Cellular Mosaics in the Rat Marginal Zone Define an Early Neocortical Territorialization

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We describe a novel neuronal group of the neocortical primordium that is formed by the pioneer neurons, unlike the Cajal–Retzius cells and the subplate component of the preplate. These pioneer neurons of the preplate and the marginal zone send transient axonal projections into the nascent internal capsule, preceding the formation of the axonal projection from the subplate neurons. In wholemount preparations, the pioneer neurons of the preplate and the marginal zone cover the prospective neocortical territory from embryonic day (E) 12 to E17. Two subpopulations of pioneer neurons (defined by differential expression of calcium-binding proteins) group into well-defined cell clusters, separated by spaces containing a lower packing density of cells immunoreactive to the corresponding calcium-binding protein. In both cases, cell clustering was consistent with fasciculation of their axons. Although both subpopulations cohabit in the same areas of the marginal zone, their clustering occurs in specific, well-delineated territories, giving a mosaic appearance to the surface of the neocortical primordium before the arrival of thalamocortical axons. We hypothesize that the fascicles of descending axons arising from defined territories of the marginal zone may intervene in the initial guidance of the subcortical projection from the subplate.

Introduction

The cerebral cortex of adult animals contains a map of diverse cortical areas, characterized by a distinctive cytoarchitecture, a specific pattern of afferent and efferent connections, and a particular function. How the differential morphofunctional properties of the cortical areas are generated during early development has been the subject of intense debate. One of the prevailing hypotheses is that of the protomap (Rakic, 1978, 1988). It takes into account the columnar organization of the cortical areas, characterized by a distinctive cytoarchitecture, and the subplate component of the preplate. These pioneer neurons (Harrison, 1910; Bate, 1976; Goodman and Shatz, 1993), whose axons pioneer the first pathways between these cells and their targets. Pioneer axons display very complex growth cones that differ significantly from the growth cones of the later-arriving follower axons (Bate, 1976; Bovolenta and Mason, 1987; Kim et al., 1991). In the cerebral cortex, the subplate neurons have the characteristics of pioneer neurons and they pioneer the corticofugal pathway (McConnell et al., 1989; Shatz et al., 1990; Kim et al., 1991; De Carlos and O'Leary, 1992). In addition, we have recently characterized a novel population of transient neurons in the preplate bearing characteristics of pioneer neurons (Meyer et al., 1998). Unlike subplate neurons, these neurons remain in the marginal zone (MZ) after the arrival of cortical plate neurons. They send axonal projections tipped by complex growth cones into the incipient internal capsule in the lateral ganglionic eminence (LGE), even before the subplate neurons are generated and thus before subplate neurons arrive to the preplate. MZ pioneer neurons arrive to their destination in the MZ by radial migration within a very simple cellular environment composed of the neuroepithelial cells of the VZ (Meyer et al., 1998).

Here we report on the peculiarities of the tangential distribution of MZ pioneer neurons, as revealed by wholemount immunohistochemistry, using antibodies that selectively stain subsets of these early cortical neurons. Firstly, we shall show that MZ pioneer neurons are more numerous than hitherto suspected. Secondly, the distribution of MZ pioneer neurons delineates a major territory in the early cerebral cortex, comprising both the hippocampal and neocortical primordia and excluding the paleocortical primordium; this presentation will limit itself to the neocortex. Thirdly, since the very early stages of development, pioneer neurons in the MZ cluster together, forming stereotyped patterns at well-delineated territories with a precise topography. Pioneer cell clustering is concomitant with fasciculation of the axons of these neurons. The presence of cortical territories defined by specific patterns of clustering of MZ pioneer neurons is the earliest histological sign of a regional cortical differentiation described so far. The topological relation-
ships among pioneer neurons in the MZ may preserve earlier topological relationships existing among their neuroblast precursors in the VZ. The clusters are formed of neurons that send fasciculated axonal projections into the incipient internal capsule, preceding the formation of the subplate projection. Because of this developmental feature, we hypothesize that they might indirectly provide a structural substrate for differential growth of thalamocortical fibers into specific cortical territories. A preliminary account of this work has been published (Soria et al., 1998).

Materials and Methods

Wistar albino rats were mated overnight, and vaginal smears examined the next morning. Embryonic day 0 (E0) was the day a sperm-positive vaginal smear was found. Timed pregnant rats were deeply anesthetized with chloral hydrate i.p., and the fetuses were extracted by cesarea. Animals used are listed in Table 1. Experimental procedures involving living animals were carried out in accordance with the guidelines set by the European Commission and the Society for Neuroscience and were approved by the Animal Care Committee of the authors’ institution. Fetuses were fixed either by immersion or, in late gestation, kept in ice until deep anesthesia and perfused intracardially at a low flow rate with a peristaltic pump. The fixative used was 4% paraformaldehyde in 0.12 M phosphate buffer.

Table 1

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<th>Calbindin</th>
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<tr>
<td>E17</td>
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Antibodies and Dilutions

Rabbit polyclonal antibodies against calbindin D-28k (1:5000) and calretinin (1:5000) (SWant, Bellinzona, Switzerland); reelin, G10 mouse monoclonal antibody, 1:1000 (generous gift of A.M. Goffinet) (de Bergveyck et al., 1998).

Whole Brain Immunocytochemistry

After 2 days in fixative, brains were carefully dissected out leaving the meninges intact. After a long preincubation in phosphate buffered saline (PBS) containing 4% bovine serum albumin (BSA) and 5% hydrogen peroxide, whole brains were immunoreacted for calbindin, calretinin or reelin using the ABC procedure (Vector Laboratories Inc., Burlingame, CA). Bound peroxidase was revealed using 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in PBS. Brains were photographed using a Leitz MZAP0 macroscope, or flattened in a Mowiol-based aqueous medium and photographed in a Zeiss Axioshot light microscope.

Some of these wholemounts were sectioned for subsequent light-microscopic examination. After the immunoreaction, the mounts were encased in 4% agar and sectioned at 100 µm in a Vibratome. Sections were coverslipped with Mowiol.

Results

Pioneer Neurons in the Upper Tier of the Preplate and the MZ Covered a Broad Cortical Territory

Most of the following figures are photomicrographs taken from the surface of flattened cortical wholemounts, using a novel immunohistochemical approach to imaging large areas of the MZ. Pioneer neurons overlaid a broad territory (covering the hippocampal and the neocortical primordia) since the initial stages of cortical development. As illustrated in Figure 1, the territory of distribution of pioneer neurons had a quite abrupt limit at its lateral border. This E12 wholemount was immuno-
stained for calbindin, and shows the frontoparietal region of the developing cortex. The ventrally located cell-poor territory corresponds to the eventual locale of the lateral olfactory tract and the insular cortex. Dorsally to the lateral olfactory tract we found the limit of a territory that included the whole neocortical surface, overlaid by calbindin-immunoreactive pioneer neurons. Pioneer neurons did not, however, restrict themselves to the prospective neocortical territory, because neurons with similar morphologies and chemical markers distributed also uniformly in the MZ of the Ammon’s horn primordium, up to the level of the future hippocampal fissure (not shown).

Pioneer Neurons did not Distribute Uniformly within the Upper Tier of the Preplate and the Marginal Zone

At definite developmental stages, striking local discontinuities in the distribution of the pioneer neurons in the tangential plane revealed a mosaic organization of the neocortical preplate (and, later, of the MZ) that, to our knowledge, has hitherto gone unnoticed. The basis of such a mosaic organization was pioneer cell clustering at certain places of the preplate and the MZ. The prominence of the mosaic organization of the preplate and the MZ suggested to us that this phenomenon might be of relevance to cortical development.

Calbindin- and Calretinin-immunoreactive Pioneer Neurons Distributed in the Marginal Zone According to Specific Patterns

Mosaic Distribution of Calbindin-immunoreactive Cells

The clusters of calbindin-immunoreactive pioneer neurons were clearly noticeable in wholemounts under the stereoscopic microscope, although in these preparations, due to the convexity of the telencephalic vesicle, sharply focused areas in the photomicrographs were rather limited in extent. In an attempt to improve the visibility of the pioneer cell clusters, we relied on composite photomicrographs of large fields of the surface of flattened wholemounts. Figure 2 shows one such mosaic reconstruction displaying calbindin-immunoreactive cells at E14. In this low-magnification photomicrograph, the ventrolateral end of the field is at the right-hand part of the photomicrograph. At this ventrolateral level, the tangential distributions of calbindin-immunoreactive cells were generally
uniform, as was the case shown in Figure 1. Looking at more dorsal regions of the cortical primordium progressively (as in the left-hand parts of Fig. 2), it was found that the distribution of calbindin-immunoreactive cells was less uniform. The pioneer neuron distribution here stood in clear contradistinction to the more uniform neuron distribution found, at the same embryonic age, in the ventrolateral territory of the pallium (Figs 2, 3B), dorsal to the future lateral olfactory tract (Fig. 1).

The earliest clusters of calbindin-immunoreactive pioneer neurons were apparent at E12. At E12, calbindin-immunoreactive pioneer neurons formed an intricate network of clusters and confluent bands of cells (Fig. 3A), where spaces free of large calbindin-immunoreactive pioneer neurons intermingled with the clustering cells. The width of the bands of clustered cells changed along development: it was $\sim 20 \mu m$ at E12, and $\sim 120 \mu m$ at E14. Accordingly, at E14, the clusters were much more apparent than at E12, as if the immunoreactive pioneer neurons had suffered lateral displacements as they concentrated into larger clusters within the MZ during this developmental period (Fig. 3B). Consequently, the boundary became readily apparent at this age between the dorsal territory containing clusters and the ventral territory where clusters did not occur: one such boundary is shown in Figure 3B with a white dotted line. At E14, clustering formed ring-like aggregates of pioneer neurons (Fig. 3C). As reported before (Meyer et al., 1998), the typical and distinctive slender forms of subpial granule cells contrasted with those of the multipolar pioneer neurons At the points of confluence of the ring-like aggregates, many immunoreactive cells formed large clusters (Fig. 3D).

Mosaic Distribution of Calretinin-immunoreactive Cells

Figure 4A,B are two partially overlapping photomicrographs forming an interrupted mosaic reconstruction of a narrow strip of the cortical surface. It was taken from an E14 wholemount immunostained for calretinin. The photomicrographs are oriented horizontally in such a way that the caudal end of the strip is located at the right-hand extreme of the composite photograph. The figure illustrates how the packing density of calretinin-immunoreactive pioneers changed progressively towards the caudal regions of the convexity of the telencephalic vesicle.

Already at E12, a mosaic pattern of calretinin-immunoreactive cell clustering was apparent in a limited territory of the neocortical primordium (Fig. 5A,B, arrows). As they clustered together, calretinin-immunoreactive pioneer neurons in the MZ formed parallel stripes (ranging in width from about 17 to 25 $\mu m$), separated by intervening bands (35–60 $\mu m$ in width) where no calretinin-immunoreactive pioneer neurons were present. At E12, the stripes of clustered cells were not confluent with each other. The morphological pattern of clustering of pioneer neurons changed with development. At E14, pioneer neurons formed tightly packed clusters and the orientation of their dendrites became less uniform (Fig. 5C). The MZ territory characterized by stripes of clustered calretinin-immunoreactive neurons...
pioneer neurons (arrows) was surrounded by uniformly distributed pioneer neurons (Fig. 5A,B,D).

Topography of the Cellular Mosaics in the MZ

Figure 6 shows an ideal three-dimensional reconstruction of the telencephalic vesicle at E12 (Fig. 6A) and at E14 (Fig. 6B). For anatomical orientation, a vertical (coronal) section at the level of the lateral ganglionic eminence (GE) is represented in both drawings. On the brain surface, we have drawn in different gray shades the territories where clusters of pioneer neurons were seen in our sample of wholemount preparations, both before and after flat mounting.

Within the comma-shaped territory comprising the medial border of the telencephalic vesicle (Fig. 6A,B), the tangential distribution of calbindin-immunoreactive pioneer neurons departed from the uniform pattern of distribution seen in the ventrolateral part of the telencephalic vesicle, down to the upper limit of the lateral olfactory tract (Figs 1, 2, 3B). To further emphasize the important point that the distribution of MZ pioneer neurons in the tangential plane was not homogeneous, we show examples of pioneer neuron distribution at E14 in each inset of Figure 6B. The arrows signal the locales in the developing brain surface where the inset photomicrographs were actually taken. The insets show that the morphological patterns of cell clustering may vary at different regions: rostrally, the calbindin-immunoreactive pioneer neurons formed large clusters (see also Fig. 2), while more caudally, clusters of calbindin-immunoreactive pioneer neurons were more spaced and formed ring-like structures (see also Fig. 3C).

With further development, at E16 (not shown), the calbindin-immunoreactive clusters covered the whole cortical surface. As we will show later, pioneer neurons initiated a descent towards the superficial cortical plate after E16, prior to the disappearance of these cells.

Calretinin-immunoreactive pioneer cells clustered in a more restricted territory, near the occipital rim of the developing cortex, and the extension of the region containing clusters did not increase beyond E14 (Fig. 6). However, the position of the territory changed from E12 to E14, most likely due to differential growth of the occipital pole of the developing cortex. The typical distribution of calretinin-immunoreactive pioneer neurons in such a territory is shown in the inset (Fig. 6B). As with calbindin-immunoreactive pioneer neurons, after E16, pioneer neurons descended into the cortical plate where they soon became unidentifiable.

An important feature of these tangential distributions, as schematically depicted in Figure 6, was the partial overlapping of the territories containing clusters of each type of pioneer neuron. This occurred within the intersection of both territories that was always present at E12 and at E14 (see, for instance, the common origin of the two large arrows in Fig. 6B; the intersection was also visible at E12, Fig. 6A). It is also worth noting that, outside such an intersection, a uniform distribution of the one type of pioneer neurons coexisted with a clustered distribution of the other type of pioneer neurons and vice versa.

In order to define the territories containing pioneer cell clusters anatomically, we relied on the comparison of the wholemount preparations with the more conventional anatomy defined by coronal sections. To this purpose, some wholemounts were sectioned after study under the stereoscopic microscope. In other cases we relied on external anatomical landmarks such as the middle cerebral artery (Fig. 5A) and the cell-poor territory corresponding to the future lateral olfactory tract (Fig. 1). It was not intended, however, to correlate the positions of the early territories with those of the possible primordia of the adult cortical areas.

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Table 2

<table>
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<td>E17</td>
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Calbindin and Calretinin Immunoreactivities Define Two Pioneer Neuronal Populations

Several observations supported our conclusion that the calbindin- and the calretinin-immunoreactive pioneer neurons belonged to two distinct neuronal populations. First, their discontinuities in tangential distributions were different, yet both classes of neurons coexisted in the same zones of the cortical primordium. In addition, the morphology of calretinin- and calbindin-immunoreactive pioneer neurons also differed: in wholemounts, calbindin-immunoreactive pioneer neurons were multipolar, whereas calretinin-immunoreactive cells were fusiform. This was reflected in obvious differences between the two neuronal populations in the major and minor diameters of the immunoreactive cell perikarya, as projected onto the brain surface (Table 2). The diameters of the reelin-immunoreactive subpial cells are given for comparison.

Axonal Fasciculation Was Concomitant with the Clustering of MZ Pioneer Neurons

High-power photomicrographs of the cortical surface in flat mounts (Fig. 7A,B) revealed fascicles of axonal processes that, during their horizontal trajectories below the pial surface, approached the clusters of pioneer neurons. In certain cases, the axons were seen to contour the silhouettes of pioneer cells very closely (Fig. 7B). No other alternative sources of immunoreactive axonal processes were found in immunostained wholemounts or in conventional immunohistochemical preparations. In particular, the distal processes of subpial granule cells were thin and axon-like, but they did not form fascicles. Although Figure 7 illustrates calbindin-immunoreactive axon fascicles, a similar axonal fasciculation was found in the wholemounts immunostained for calretinin (not shown).

Temporal Evolution of the Clustering of Marginal Zone Pioneer Neurons

The time-course of the presence of pioneer neuron clusters followed the ephemeral character of these neurons. With advancing development, at E17, pioneer neurons started a downward movement in the MZ, plunging into the cortical plate as described (Meyer et al., 1998) until they disappeared. Such a phenomenon could be identified in the wholemounts of E17 brains (Fig. 8). These pairs of photomicrographs (Fig. 8A,B; C,D) show calretinin-immunoreactive cells in the MZ at two different planes of focus. Focusing on the surface of the MZ (Fig. 8A,C) revealed the slender, bipolar forms of the subpial granule neurons, the future Cajal–Retzius cells (Meyer et al., 1998). Subpial granule neurons were smaller than pioneer neurons and the difference between their major and minor diameters was apparent (Table 2). Deep in the MZ (Fig. 8B,D) were the clusters of calretinin-immunoreactive pioneer neurons. At this developmental stage, clustering of these neurons was even more apparent than at E12 or E14 (cf. Fig. 4). Deepening of calretinin-immunoreactive pioneer neurons occurred in the whole neocortical primordium, but clustering was exclusively present in the temporo-occipital region of the telencephalic vesicle where, as described above, clustering was apparent earlier in development. Similarly, calbindin-immunoreactive pioneer neurons also deepened with development (not shown) and, in this case, all the prospective neocortical territory showed pioneer cell clusters.

Discussion

Pioneer Neurons of the Upper Tier of the Preplate and of Marginal Zone Delimit a Large Common Territory in the Dorsal Telencephalon

One important feature of pioneer neurons in the upper tier of the preplate and the MZ is their tangential distribution. These pioneer neurons are specific to the cortex, and they mark the limit between the rudimentary cerebral cortex and olfactory bulb during early corticogenesis (Meyer et al., 1998). Pioneer neurons are seen laterally down to the dorsal border of the lateral olfactory tract territory. This territory is identifiable in wholemounts well before olfactory axons invade it during development (López-Mascaráque et al., 1996; Sugisaki et al., 1996) and also includes the place of the future insular cortex.
Thus, the presence of pioneer neurons in the early preplate indicates the basic uniformity of a presumptive cortical territory in the convexity of the telencephalon that includes the neo- and archicortical primordia but excludes the olfactory bulb and the paleocortical primordia (Meyer et al., 1998). Within such a basically uniform territory, a discontinuous areal pattern consisting of territories showing clustered pioneer neurons is superimposed.

A Mosaic Organization of the Preplate and the Marginal Zone is Revealed by Antibodies to Calcium-binding Proteins
A striking feature of pioneer neurons in the MZ is their shift from a predominantly uniform distribution into a clustered distribution within discrete territories. Clustering is present from the earliest stage of the preplate, but it evolves as a dynamic developmental process: the area containing clustered calretinin-immunoreactive neurons does not change much in size over time, while clustering of calbindin-immunoreactive neurons advances from caudal to rostral to cover with cell clusters the complete neocortical surface by E16. The gradient is opposite to the general rostral to caudal gradient of maturation in the cerebral cortex. This phenomenon has not been described previously, and its functional implications need to be elucidated.

Recent studies of cortical development have used the antibody TuJ1 in wholemounts that recognizes the neuron-specific marker class III β-tubulin (Easter et al., 1993; Richards et al., 1997). These studies did not reveal discontinuities in the tangential distribution of TuJ1-immunoreactive neurons in either the preplate or MZ, probably because the TuJ1 antibody is a general neuronal marker and, as such, it may not be able to reveal subtle variations in the tangential distribution of subclasses of
MZ neurons. Our finding that the calcium-binding proteins calretinin and calbindin stain distinct subpopulations of pioneer neurons allowed us to study an early cortical regional territorialization in more detail, showing patterns that would have remained hidden if all subpopulations were stained together by a common histochemical marker.

The patterns of distributions of neurons in the MZ differ from a random distribution and define a cellular compartmentalization in the neocortical MZ that occurs earlier than the arrival of afferent axons.

The developmental timing of this cellular compartmentalization of the developing neocortex does not correlate with those of published patterns of gene or protein expression in the same region (Horton and Levitt, 1988; Barbe and Levitt, 1991; Arimatsu et al., 1992, 1994; Ferri and Levitt, 1993; Cohen-Tannoudji et al., 1994; Frantz et al., 1994; Bulfone et al., 1995; Sheppard et al., 1995; Briata et al., 1996; Gulisano et al., 1996; Pimenta et al., 1996; Suzuki et al., 1997; Nothias et al., 1998; Smith Fernández et al., 1998). Similarly, no relationship between the mosaic organization of the MZ and local differences in early proliferative activity in the VZ has yet been found. Local differences in proliferative activity occurring at more advanced stages of corticogenesis are known to intervene in the regionalization of the neocortex (Dehay et al., 1993; Miyama et al., 1997; Polleux et al., 1997a,b).

**Figure 6.** A summary of the topography of the specific patterns of distribution of calbindin- and calretinin-immunoreactive pioneer neurons. The drawings are ideal three-dimensional views of rat brains at E12 (A) and E14 (B), based on drawings by Altman and Bayer (Altman and Bayer, 1995). A vertical, vertical section passing through the ganglionic eminence (GE) is shown in both drawings. In (B), the part of the telencephalic vesicle rostral to the vertical section is rendered transparent to facilitate the three-dimensional interpretation of the drawing. The visible limits of the transparent brain surface are drawn as dashed lines. Shaded areas indicate the places were pioneer cell clustering was encountered. The patterns of clustering at E14 are shown in the accompanying photomicrographs, and the large arrows indicate the cortical territories were the pictures were taken. The most dorsal shaded area corresponds to the territory where calbindin-immunoreactive pioneer neurons cluster together. At E14, the morphological patterns of clustering differ at different locales within this territory (insets). The more ventral, rounded shaded area corresponds to the territory where calretinin-immunoreactive cells cluster together. Observe an area of intersection of both territories. At the intersection, both classes of pioneer neurons form clusters, each with their typical pattern of clustering.

**Cellular Mosaics and the Protomap Hypothesis of Cortical Specification**

In the protomap hypothesis, the characteristics of each cortical area are determined by the genetic commitment of VZ cells, which are guided by radial fibers to the subplate and cortical plate (Rakic, 1988). The radial glia scaffold has been considered an essential component of this model, since it preserves in the cortical plate the topological relationships between neurogenic units in the neuroepithelium along the progress of radial migration (Rakic, 1972). The radial guide concept derived from studies of middle and late stages of primate cortical development. However, it remains to be determined whether radial glia participates in the arrival of pioneer neurons to the MZ since this happens at the very early stages of cortical development. At these
early stages, radial migration is sufficiently guaranteed by the strict radial arrangement of the columnar epithelial ventricular cells (Rakic, 1978).

Radial migration is not the only mechanism of migration involved in building up the neuronal populations of the cerebral cortex and hippocampus (DeDiego et al., 1994; De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997, Meyer et al., 1998; Lavdas et al., 1999; Zhu et al., 1999). Tangential migration affects precisely the GABA-ergic nonpyramidal neurons (Anderson et al., 1997; Tan et al., 1998), and radial and tangential migrations coexist during cortical development (Tan et al., 1998).

The early formation of cortical territories defined by clustering of pioneer neurons in the MZ as described in this paper is an intrinsic property of the cortex. Since it first appears well before thalamic axons have reached the cortex, it does not depend upon the arrival of the thalamic fibers. Because of the close temporal and spatial links between VZ and preplate during the earliest phase of cortical development, the topography of MZ pioneer neurons may reflect a hypothetical early topography of their mother neuroblasts within the VZ. Therefore, our observations are compatible with the possible existence of a mosaicism in the VZ, as the mosaicism in the MZ that we describe might be an early transformation of a mosaicism in the VZ.

The idea of mosaicism of the germinal ventricular zone that is reproduced in the cortical plate was developed by Rakic (Rakic, 1978), who considered this feature a relevant component of his protomap hypothesis. Therefore, data showing that the cortex input offer real support to the protomap hypothesis. This has been done in a recent study of gene expression in the developing cortex (Miyashita-Lin et al., 1999): Gbx-2 mutant mice, whose thalamic differentiation is disrupted to the point that the thalamocortical projection is missing, still maintain specific gene expression in defined territories of the cerebral cortex. Such genes are Cadherin-6 (Suzuki et al., 1997), EphA7, Id2 (Bulfone et al., 1995), and RZK-beta (Rubenstein and Rakic, 1999a,b).

Another recent study (Donoghue and Rakic, 1999) describes regional patterns of expression of Eph receptors and their ligands, the ephrins. At E65, before the arrival of thalamocortical axons in primate neocortex, EphA3 is expressed in the cortical plate (CP) of the extrastriate cortex and in the subventricular zone, while EphA4, EphA5, EphA6 and EphA7 are expressed in diverse embryonic layers of the developing cortex with clear regional preferences. In particular, EphA6 is present within the presumptive visual cortex, in the deep CP and the subplate. Thus, an intrinsic cellular compartmentalization exists within the cortex from the early stages of corticogenesis. Since these molecules are known to participate in axonal guidance, their region-specific expression may play a role in the guidance of thalamocortical axons to appropriate regions of the cerebral cortex.

In the present investigation, we were looking for differential patterns of distribution of neurons in the MZ sharing the property of sending early corticofugal axonal projections. The novel finding is that we see signs of mosaicism at a very early stage of corticogenesis, not only before thalamic axons arrive but also before the subplate and the CP are formed.

The present results indicate a cellular substrate that is specific

Figure 7. Fascicles (large arrows) formed by axons originating from calbindin-immunoreactive pioneer neurons at E14. The axonal fascicles group with the clustered neurons, and take a close contact with the cell bodies and dendrites of pioneer neurons. Scale bars = 10 µm.
to the MZ of the neocortex and is independent of the arrival of thalamocortical axons to the subplate and CP. It is thus formed as a consequence of some unknown mechanism intrinsic to the neocortical primordium. It takes the form of topographically defined specializations in the upper tier of the preplate and in the MZ based on clustering of MZ pioneer neurons in specific territories of the developing cortex.

The Identity of Pioneer Neurons

In the present investigation, pioneer neurons in the rat MZ were revealed by their expression of the calcium-binding proteins calbindin and calretinin. Wholemounts provided the opportunity to seeing virtually the whole population of pioneer neurons in the MZ. Such a large sample revealed that calretinin and calbindin label two different subpopulations of neurons. Both

Figure 8. Wholemounts of E17 brains immunostained for calretinin. (A,B) and (C,D), are pairs of photomicrographs taken at different planes of focus. Large arrows show orientation: they indicate the same pioneer neuron in each of the photographic pairs. Cells in the images focused on the cortical surface (A,C) are subpial granule neurons. The photomicrographs in (B) and (D) show clusters of pioneer neurons placed deep within the MZ. Scale bars = 25 µm.
subpopulations are intermingled in the MZ, and the differential patterns of clustering of both types of neurons coexist in common areas of the MZ. However, both neuron populations have the common property that they extend pioneer axons.

In the present study, as in a previous paper (Meyer et al., 1998), we used the name of pioneer neurons for the early projecting neurons of the preplate and the MZ (Meyer et al., 1998). Pioneer neuron is a term introduced by Harrison (Harrison, 1910) [see also Cajal (Cajal, 1890)] that defines those neurons extending pioneer axons, i.e., those that explore the tissue to prepare an axonal projection and thus pave the way for follower axons. Simple organisms are excellent models for the experimental study of pioneer neurons (Bate, 1976; Goodman and Meyer et al., 1998, 1999) of fasciculate descending axons in developing neural systems of mammals (Yaginuma et al., 1990; Bovolenta and Mason, 1987; McConnell et al., 1989; Shatz et al., 1990; De Carlos and O’Leary, 1992; Koester and O’Leary, 1994). A recent usage of the term pioneer (Supèr et al., 1998) is based exclusively on the early appearance of the cells such as radial glia, Cajal–Retzius cells or subplate cells, rather than on its original meaning (Harrison, 1910; Bate, 1976). In the developing cerebral cortex, we would like to reserve pioneer for neurons that extend axons that pioneer a corticofugal connection. This would restrict the name pioneer to the subplate neurons and the pioneer neurons in the MZ. The evidence for pioneer neurons in the MZ is still limited, but includes the important fact that they project to the forming internal capsule in the lateral ganglionic eminence before subplate neurons do, while Cajal–Retzius cells or their precursors do not project axons subcortically.

Previous results showed that subplate neurons pioneer the efferent pathway from the cortex (McConnell et al., 1989; Kim et al., 1991; De Carlos and O’Leary, 1992; Ghosh and Shatz, 1993; Molnár and Blakemore, 1995; Méton and Godemont, 1996; Molnár et al., 1999). This is based on the fact that the DiI injections fail to label most of the MZ cells. Thus, the axons of the MZ pioneer neurons would extend axons (Molnár and Blakemore, 1995; Métin and Godemont, 1996) toward the subplate of the cortex. Although all pioneer neurons in the MZ may send axons (Molnár and Blakemore, 1995; Métin and Godemont, 1996) to the subplate, the upper region of the MZ is still limited, but includes the important fact that they project to the forming internal capsule in the lateral ganglionic eminence before subplate neurons do, while Cajal–Retzius cells or their precursors do not project axons subcortically. The present results suggest that our pioneer neurons are genuine MZ cells. This is the observation that, on wholmounts stained for calbindin or calretinin, the immunoreactive cells appeared as large neuronal populations that occupied the most superficial positions, a feature verified by making coronal sections from the same in toto immunostained brains. Immunoreactive cells were limited to a very narrow layer of tissue below the pial surface, in such a way that staining of the subplate cells was unlikely. Only after the CP appeared below the MZ did pioneer neurons enter to some extent the superficial part of the cortical plate, yet they maintained an MZ position. The second argument derives from bromodeoxyuridine (BrDu) incorporation experiments combined with immunocytochemical markers (Meyer et al., 1998) (also A. Fairén et al., unpublished data) that showed that the pioneer neurons complete their last round of DNA replication at E11, before the subplate neurons are generated. The third argument is based on our immunocytochemical observations (Meyer et al., 1998, 1999) of fasciculate descending axons exiting from neurons in the upper subplate as early as at E13, at anatomical levels where the subplate component of the preplate could not yet be recognized. Note that other researchers described the first corticofugal projection at E14 using Dil labeling (De Carlos and O’Leary, 1992; Molnár et al., 1998). Such a difference in timing may be due to the different methods (immunocytochemistry, Dil labeling) used in these studies. Méton and Godemont found the earliest corticofugal projections in hamsters by E11.5 (Métin and Godemont, 1996). In this case, the conclusion is that extending corticofugal pioneer projections from subplate neurons to the subplate and ascending axons from the dorsal thalamus also form fascicles. Such projections descending through the intermediate zone to the thalamic ventricular zone, where they form complex growth cones, but the possible functional significance of these transient projections is unknown. Pioneer neurons also extend an axonal projection descending through the intermediate zone to the forming internal capsule. Later in development, after the cortical plate has emerged within the preplate, the axons of pioneer neurons were seen only until the subplate and the upper region of the forming internal capsule (Meyer et al., 1998). The illustration of a rich corticofugal projection from subplate cells as compared to a minor contribution from MZ cells (Molnár et al., 1998; their Fig. 7E, Dil labeling) suggests two possible scenarios. First, the preplate may contain both MZ and subplate neurons, but most of the projecting neurons belong to the subplate component of the preplate. The present results do not favor this possibility, since we find that the pioneer neurons in the MZ are more numerous than hitherto suspected. Second, the projection from the MZ pioneer neurons may have retracted during development, so that the Dil injections fail to label most of the MZ cells. Thus, the axons of the MZ pioneer neurons send a transient corticofugal pioneer projection, preceding the initial elongation of the pioneer axons emanating from the subplate neurons. This suggests that the role of the MZ pioneer axons might well be to provide the initial guidance for the axons emanating from the subplate neurons. Further experiments are needed to explore such an intriguing possibility. It is of interest to note that, out of the initial clustering of pioneer neurons in the MZ correlates with axonal fasciculation, and to recall that descending axons from subplate and ascending axons from the dorsal thalamus also form fascicles. (Molnár, 1998; Molnár et al., 1998).

Several studies suggest that the ordered pattern of early thalamocortical axons could be a sign that these axons play the role of instructor in cortical specification (Stanfield and O’Leary, 1985; O’Leary and Stanfield, 1989; Catalano et al., 1991, 1996; Schlagger and O’Leary, 1991, 1994). The present results, which reveal the existence of a massive population of projection neurons in the MZ with a defined systematics of anatomical distribution, suggest an alternative possibility, namely that the cortex may form a more precocious scaffold to guide thalamic axons (Molnár and Blakemore, 1995; Métin and Godemont, 1996; Molnár et al., 1998; Donoghue and Rakic, 1999). The fact that the tangential distribution of MZ pioneer neurons is not uniform, because pioneer cells forming axonal fascicles cluster together in certain cortical territories, suggests that the extension of pioneer axons could start in specific places of the cortical surface. Although all pioneer neurons in the MZ may send descending axons, it is also true that axonal fasciculation only occurs within the specific territories where pioneer cells cluster together. The present results suggest, therefore, the existence of an anatomical framework for a spatiotemporally ordered axonal projection from the early neocortical primordium that may be involved in the ordered arrival of thalamic axons to the cortex.

Notes
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