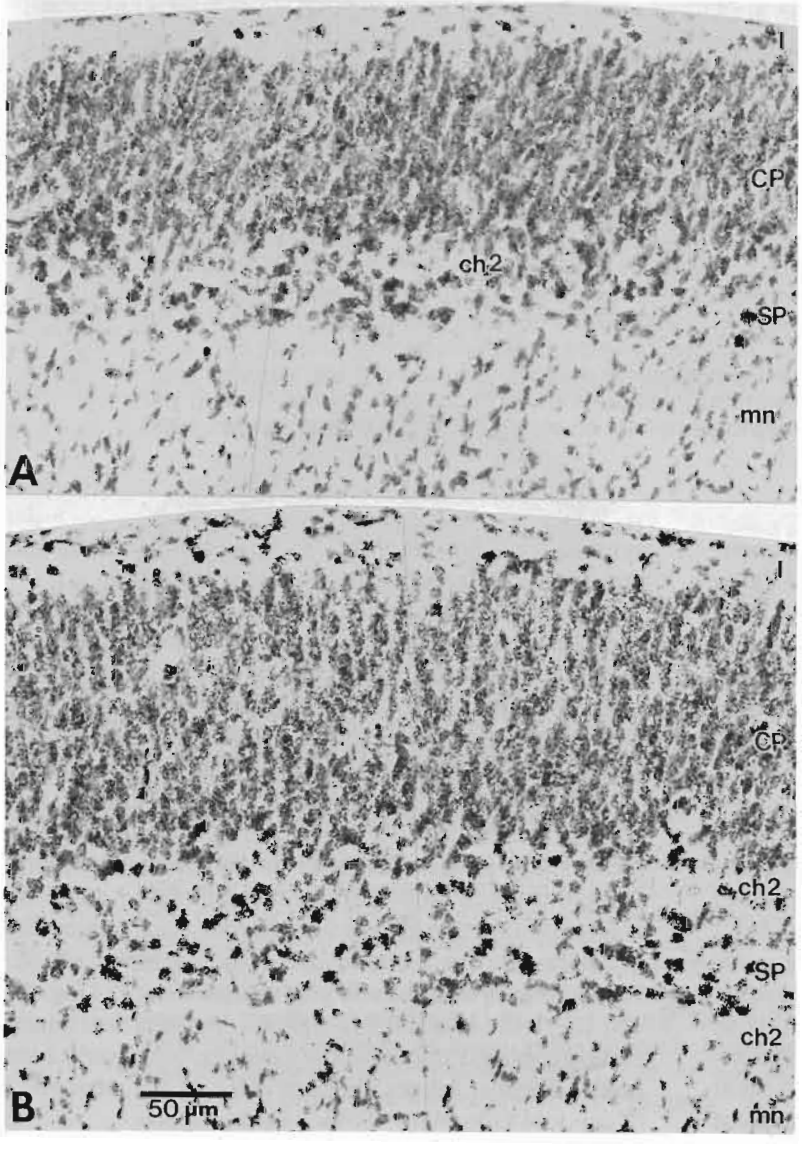

FIG. 5–4. A continuation of the sagittal series of the dorsomedial neocortex begun in Fig. 5–3 (6 μm paraffin sections, hematoxylin stain). In **A** (E19), four days after an E15 [^{3}H]thymidine injection, more heavily labeled cells accumulate in the subplate (SP) and it stands out as a separate layer beneath the cortical plate (CP). In **B** (E20), the subplate is even more definite.

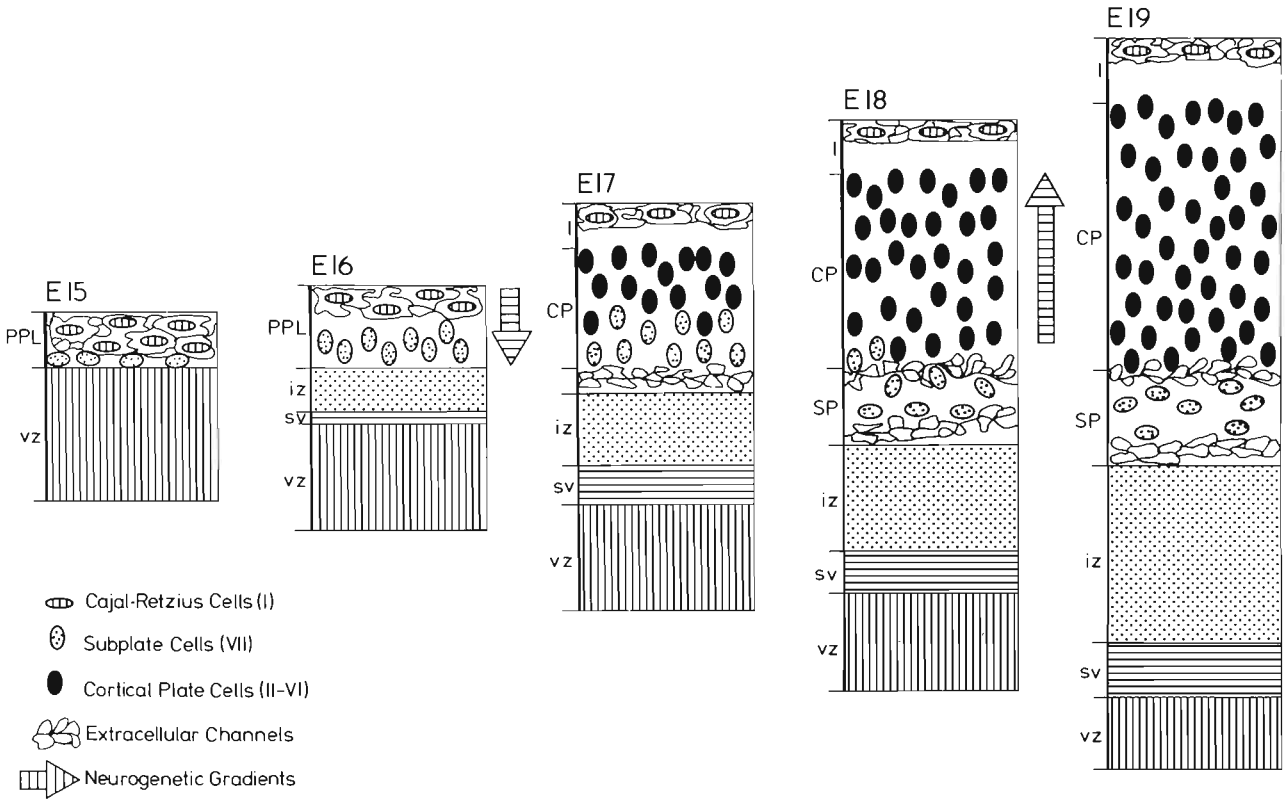

FIG. 5–5. Summary diagram of the main points made in this chapter. The layers of the ventrolateral neocortex are represented each day from E15 (*left*) to E19 (*right*). The first cell populations to accumulate outside the neuroepithelium (or ventricular zone, vz) reside in the primordial plexiform layer (PPL) with older cells superficial, younger cells deep (*arrow outside* E16 strip). From E17 to E19, the PPL is progressively segregated into superficial (Cajal-Retzius, *striped ovals*) and deep (subplate, *dotted ovals*) populations as cortical plate neurons (*solid black ovals*) settle in between them with older neurons deep, younger neurons superficial (*arrow outside* E18 strip). The Cajal-Retzius neurons settle early and permanently among the superficial extracellular channels. In contrast, subplate neurons temporarily reside in the cortical plate before settling permanently in the subplate among the deep extracellular channels. From Bayer and Altman, 1990.

Shatz and Luskin, 1986). Like the Cajal-Retzius neurons, subplate neurons (dotted ovals, Fig. 5–5) also have early synaptic contacts (Meller et al., 1969; Molliver and van der Loos, 1970; Molliver et al., 1973; König et al., 1975; Kristt and Molliver, 1976; Rickmann et al., 1977; König and Marty, 1981; Kostovic and Rakic, 1990). Since specific thalamic afferents form a dense plexus in the subplate before invading the cortical plate, it has been proposed that the subplate acts as a temporary target for afferents ultimately destined to contact pyramidal cells (Rakic, 1983; Crandall and Caviness, 1984; Shatz and Luskin, 1986; Chun et al., 1987; Friauf et al., 1990; Kostovic and Rakic, 1990). Friauf et al. (1990) showed that embryonic subplate neurons can be excited by stimulation of the optic radiation in cats; after iontophoresis of biocytin, these neurons were shown to have extensive axonal arborizations in the cortical plate, strongly suggesting that the subplate might represent a crucial but transient synaptic link between the thalamocortical axons and their ultimate target cells in the cortical plate.

Presumably responding to the altered milieu in the deep fiber plexus, subplate neurons change their orientation from radial (pyramidal-like) to horizontal (polymorphic) (Fig. 5–5). The developmental Golgi studies of Cajal (1911), Stensaas (1967c), and Marin-Padilla (1971) indicate that subplate neurons have axons in layer I. The subplate neurons may be extruding their axons in a superficial direction during the radial shape phase when they are in the cortical plate Once they are couched in the subplate, dendritic growth within the plexus of afferent fibers gives the subplate neurons a characteristic polymorphic shape. Subplate neurons are still polymorphic on P5 (Fig. 3–1D, E). In the adult rat brain (P60), sparsely distributed early-generated polymorphic cells are commonly seen in the deepest layers (VIb or VII) of the neocortex (Reep and Goodwin, 1988).

5.3 TRANSIENT POSITIONS OF THE SUBPLATE NEURONS AND MORPHOGENESIS OF THE CORTICAL PLATE

Our observations support the view that the cortical plate splits the primordial plexiform layer into a superficial layer I containing Cajal-Retzius cells and a deep subplate containing polymorph cells (Marin-Padilla, 1978; Wolff, 1978; Raedler et al., 1980). Two assumptions are implicit in that view. First, that the subplate neurons settle immediately and permanently in a subplate layer before neurons in the cortical plate migrate above them. Second, that all neurons in the cortical plate are destined to settle there permanently. Our autoradiographic evidence casts doubt on these assumptions because in rats surviving to E17 after a [^{3}H]thymidine injection on E15, the peak day of subplate neurogenesis, heavily labeled cells are located in the cortical plate rather than in the subplate, which does not become distinct until 2 days later.

We offer the hypothesis that the subplate neurons temporarily reside in the cortical plate (E16-E17 strips,

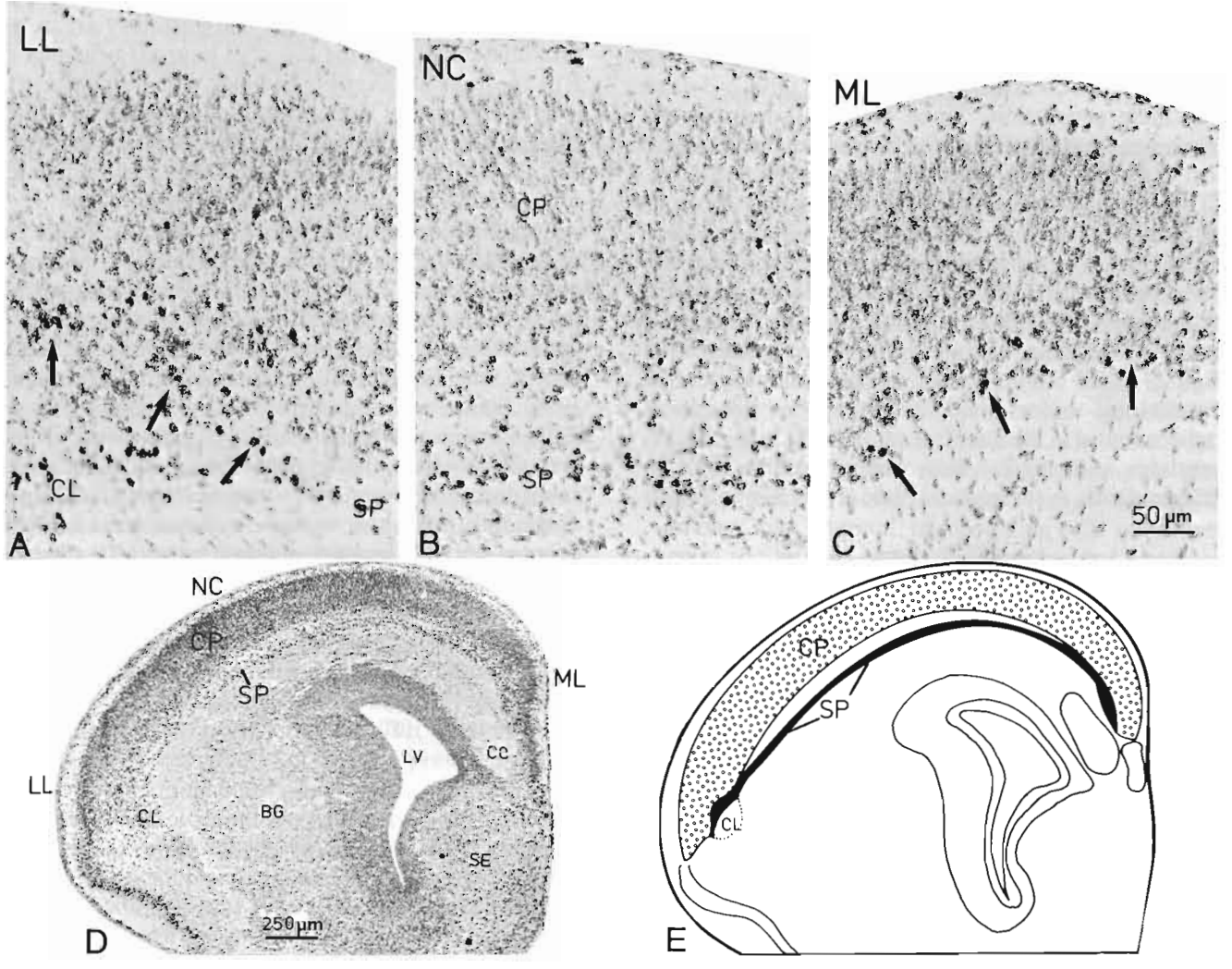

FIG. 5–6. Labeling patterns in an anterior coronal section of an E21 rat embryonic brain that was exposed to a single [^{3}H]thymidine injection on E15. Since an E15 injection will intensely label slightly over half of the neurons in the subplate (SP), it stands out as a layer of intensely labeled cells in the low magnification view in **D,** and is represented as a *solid black area* in **E.** A high-magnification view **(B)** of the neocortex (NC) shows that the subplate is separated from the deep parts of the cortical plate (CP) by a cell sparse zone. In contrast, high-magnification views of the lateral limbic (LL) **(A),** and medial limbic (ML) **(C)** cortices show that cells of the same labeling intensity as those in the subplate (*arrows*) are in the deep cortical plate rather than in a separate layer. In ventrolateral areas **(A),** the claustrum (CL) also has intensely labeled cells due to prominent neurogenesis on E15. (6 µm paraffin section, hematoxylin stain.) From Bayer, 1990b.

Fig. 5–5). Luskin and Shatz (1985b) hinted at that possibility in their work on the cat neocortex but called the accumulation of radially aligned cells the *upper subplate,* a term also used by Kostovic and Rakic (1990) in their study of the primate neocortex. There are two observations that support our hypothesis. First, the young neurons settling in the early cortical plate on E17 (Fig. 5–3B) have labeling patterns that are consistent with a peak of cell origin on E15, matching the neurogenetic pattern found in the dorsomedial subplate (MO, Fig. 3–2B). Second, our recent findings with sequential survival thymidine autoradiography indicate that neocortical neurons that will reside in layers VI–II do not directly move to the cortical plate. They first accumulate in bands in the subventricular and intermediate zones (Chapter 7) for approximately 1–2 days and do not reach the cortical plate until 2–3 days after their generation. Since layer VI neurons are mainly generated on E16 in the dorsomedial neocortex, most of them will not reach the cortical plate until E18.

Two observations in this chapter support the suggestion of Luskin and Shatz (1985b) that the subplate may provide the "morphogenetic foundation" for the cortical plate. First, subplate neurons do not leave the cortical plate until E18, after substantial numbers of permanent resident neurons have arrived and settled (Figs. 5–3 and 5–4). Second, the pronounced ventrolateral/oldest-to-dorsomedial/youngest neurogenetic gradient found in the subplate on E14–E15 (Fig. 3–2B) correlates with similar neurogenetic and morphogenetic gradients in the cortical plate (Figs. 3–8 to 3–10).

5.4 SUBPLATE AND CORTICAL PLATE BLENDING IN THE LIMBIC CORTEX

Curiously, a separate subplate does not form below the medial and lateral extremes of the cortical plate where the limbic cortical areas will differentiate. Kostovic and Rakic (1990) also noted the same phenomenon in Nissl-stained sections of the developing primate neocortex. A low magnification view of an E21 rat embryo exposed to [^{3}H]thymidine on E15 (Fig. 5–6D) shows that the subplate (SP) stands out as a band of intensely labeled cells. Higher magnification views show that the subplate is especially prominent in central areas that will differentiate into neocortex (Fig. 5–6B). Both medially (Fig. 5–6C) and laterally (Fig. 5–6A) however, the intensely labeled cells (*arrows*) are situated in the deep cortical plate rather than in a separate subplate (shown in the drawing in Fig. 5–6E).

The data in Chapters 3 and 15 indicate that some cells in layer VI originate unusually early in the lateral and medial limbic areas. For example, in posterior parts of the agranular insular and gustatory areas, approximately 20% of the layer VI neurons originate on E14 (*bottom graphs,* Fig. 15–4A, B). Similarly, it is only in the medial limbic cortex that between 30–50% of the layer VI cells are born on or before E15 (CG, Fig. 3–8). These early cells may be the "subplate" neurons that infiltrate the cortical plate.

Subplate/cortical plate blending in limbic areas can be related to anatomical studies. The distribution of dopamine axons in the cortex, especially those from the ventral tegmental area, terminate predominantly in limbic rather than neocortical areas (Simon et al., 1976; Carter and Fibiger, 1977; Bjorklund and Lindvall, 1978; Fallon and Moore, 1978; Gerfen and Clavier, 1979; Porrino and Goldman-Rakic, 1982; Saper, 1982; Descarries et al., 1987; Kalsbeek et al., 1987). The small dopamine projection to the neocortex comes predominantly from the ventral tegmental area and terminates only in the very deepest layer (VII) (Descarries et al., 1987), just where remnants of the subplate are found in adult brains. The ventral tegmental area projection spreads out in the deep layers (V–VI) of the limbic cortical areas (Descarries et al., 1987; Foote and Morrison, 1987), just what would be expected if subplate neurons infiltrate the deep layers in the limbic cortices.