Neuronal Clones in the Cerebral Cortex Show Morphological and Neurotransmitter Heterogeneity during Development

The mammalian cerebral cortex, although a structure of great complexity, is characterized by a high degree of organization where the proportions, spatial relationships, and properties of the various cell types are rigidly controlled. The mechanisms responsible for the creation of such a rigid distribution of cell types are not known. Lineage studies in adult rats have suggested that each of the cortical progenitor cells lining the telencephalic ventricles during embryonic development gives rise to progeny of the same phenotype (homogeneous clones). However, the possibility that homogeneous clones are the result of complex processes affecting both the final number and the phenotype of clonally related cells during development has not been investigated. In the present study, we followed the development of cortical cell lineages labeled with retroviral injections at embryonic day (E) 16 in rats of 7, 14, or 21 d of age using electron microscopy and immunocytochemistry for the neurotransmitters glutamate and GABA. We found that a significant number of cortical clones at postnatal day (P) 7 and P14, and fewer at P21, showed mixed pyramidal/nonpyramidal cell composition. We sometimes observed that "mixed" neuronal clones contained cells immunoreactive for both glutamate and GABA. In the general population of cortical cells, "bireactive" neurons represented 3.7% of all neurons at P7, 1.5% at P14, and 0.6% in adult rats. Although the change in the composition of neuronal clones between the third week of postnatal life and adulthood may be due to changes in the phenotype of some developing neurons, we would like to suggest that it is probably due to selective neuronal cell death.

All neuronal cell types in the cerebral cortex as well as the glioblasts that generate the cortical astrocytes and oligodendrocytes arise from the seemingly homogeneous ventricular zone cells in the embryonic telencephalic ventricles (Sidman and Rakic, 1973; Levitt et al., 1981; McConnell, 1988). Neurons in the cerebral cortex are divided into two classes—the pyramidal and nonpyramidal cell types. The former are the projection neurons, while the latter are the cortical interneurons. Apart from morphological differences (Szentagothai, 1973), the two cell classes show distinct neurochemical and physiological properties (Gilbert, 1983; Parnavelas et al., 1989). Pyramidal neurons utilize the amino acid L-glutamate (Glu) to exert excitatory actions in the target sites (Fagg and Foster, 1983; Streit, 1984; Dori et al., 1989, 1992) while nonpyramidal cells exert local inhibitory effects by releasing GABA (Fagg and Foster, 1983; Sillito, 1984). In the mature cortex, the two neuronal populations can be reliably marked by the presence of the neurotransmitters Glu or GABA.

A number of studies have clearly shown that the organization (Gilbert, 1983) and both the proportions and the spatial relationships (Rockel et al., 1980; Hendry et al., 1987) of the two neuronal cell types in the cortex are rigidly controlled. The question of how this rigid organization is achieved and at what point during development the final phenotype of each cell is specified has attracted the attention of many investigators (for reviews, see McConnell, 1988, 1991). However, the process involved in the choice of neurotransmitter substances by developing cortical neurons has not yet been elucidated. Immunocytochemical studies have demonstrated Glu immunoreactivity in the developing rat cerebral cortex around the time of birth (Erdo and Woolf, 1989; Dori and Parnavelas, 1996) and GABA immunoreactivity as early as embryonic day (E) 13 (Van Eden et al., 1989; Cobas et al., 1991). Are these transmitter substances segregated in the appropriate neuronal population from the time they first appear in the cortex? Several studies have utilized retroviral vectors as lineage markers to investigate whether lineage plays a role in the determination of cell phenotype in the cerebral cortex. These lineage studies carried out in adult animals, and using electron microscopical and/or immunocytochemical criteria to determine cell phenotype, have shown that discrete clusters of clonally related neurons are homogeneous comprising either all pyramidal or all nonpyramidal neurons (Parnavelas et al., 1991; Usken et al., 1993; Mione et al., 1994). However, such studies do not tell us whether clonally related neurons acquire their mature phenotypes early in development or over a period of time, during which some may be expressing both pyramidal and nonpyramidal traits. In addition, several developmental events are taking place in the rat cerebral cortex during the first few weeks of life. These include migration (which is likely to expose late generated neurons to different environmental cues), synapse formation, and naturally occurring cell death (Berry, 1974, Uylings et al., 1990; Ferrer et al., 1992). What effect do these processes have on the composition of neuronal clones in the cerebral cortex?

Here we have investigated the phenotype of clonally related neurons, labeled by retrovirus injections in the telencephalic ventricles of E16 rat embryos, at postnatal days (P) 7, 14, and 21. Analysis, using Glu and GABA immunocytochemistry as well as electron microscopy, revealed a picture markedly different from that seen in adult animals in that it showed a significant number of clones containing neurons of different phenotypes. Another finding, also characteristic of these stages of cortical development, was the presence of a number of neurons immunoreactive for both Glu and GABA.

Materials and Methods

The technique of retrovirus-mediated gene transfer was used to label clusters of clonally related cells in the cortex of developing rats. The method of production of the retrovirus, the surgical procedures, and the tissue processing technique have been published in detail recently (Luskin et al., 1993; Mione et al., 1994). Briefly, a solution of the BAG retrovirus containing the reporter gene encoding for Escherichia coli β-galactosidase (Sanes et al., 1986; Price et al., 1987) was injected into the telencephalic ventricles of rat embryos at E16 (the first day of gestation was designated as E1). Following birth, the rats were allowed to survive for 1, 2, or 3 weeks, at which time they were perfused with a mixture of aldehydes. Following perfusion, brains were removed and sectioned serially using a Vibratome at 100 µm. These sections were subsequently incubated in X-gal to reveal β-galactosidase-positive (β-gal+) cells. Sections that contained labeled cells were processed for electron microscopy and flat embedded in Araldite in serial order.
Selection of Cell Clusters

The β-gal+ cells were mapped in camera lucida drawings of serially arranged sections. Labeled cells were assigned to a layer according to Krieg (1946). In accordance with recent reports (Luskin et al., 1993; Mione et al., 1994), clusters of closely spaced β-gal+ cells that were contained within a 500 μm strip of cortex and separated from any other labeled cells by at least 500 μm were considered to be members of the same clone. The precise spatial organization of each group of labeled cells was mapped using a computer-aided microscope system (Neotrace, Interaction Co., Cambridge, MA).

In view of the possibility of widespread dispersion of cortical clones (Walsh and Cepko, 1992), only hemispheres containing a limited number (1–7) of widely spaced clusters of labeled cells were used. The aim was to reduce the risk of “lumping” error, that is, the possibility of including labeled cells from different progenitors in a single clone (for statistical analysis, see Mione et al., 1994).

Clones of labeled cells that were made up exclusively of glia, as determined by light microscopical appearance (Price and Thurlow, 1988; Luskin et al., 1993), were not included in the analysis. Of the remaining nonglial clones, the vast majority comprised only neurons, but a small number included both neurons and astrocytes. The astrocytes identified as part of these mixed clones displayed characteristic light microscopical features (Luskin et al., 1995). They either showed a “halo” of reaction product surrounding a lightly stained cell body or short, wavy processes emanating from the cell body often coming in contact with a blood vessel.

Postembedding Immunocytochemistry

Every β-gal+ cell of every clone selected for analysis was sectioned so as to obtain about 10 semithin (0.5 μm thick) sections for immunocytochemistry and a number of ultrathin sections. Prior to processing the semithin sections for immunocytochemistry, a drawing of each section was made with the camera lucida, documenting the location of the β-gal+ cells and features of the surrounding tissue; this is essential because the X-gal reaction product disappears during the subsequent processing. Following that, the resin was removed by a mixture of absolute alcohol and propylene oxide saturated in NaOH. Endogenous peroxidase activity was blocked with 2% sodium metaperiodate, and sections were incubated with normal goat serum (NGS) to decrease nonspecific antibody binding; the sections were then incubated overnight in room temperature with anti-Glu (Arnel) and anti-GABA (Sigma) primary antibodies diluted 1:1500 in phosphate-buffered saline (PBS) containing 0.2% Triton X-100 and 3% NGS. After washing with PBS, the sections were incubated with biotin-conjugated goat anti-rabbit secondary antibody (Vector Lab) diluted 1:200 in PBS for 3 hr. After more washes with PBS, the third layer (avidin-biotin complex, Vector) was applied for another 3 hr. The avidin-biotin complex had been prepared in PBS 30 min before it was applied. After a brief wash with PBS, the sections were preincubated with a 0.05% diaminobenzidine (DAB) solution in 0.1 M Tris buffer pH 7.2–7.4 for 10 min, then incubated for another 15 min in DAB solution containing 0.015% H2O2. Following immunocytochemical staining, the sections were dehydrated and mounted with DPX.

Cell Identification

Using the camera lucida and by comparing the drawings of the unstained sections with the immunocytochemically stained slides, the cells of interest were distinguished from the surrounding tissue. These cells were then characterized according to their immunoreactivity as GABA+ or GABA− and Glu+ or Glu−. Glu+/GABA− cells were identified as pyramidal neurons while Glu−/GABA+ cells were identified as nonglial neurons (Mione et al., 1994). Confirmation of their identity was achieved with electron microscopy using widely recognized ultrastructural criteria (Peters, 1985; Parnavelas et al., 1989). Pyramidal cells typically possess modest amounts of cytoplasm and a large, pale nucleus, round or oval in shape; only the large pyramidal cells of layer V have deeply indented nuclear envelopes (Peters and Palay, 1962). Nonglial cell nuclei appear more electron dense, and their nuclear envelope is invariably indented. The amount of cytoplasm varies among nonpyramidal cells; those with small somata contain a few loosely dispersed organelles, while those with larger somata show an abundance of perinuclear cytoplasm that includes a rich complement of organelles and frequent cisternae of granular endoplasmic reticulum organized in parallel arrays. However, the fine feature that distinguishes the two cell types unequivocally is the type of axosomatic synapses they receive. Pyramidal cells have been shown to receive exclusively symmetrical axosomatic synapses, while nonpyramidal neurons receive both symmetrical and asymmetrical synapse types.

Results

A total of 229 cells were identified: 143 were pyramidal neurons, 41 were nonpyramidal cells, 6 neurons were immunoreactive for both Glu and GABA (bireactive), and 39 cells were astrocytes. Of the six bireactive neurons, two in the P7 material could not be classified using morphological criteria because of their immature appearance. The other four bireactive cells were observed in the P14 tissue; two were pyramidal and the other two had features typical of nonpyramidal cells. All these cells made up 55 clones, 23 of which were composed exclusively of pyramidal neurons (Fig. 1), 10 were composed of nonpyramidal neurons, and 22 included more than one cell type (Table 1; Figs. 2–4). Of these 22 mixed clones, 4 comprised pyramidal neurons and a number of astrocytes. We do not know at present whether these pyramidal neurons and astrocytes originate from the same progenitor cell or whether the “mixing” is due to the fact that astrocytes continue to proliferate and migrate well into postnatal life.

Postnatal Day 7

Of the 14 clones examined with immunocytochemical criteria at this stage of development, 9 were homogeneous, for instance, they were comprised of cells that were either all Glu− or all GABA− immunoreactive. The remaining five clones were mixed (Table 1). Two cells in these clones were bireactive. One additional two-cell clone, situated in layers II/III, was examined with GABA immunocytochemistry and electron microscopy. The identity of the cells was not established as they both showed a rather immature ultrastructural appearance; they were both both GABA− negative.

Postnatal Day 14

Twenty clusters of clonally related cells were examined at P14. Eight of the clones were homogeneous, as established by immunocytochemistry and electron microscopic appearance; one, found in layer II–IV, contained two nonpyramidal neurons, both of which were bireactive. The remaining 12 clusters were mixed (Table 1; Figs. 2, 3). Two of the mixed clones contained a pyramidal neuron that was bireactive (Fig. 4a, a).

Postnatal Day 21

Twenty clusters of clonally related cells were studied at P21. Fifteen of these clones were homogeneous (Fig. 1), as established with immunocytochemistry as well as with electron microscopy. The remaining five clones were mixed (Table 1). An additional clone was composed of four cells situated in layer IV; three of the cells were pyramidal neurons and the other appeared as a dying cell, the phenotype of which could not be determined.

Coexistence of Glutamate and GABA Immunoreactivity in Cortical Neurons

Prompted by the finding of bireactive cells in clusters of clonally related neurons, we counted the number of neurons that showed immunoreactivity for Glu, GABA, or both in 0.5 μm thick sections of cerebral cortex of developing (7 and 14 d old) and adult rats. The area of the cortex evaluated was approximately 15,000 μm² at each age, and included samples of roughly equal size from all layers and comparable cortical regions. One thousand five hundred seventy-five cells were studied immunocytochemically in 7-d-old rats; 91.6% were immunoreactive for one or the other neurotransmitter, while
8.4% were negative for both antibodies. Seventy-eight percent of the stained cells were Glu positive, 18.3% GABA positive, and 3.7% were bireactive. Of the 1407 cells examined at P14, 84.7% were labeled, while the remaining 15.3% immunonegative; 79.4% of the stained cells were Glu positive, 18.5% GABA positive, and 2.1% were bireactive (Fig. 4b,b2). In adult rats, 74.8% of the 1708 cells examined were immunoreactive for Glu or GABA. Of the stained cells, 78.1% were Glu positive, 21.1% GABA positive, and only 0.8% of the labeled cells were bireactive. Electron microscopical examination of a random sample of 30 nonimmunoreactive cells at each of the three ages showed that they had the ultrastructural appearance of glial cells (Parnavelas et al., 1983).

**Discussion**

In the present study, progenitor cells in the ventricular zone were labeled using a recombinant retrovirus injected into the telencephalic ventricles of rat embryos at E16, and the clusters of clonally related neurons that originated from these cells were examined 1, 2, and 3 weeks postnatally. Many clones were found to be composed of only one cell type, but a substantial number at all three ages were composed of cells of two different types. The number of mixed clones reached a peak in 2-week-old animals, when more than 50% were composed of neurons of different phenotypes. While a number of reports (Price and Thurlow, 1988; Walsh and Cepko, 1988) have documented the presence of clones containing both pyramidal and nonpyramidal cells, studies in the adult rat cerebral cortex have shown that clusters of clonally related cells are homogeneously composed of either all pyramidal or all nonpyramidal neurons (Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994). Here, we used the same immunocytochemical and ultrastructural criteria applied to the study of adult animals to examine the development of the neuronal lineages in the cortex during the first 3 weeks of life. We relied especially on Glu or GABA content in the 7-d-old animals, because earlier studies (Parnavelas and Lieberman, 1979) had shown that not all neurons in the cortex have acquired their characteristic ultrastructural features at this stage of development.

The expression of GABA immunoreactivity in the developing cerebral cortex of the rat has been studied in considerable detail (Van Eden et al., 1989; Cobas et al., 1991). According to these studies, GABA-containing neurons first appear in the primordial plexiform layer at the onset of cortical neurogenesis. Beginning at E16 and throughout the remaining period of gestation, GABA-containing neurons appear throughout all layers of the developing cerebral anlage including the cortical and subventricular zones. After E19, while the number of GABA-immunoreactive neurons in the cortical plate increases, the number of these cells in the marginal zone and the layers below the cortical plate diminishes.

**Table 1**

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 P</td>
<td>7 P</td>
<td>7 P</td>
</tr>
<tr>
<td>5 P</td>
<td>5 P</td>
<td>7 P</td>
</tr>
<tr>
<td>4 P</td>
<td>4 P</td>
<td>7 P</td>
</tr>
<tr>
<td>3 P</td>
<td>3 P</td>
<td>6 P</td>
</tr>
<tr>
<td>2 P</td>
<td>3 P</td>
<td>4 P</td>
</tr>
<tr>
<td>2 NP</td>
<td>2 P</td>
<td>4 P</td>
</tr>
<tr>
<td>2 NP</td>
<td>2 NP</td>
<td>3 P</td>
</tr>
<tr>
<td>2 NP</td>
<td>2 NP (B)</td>
<td>3 P</td>
</tr>
<tr>
<td>1 P1 BR</td>
<td>1 P (BR/1 NP)</td>
<td>3 P</td>
</tr>
<tr>
<td>1 NP1 BR</td>
<td>3 P (1 BR/1 NP)</td>
<td>2 P</td>
</tr>
<tr>
<td>1 P1 NP</td>
<td>1 P1 NP</td>
<td>2 P</td>
</tr>
<tr>
<td>1 P1 NP</td>
<td>1 P1 NP</td>
<td>3 NP</td>
</tr>
<tr>
<td>2 P12 A</td>
<td>2 P1 NP</td>
<td>2 NP</td>
</tr>
<tr>
<td>1 P1 NP</td>
<td>2 NP</td>
<td></td>
</tr>
<tr>
<td>1 P1 NP</td>
<td>2 P12 NP</td>
<td></td>
</tr>
<tr>
<td>1 P12 NP</td>
<td>2 P12 NP</td>
<td></td>
</tr>
<tr>
<td>1 P12 A</td>
<td>1 P12 A</td>
<td></td>
</tr>
<tr>
<td>1 P12 A</td>
<td>6 P12 A</td>
<td></td>
</tr>
<tr>
<td>1 P1 NP</td>
<td>1 P12 NP</td>
<td></td>
</tr>
<tr>
<td>1 P1 NP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P, pyramidal neurons; NP, nonpyramidal neurons; A, astrocytes; BR, bireactive neurons (Glu + GABA).
Figure 2. An example of a two-cell mixed clone in layer V of a 14-d-old rat. The first cell (arrows in a–c) shown in a section stained histochemically for β-gal (a) and in two consecutive sections stained immunohistochemically for GABA (b) and for glutamate (c). In a, the cell appears heavily labeled, a feature characteristic of nonpyramidal neurons. This was confirmed in the subsequent sections as the cell appeared GABA positive (b) and glutamate negative (c). The second cell of the clone (arrows in d–f) shows features typical of many pyramidal neurons. Specifically, β-gal staining consists of a ring of reaction product outlining the round nucleus and spots of reaction product near the origins of two of the dendrites. Consistent with this appearance, the cell was found to be GABA negative (e) and glutamate positive (f). Various symbols are used to show the positions of blood vessels in the serial sections. The same symbol is used to indicate the same blood vessel as it appears in the three consecutive sections. These vessels as well as other nearby cells (arrowheads in a–c) are used as landmarks to identify the cell in question. Magnification, 580x.

significantly. By the end of the first postnatal week, GABA-containing neurons show adult-like density and distribution throughout the cortex. Glutamate-containing neurons first appear in the deep layers around the time of birth and subsequently throughout the cortical thickness following the inside-out development of the cortex (Götz and Bolz, 1994; Dori and Parnavelas, 1996). However, none of the earlier studies examined the possibility that the two amino acids are colocalized in cortical neurons during development. Although the degree of coexistence at P7 was less than 4%, it was significantly higher than in older animals. It is not known to what extent GABA and Glu are colocalized in even younger animals.

Colocalization of excitatory and inhibitory neurotransmitters has also been reported in adult brain: a preparation of cortical synaptosomes has been shown to release both Glu and GABA (Docherty et al., 1987); in hippocampal mossy fiber nerve terminals, Glu and GABA have been colocalized immunocytochemically at the ultrastructural level (Sandler and Smith, 1991); retrogradely transported [3H]-d-aspartate has been colocalized with immunohistochemically detected GABA in layer I of monkey striate cortex (Kisvarday et al., 1989), and in striatopallidal and striatonigral neurons in the rat (White et al., 1994). In all these examples, the two neurotransmitters have been attributed with a role in synaptic transmission, whereas it is likely that during development they play different roles. A neurotrophic function has been attributed to GABA (Lauder, 1993), and it may be that during cortical development GABA is expressed by a larger number of cells than in older animals. In accordance with this hypothesis, a slight decrease in the number of GABA-containing cells has been reported to occur between the first postnatal...
week and adulthood in the mouse neocortex (Del Rio et al., 1992). These neurons have also been associated with transient transmitter expression during development (Parnavelas and Cavanagh, 1988). In cultures of cortical progenitor cells prepared from E16 rat embryos, approximately 15% of MAP2-immunoreactive cells expressed both GABA and Glu immunoreactivities (Vaccarino et al., 1995). Surprisingly, all hippocampal neurons cultured from P5 in serum-containing medium expressed high levels of both Glu and GABA; in contrast, when these cells were cocultured with glial cells, the levels of GABA in pyramidal and Glu in nonpyramidal neurons were selectively reduced (Mattson and Kater, 1989). It is interesting to note that the regression of bireactivity that we observed with increasing age coincides with the increase of the glial population of the cortex; this increase obviously results in a more frequent association of neurons with glial cells. A similar plasticity has been observed in cultures of rat sympathetic neurons (Furshpan et al., 1976; Reichardt and Patterson, 1977). When these neurons were grown alone under certain conditions they became predominantly adrenergic, but when...
they were cultured together with non-neuronal cells they developed a primarily cholinergic phenotype. In this system Furshpan et al. (1976) demonstrated that single neurons can secrete both neurotransmitters, while Reichardt and Patterson (1977) speculated that young neurons responding to cholinergic inducer substances might pass through a “bifunctional” stage. Thus, there are indications that the choice of the appropriate neurotransmitter, although it may be genetically determined, may be challenged during the early stages of a neuron’s life. At least for some developing neurons, maturation of a specific phenotype may involve a “trial” expression of one or the other (or both) neurotransmitter, and the bioactive neurons observed during the first 3 weeks of life may represent cells in the process of changing their phenotype. It is interesting to note that GABAergic cells observed in the upper cortical plate during the early stages of the intermediate fetal period in the rhesus monkey exhibit pyramidal morphology (Schwartz and Meinecke, 1992). This observation supports the notion that projection neurons and interneurons of the cerebral cortex share phenotypical traits in early development.

It is more difficult to envisage that cortical neurons undergo morphological transformation after the third postnatal week, because that would involve a change of a full complement of nuclear, cytoplasmic, dendritic, and synaptic features (Parnavelas and Lieberman, 1979; Miller, 1988). However, it is possible that an individual neuron in a clone that may mistakenly express a phenotype different from that of its siblings eventually dies, resulting in a homogeneous clone. Although we do not have direct evidence to support this interpretation, naturally occurring cell death is at its peak in the cerebral cortex during the first 3 weeks of postnatal life (see Ferrer et al., 1992, for review); at the same time we observed clones containing both pyramidal and nonpyramidal neurons. Nonpyramidal cells seem to be selectively affected in the process of naturally occurring cell death in the cortex (Parnavelas and Cavanagh, 1988; Miller, 1994). No consistent mechanisms have been proposed to explain cell death in the cortex. A lack of target-mediated neurotrophic factors, which are held responsible for naturally occurring cell death in the peripheral nervous system (see Rohrer, 1990, for review), is less likely to be involved in the cortex due to its complex organization and, perhaps, autocrine production of growth factors (Ernfors et al., 1992; Kokaia et al., 1993; Miranda et al., 1993). Cell death could turn an original number of mixed clones into smaller homogeneous ones or single cells, depending on the original size of the clone and the number of the cells eliminated. In support of this interpretation is the finding of a significant increase of single β-gal+ cells between P7 and adulthood (Mione et al., 1994). Given the fact that homogeneity seems to have been reached by P28 (Mione, unpublished observations), the process responsible for having only homogeneous clones in the adult cortex should be concluded by the end of the fourth week.

Notes
We thank Dr. M. Lukin for providing the retrovirus-producing cell line, and Peter Boardman and Brett Harris for technical help. We acknowledge the financial support provided by the Medical Research Council.

Address correspondence to A. Lavdas, Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK.

References


