CELL LINEAGE OF PYRAMIDAL AND NONPYRAMIDAL NEURONS
IN THE RAT CEREBRAL CORTEX

by

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ABSTRACT

Cell Lineage of Pyramidal and Nonpyramidal Neurons in the Rat Cerebral Cortex

We have utilized the techniques of retrovirally-mediated gene transfer, immunocytochemistry, and electron microscopy to study the generation of the two classes of cells in the rat cerebral cortex, the pyramidal and nonpyramidal neurons. These two neuronal types have different functions in the brain, pyramidal neurons are excitatory (utilizing glutamate) and project outside of the cortex, while nonpyramidal neurons are inhibitory (utilizing GABA) and terminate locally. An important question is whether these two neuronal categories arise from the same ventricular zone progenitor or whether, by the time of corticogenesis, the two are generated by different progenitors.

The replication-defective retroviral vector used contained an E-coli β-galactosidase gene insert which could be detected histochemically. The viral constructs were injected in low titers into the telencephalic ventricles of fetuses during the period of corticogenesis (E14-E21) where they infected ventricular cells. The beta-galactosidase gene was used as a heritable marker since it is passed on to the progeny of these cells. The fetuses were then allowed to continue their development until either two weeks postnatally or until adulthood (1-3 months). Animals were then perfused,
the brains serially sectioned, then stained histochemically β-
galactosidase utilizing X-gal as the enzymatic substrate. The
sections were then processed for electron microscopy and flat
embedded in Araldite.

Sections containing stained cells were serially drawn
using camera lucida and clusters of clonally-related cells
were defined. Cells in each cluster were then identified using
postembedding immunocytochemistry to localize GABA or
 glutamate in semithin sections. In some cases the findings
were confirmed by light and/or electron microscopy.

Our results showed two different outcomes for the two age
groups studied. In the adult brains, the vast majority of
neurons in a group were composed of only one cell type, either
all pyramidal or all nonpyramidal neurons. The two week old
animals, on the other hand, showed very different results.
Many of the clones contained both pyramidal and nonpyramidal
neurons. The possible explanations for this discrepancy
between young and adult animals such as cell death and cell
transformation are discussed.
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1. INTRODUCTION

The mammalian cerebral cortex is subdivided into a number of areas, each characterized by its own individual connections, cytoarchitecture, and physiological characteristics. In addition, it is composed of two main classes of cortical neurons, pyramidal and nonpyramidal cells which display differing neurochemical and physiological properties, although throughout the cortex, the proportion of the two classes of neuron remains the same (Rockel et al., 1980; Hendry et al., 1987). Pyramidal neurons, the projection neurons of the cortex, are excitatory and utilize glutamate or aspartate as their neurotransmitters (Fagg and Foster, 1983; Streit, 1984). Nonpyramidal neurons, on the other hand, are cortical interneurons and exert an inhibitory influence on neighbouring cells by utilizing GABA (Fagg and Foster, 1983; Sillito, 1984).

An important question is how the complex organization of the cortex develops at the cellular level. How do the two types of cortical neurons with very different functions, the pyramidal neuron (projection) and nonpyramidal neuron (interneuron), arise? The technique of retrovirally-mediated gene transfer has enabled researchers to probe these questions and begin to understand these complex problems in neocortical development.
1.1. Cytology of the Rat Cerebral Cortex

The cerebral cortex in the rat, similar to other mammalian cortices, shows 6 layers in Nissl-stained sections. Layer I, immediately beneath the pial surface, can be easily recognized by its general lack of neurons and abundance of traversing dendritic elements. Layer II initially contains an area of tightly packed neurons, under this initial zone, the neurons tend to be fewer in number and more loosely packed. In the rat, unlike the primate, there is no obvious division between layers II and III so the two are usually considered as one layer; these layers contain round and elongated neurons. The beginning of layer IV, the granular layer, is distinct; the neurons in this layer are small, abundant and fairly tightly packed. Beneath layer IV are the medium and large neurons of layer V, the largest neurons in the neocortex. The cells here tend to be less numerous than in layer IV and more loosely packed. The deepest layer, layer VI, is characterized by an initial zone of small closely packed neurons followed by a zone containing fewer cells. Directly beneath layer VI is the white matter, which is made up of cortical afferent and efferent axons.

1.2. Neuronal Cell Types

The cerebral cortex contains an array of different neurons which are usually divided into two main morphological
and functional groups, the so-called, pyramidal and nonpyramidal neurons. The axons of pyramidal neurons typically project to distant cortical or subcortical areas, and can be further classified according to their projection site (Jones, 1984). The axons of nonpyramidal cells, on the other hand, tend to terminate locally, influencing cells in the general vicinity; they are, therefore, termed local circuit neurons or interneurons. The dendrites of pyramidal cells give rise to spines, while the dendrites of nonpyramidal neurons have very few or no spines at all. Furthermore, the two cell classes have very different functional properties. Pyramidal neurons form asymmetrical synapses with their targets and contain glutamate and/or aspartate, the excitatory transmitters (Gilbert, 1983), while axons of nonpyramidal neurons form symmetrical synapses and contain \( \gamma \)-aminobutyric acid (GABA), a well-known inhibitory neurotransmitter (Houser et al., 1984). In fact, the major sources of asymmetrical synapses in the cortex are pyramidal neurons, while the major source of symmetrical synapses in the cerebral cortex are the intrinsic cortical nonpyramidal neurons (Peters and Jones, 1984; Peters, 1987; White, 1989).

1.2.1. Pyramidal Neurons

Pyramidal neurons are by far the most numerous cell type in the cortex with estimates ranging from 2/3 (Parnavelas et al., 1977a; Rockel et al., 1980; Winfield et al., 1980), to 85% of all cortical neurons (Peters, 1985). The morphological
features of pyramidal neurons have been described by a number of authors (Winkelmann et al., 1973; Parnavelas et al., 1977a; Werner et al., 1979). The most striking feature of these cells is the triangular shape of their cell body, although other shapes exist as well, and the apical dendrite which arises from the apex of the soma. This dendrite ascends the cortical layers and often forms terminal tufts in layers I and II. The apical dendritic tree typically contains an abundance of dendritic spines which are the focus of asymmetrical axonal terminations (White and Hersch, 1981, 1982). It is reported that all spines receive at least one, if not more, asymmetrical synapses and some spines also receive symmetrical synapses (Peters and Kaiserman-Abramof, 1969, 1970). Pyramidal neurons tend to have a larger diameter than nonpyramidal neurons, and they usually possess a large, pale nucleus which is typically round or oval in shape. These cells also contain more modest amounts of cytoplasm than nonpyramidal neurons.

Dendrites can emerge from the base of the pyramidal cell body, termed basal dendrites, which pass horizontally to make lateral connections. The axon of pyramidal neurons typically emerges from the base of the cell body and descends to join the white matter below layer VI; while on route it gives off several collateral branches. These branches can run horizontally, vertically or locally, close to the point of origin. Electron microscopic analysis has shown that the local axon collaterals of pyramidal neurons form only asymmetrical synapses (LeVay, 1973; Parnavelas et al., 1977b; Somogyi, 1978). They terminate onto dendritic spines and shafts of
mainly pyramidal neurons but they can also contact nonpyramidal cells.

A feature of pyramidal neurons which distinguishes them from nonpyramidal cells is that they receive exclusively symmetrical (presumptive inhibitory) axosomatic synapses (Parnavelas et al., 1977b; Peters, 1985; Peters et al., 1991), the vast majority of which are GABAergic (Ribak, 1978; Hendry et al., 1983; Freund et al., 1983; Farinas and DeFelipe, 1991). Nonpyramidal neurons receive both symmetrical and asymmetrical synapses on their somata. In contrast to the axosomatic innervation, the pyramidal cell dendritic tree receives both symmetrical and asymmetrical axodendritic synapses, although, as mentioned earlier, the vast majority of axonal synapses onto pyramidal neurons are asymmetrical (Parnavelas et al., 1977b; Peters, 1985).

Pyramidal neurons do not always follow the stereotypical pattern. The characteristics of the cells are different for the different cortical layers, although within a layer cells tend to be relatively similar. Because of this, rather than describe the different variations of pyramidal cell, it is more appropriate to describe the predominant pyramidal cell type(s) for each layer of the cortex.

Layer I is completely devoid of pyramidal neurons although it does contain some nonpyramidal cells. The pyramidal neurons of layer II/III are small to medium with round or irregularly shaped nuclei. The apical dendrites of some of these cells near the border with layer I tend to be oriented obliquely rather than vertically (Parnavelas et al.,
1977a; Werner et al., 1979). The pyramidal neurons of layer IV are usually small, tightly packed, and lack a well developed apical dendrite. These are in sharp contrast to the neurons of layer V which are medium to large and loosely packed. Some of the neurons, especially in the areas devoted to motor processing, are enormous with a large apical dendrite and basal skirt. Layers V and VI also contain some pyramidal neurons with their apical dendrite pointed either at an angle to the pial surface or even in the opposite direction to the pia, towards the white matter. These are the so-called inverted pyramidal neurons (Van der Loos, 1965; Parnavelas et al, 1977a). It has also been reported in the cat and monkey that layer V may have pyramidal neurons with two or more apical dendrites, although they are very rare and have not been described in the rat (Deschenes et al., 1979; Yamamoto et al., 1987a,b). Layer VI contains small pyramidal neurons, some with irregularly shaped nuclei. Most of the apical dendrites of these neurons extend only to layer IV; however, some continue their ascent to eventually terminate in layers II and I.

1.2.2. Nonpyramidal Neurons

Unlike pyramidal neurons, nonpyramidal cells are a much more heteromorphic group. The vast majority utilize the same neurotransmitter, but they vary in form (Peters et al., 1982; Somogyi et al., 1984; Somogyi and Hodgson, 1985), contain different peptides (Hendry et al., 1984), and indeed, express
different surface molecules (Naegele and Katz, 1990; Zaremba et al., 1990). Therefore, it seems likely that different nonpyramidal neurons perform different functions. The defining characteristic of this diverse group, is, as mentioned previously, that they receive both symmetrical and asymmetrical axosomatic synapses (Parnavelas et al, 1977b; Peters and Fairen, 1978; Peters and Kara, 1985b). Normally, these cells lack an apical dendrite and unlike pyramidal neurons, whose axon can project to distant areas, the axon of nonpyramidal neurons tends to arborize locally. Under the electron microscope nonpyramidal neurons appear more electron dense than pyramidal neurons and their nuclear envelopes frequently have indentations. Small nonpyramidal neurons usually contain little perinuclear cytoplasm, while those with larger somata contain large amounts of cytoplasm with a rich complement of organelles and frequent cisternae of granular endoplasmic reticulum organized in parallel arrays.

Most of the inhibitory synapses in the cortex are due to the intrinsic nonpyramidal neurons (Montero and Zempel, 1985; Gabbott et al., 1986; Montero, 1986), and therefore, these neurons are extremely important for the proper functioning of the cortex. Inhibition is vital in the operation of the cerebral cortex as single unit recordings in the cat have shown. In these experiments GABAergic neurotransmission was blocked during electrophysiological recording of single cells. The results showed striking changes in the configuration and selectivity of receptive fields (Sillito, 1975a,b; Tsumoto et al., 1979).
Many schemes have been used to categorize this heteromorphic collection of cells. A frequently used scheme in the rat cerebral cortex is that of Feldman and Peters (1978) which divides nonpyramidal neurons into three main categories: multipolar, bitufted, and bipolar.

Multipolar neurons are the most numerous nonpyramidal cell type; most estimates report them to be approximately 60% of nonpyramidal neurons in the rat visual cortex. The cell body can vary in shape and size but the main feature is the dendrites which typically radiate out from the soma in all directions. They are found in all layers of the cortex and there are both spiny and non-spiny varieties. Although these cells receive both symmetrical and asymmetrical axosomatic synapses, their axons form exclusively symmetrical synapses with the dendrites, cell body, and axon hillock of both pyramidal and other nonpyramidal neurons.

Bitufted neurons are thought to account for approximately 20% of nonpyramidal neurons in the rat visual cortex. The cell body of these cells is slightly elongated with the dendrites emanating from two tufts, one pointing towards the pial surface, the other towards the white matter. They are found predominately in layers IV and V and can be sparsely spinous or non-spiny. The axon of a bitufted neuron emerges from a primary dendrite and can either form a local plexus near its cell body or travel several layers before forming collaterals. Like the multipolar cells described above, bitufted neurons always form symmetrical synapses. These can be formed with a wide variety of neuronal elements.
such as the cell body or dendrites and also with either pyramidal or nonpyramidal neurons (Parnavelas et al., 1977b; Peters and Fairen, 1978; Peters and Kara, 1985b).

Chandelier cells are usually considered a subset of bitufted neurons and are encountered primarily in layer II/III. They are a truly unique cell type as they make distinct synapses, while most cells in the cortex contact a wide variety of postsynaptic sites. As with other nonpyramidal neurons, these cells receive both symmetrical and asymmetrical axosomatic synapses; however, unlike the previously described multipolar and bitufted neurons, the axons of chandelier cells form symmetrical synapses exclusively with the axon initial segments of pyramidal neurons (Somogyi et al., 1979, 1982; Peters et al., 1982). The initial segment of an axon is thought to be the position where the action potential is initiated, therefore, chandelier cells are strategically located to influence the more numerous pyramidal neurons. These selective axo-axonic synapses are accomplished by vertical rows of boutons which are lined up with the initial segment. It is this which gives these cells their distinctive shape. Immunocytochemistry and electron microscopic analysis show that chandelier cells are likely to use GABA as their predominant neurotransmitter (Peters et al., 1982; Freund et al., 1983).

The third type of nonpyramidal neuron encountered in the rat cortex are bipolar cells. The perikarya of these neurons are spindle or oval shaped with usually just a single dendrite extending from two opposite poles of the cell. These dendrites
can span just one or two, and sometimes several layers. Bipolar neurons are found in all layers, but are especially numerous in layers II-IV (Feldman and Peters, 1978; Peters and Kimener, 1981). Although it is known that the axon of a bipolar neuron usually emerges from a primary dendrite, the kind of synapse it forms is as yet unclear. Early research suggested they formed exclusively symmetrical synapses (Parnavelas et al., 1987b), as do the majority of other nonpyramidal neurons. However, more recent studies indicate there may be two types of bipolar neuron, one which forms asymmetrical connections, typically with dendritic spines of pyramidal neurons (Peters and Kimmer, 1981; Fairen et al., 1984; Peters and Harriman, 1988), and another that forms symmetrical synapses (Peters and Kimmer, 1981; Peters and Harriman, 1988). As yet however, the issue is still unresolved.

In summary, layer II contains a preponderance of multipolar, chandelier and bipolar neurons; the contents of layer III are very similar to layer II (multipolar, chandelier, and bipolar); layer IV contains predominately multipolar, bitufted, and bipolar neurons; layer V contains multipolar and bitufted cells; and, finally, layer VI contains mostly multipolar neurons.

Although other types of nonpyramidal neurons exist in other mammalian species such as basket cells and double bouquet neurons, there is some dispute whether they exist in the rat cerebral cortex. In addition, the spiny stellates so prevalent in layer IV of some mammals, are virtually nonexistent in the
As mentioned previously, layer I contains no pyramidal neurons and very few nonpyramidal cells. Although little is known about them, at least four different types of nonpyramidal neurons have been described in layer I, including horizontal cells, multipolar cells, vertical cells and neurons lacking an axon (Bradford et al., 1977). Many of the neurons of this layer are unusual in appearance and are very different from the cells of the rest of the cortex.

1.3. Neurotransmitters of Intrinsic Cortical Neurons

Extensive investigations over the last twenty years have yielded a great deal of information on neurotransmitters in the rodent cerebral cortex. Since the focus of this paper has been pyramidal and nonpyramidal neurons, this section will also concentrate on the major transmitters used by these two neuronal cell types. In addition, since the rat visual cortex has been a major focus of study in cortical neurotransmitters, this section will also emphasize this cortical area, although information about the entire cerebral cortex is included wherever possible.

1.3.1. GABAergic Neurons

γ-aminobutyric acid (GABA) is thought to be the major inhibitory neurotransmitter in the brain, especially in the cerebral cortex (Krnjevic, 1974; Krnjevic and Schwartz, 1967),
and cerebellum. In the cortex, GABA arises almost exclusively from the intrinsic nonpyramidal neurons (Emson and Lindvall, 1979). It is synthesized from L-glutamic acid by the enzyme glutamic acid decarboxylase (GAD) in the axon terminals, and is later transported and stored in the synaptic vesicles. Neurons expressing GABA are generated between embryonic days 14 (E14) and embryonic day 20 (E20) (Miller, 1985). During the last week of gestation the GABAergic neurons are progressively observed in the ventricular, subventricular, intermediate, and marginal zones, and finally the cortical plate as the neurons migrate to their final positions (Van Eden, 1989).

In the cerebral cortex antibodies to GAD mark the cell bodies and axon terminals of nonpyramidal neurons, and are distributed in all layers fairly evenly, although the staining in layer IV is slightly more pronounced (Lin et al., 1986). Most of these neurons appear to be of the multipolar or bitufted variety, although some horizontal neurons of layer I are also stained (Ribak, 1978).

Estimates of the number of GABAergic neurons in the rat cortex do not vary significantly. Most estimates suggest 15-20% of cortical neurons contain GABA, determined either with GAD or GABA immunohistochemistry (Gabbott and Somogyi, 1986; Lin et al., 1986, Meinecke and Peters, 1987). Meinecke and Peters (1987) reported that only a small proportion of the bipolar types were GABA negative. These authors also reported that electron microscopic analysis revealed that astrocytes are frequently GABA immunoreactive; it has been suggested that astrocytes play a role in the metabolism of excess GABA.
Immunocytochemical studies using antibodies against choline acetyltransferase (ChAT), the enzyme which synthesizes ACh, or acetylcholinesterase (AChE), have shown that the rat cerebral cortex contains a population of CHAT positive intrinsic cortical nonpyramidal neurons (Eckenstein and Thoenen, 1983; Eckenstein and Baughman, 1984) which first express the transmitter around postnatal day 11 (Dori et al., 1989b).

The intrinsic cholinergic neurons in the rat cerebral cortex were first visualized with ChAT immunocytochemistry by Eckenstein and Thoenen (1983); most of these neurons were later found to also contain the peptide, vasoactive intestinal polypeptide (VIP) (Eckenstein and Baughman, 1984; Eckenstein et al., 1988). Subsequent studies have shown that ChAT-labelled neurons are spread in layers II to VI, although most are concentrated in layers II and III; in addition, labelled neurons are exclusively nonpyramidal cells, most are bipolar in morphology (Houser et al., 1985; Parnavelas et al., 1986; Eckenstein et al., 1988). Electron microscopic studies of ChAT-labelled neurons show that the majority of synapses in the visual (Parnavelas et al., 1986) and in the motor and somatosensory areas (Houser et al., 1985) are symmetrical; postsynaptic targets are usually dendritic, but they can also contact the perikarya of unlabelled neurons (Parnavelas et al., 1986).
Radioimmunoassay and immunohistochemical studies have localized some peptides in cortical neurons in all mammalian species examined. Most peptide containing neurons are also immunoreactive for GABA or GAD (Jones and Hendry, 1986), and most are nonpyramidal neurons, however, a few labelled cells have been pyramidal. These peptides include somatostatin (somatotropin release inhibiting factor (SRIF)), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), and neuropeptide Y (NPY). The distribution, synaptic organization, morphology and development of these peptide-containing neurons in the rodent have been extensively studied (for reviews see, Parnavelas and McDonald, 1983; Emson and Hunt, 1984; Jones and Hendry, 1986). Peptide-containing neurons are reported as forming predominantly, indeed some authors report exclusively, symmetrical synapses (Hendry et al., 1983, 1984).

1.3.2. Glutamate and Aspartate

Glutamate (Glu) or glutamic acid, an amino acid prevalent in the body and brain as a metabolic by-product, is also thought to act as a neurotransmitter in many areas of the brain including the cerebral cortex (Johnson, 1978; Cotman et al. 1981; Fagg and Foster, 1983). Evidence suggests that glutamate is utilized by pyramidal neurons only, whose axons form exclusively asymmetrical synapses (DeFelipe et al., 1988; Conti et al., 1989; Dori et al., 1989). Glutamate generally produces depolarization, hence excitation (Krnjevic and Phillis, 1963, Krnjevic, 1974), in the target neuron in the
contralateral cortex or subcortical areas. Immunocytochemical analysis has shown Glu-immunoreactive neurons in layers II to VI of the rat visual cortex, with the majority present in layers II/III and V/VI (Dori et al., 1989). Glu immunoreactivity can first be detected in the rat visual cortex by postnatal day 3, exclusively in layers V and VI; by postnatal day 10, Glu staining can also be found in the upper layers (Dori et al., 1989).

Aspartate, like glutamate, is present throughout the brain and is also thought to act as an excitatory neurotransmitter (Krnjevic and Phillips, 1963; Baughman and Gilbert, 1981; Hicks, 1987). In the rat visual cortex, Asp immunoreactive neurons are found throughout layers II-VI, although the concentration is slightly higher in layers II and III (Dori et al., 1989). Unlike glutamate, which is thought to be utilized by pyramidal neurons only, approximately 10% of Asp-immunopositive neurons appear to be nonpyramidal (Dori et al., 1989).

1.4. Neocortical Connections

1.4.1. Afferent Connections

The afferent connections of the rodent neocortex can be grouped into two classes, subcortical and corticocortical afferents. The subcortical afferents originate predominately from the thalamus although other tracts also arrive from the basal forebrain, midbrain, and brainstem. The corticocortical
afferents consist of callosal and ipsilateral fibers.

1.4.1(a) Thalamic afferents

The thalamus projects to all cortical areas, however, the density of the projection varies in different cortical regions. In adult rodents, the main termination sites of the thalamocortical fibers are layers III and IV, although other laminae may also receive a more sparse projection. An important feature of the thalamocortical afferents is that they retain a highly topographic organization from the thalamus to all cortical sensory regions.

1.4.1(b) Corticocortical afferents

The main projection to all cortical areas arises from the corticocortical afferents. All regions of the cortex are richly interconnected to both ipsilateral and contralateral cortical areas, although the pattern, and density of projection varies among different species and different cortical regions. Generally, pyramidal neurons in the superficial cortical layers project to layer IV of another cortical area, while pyramidal neurons in the deep layers project predominately outside layer IV to other cortical destinations.

1.4.2. Other afferents
Noradrenergic input

The entire cerebral cortex receives an extensive projection of noradrenaline (NA) from neurons located in the locus coeruleus (Dahlstrom and Fuxe, 1964); studies utilizing retrograde transport of HRP have shown this projection to be topographically organized (Waterhouse et al., 1983b). Biochemical and histochemical studies in the rat have shown that the intensity of NA innervation varies in different cortical regions (Levitt and Moore, 1978; Palkovits et al., 1979). The frontal and cingulate cortical areas have the highest concentration, followed by the visual, then the auditory, and finally by the sensorimotor cortices. In addition, the density of innervation of the various layers also varies from one cortical area to another. For example, in the rat visual cortex, the more superficial layers are more densely innervated than the infragranular layers (Morrison et al., 1978; Papadopoulos et al., 1989a), while in the somatosensory and motor cortices the infragranular layers are more densely innervated (Morrison et al., 1978; Molliver et al., 1982). However, the intracortical pathway of noradrenergic axons appears to be highly preserved in different cytoarchitectonic areas and in different species (Lindvall and Bjorklund, 1984). Generally, in layer I and VI, the NA axons run tangentially; in layers II and III the fibers are radially oriented while in layers IV and V the fibers follow a more circuitous and oblique pathway.
(2) Dopaminergic input

The dopaminergic (DA) innervation of the rat cerebral cortex is much more restricted than the NA innervation. Biochemical and anatomical studies have shown that the cortical DA-containing axons originate in the ventral midbrain tegmentum and project to restricted fields in the prefrontal cortex, anterior cingulate cortex, suprarhinal and perirhinal cortices, and entorhinal cortex (Thierry et al., 1973,). More recent immunohistochemical studies have shown the presence of other, less intense, dopaminergic fields in the rat retrosplenial, sensorimotor and occipital cortices (Berger et al., 1985). Laminar organization of DA innervation also varies in different cortical regions (Lindvall and Bjorklund, 1984; Berger et al., 1985; Descarries et al., 1987). For example, in the anterior cingulate cortex the dopaminergic fibers innervate only the supragranular layers (Lindvall and Bjorklund, 1984), while in the prefrontal cortex, DA-containing axons are found throughout layers II to VI, although the highest concentration is in the infragranular layers. The rodent primary visual cortex contains very few DA-containing axons, most of which are concentrated in layer VI (Phillipson et al., 1987; Papadopoulos et al., 1989b). Examination of DA-containing terminations, using serial ultrathin sections, have shown that these afferents usually terminate onto dendritic shafts or spines and form asymmetrical synapses (Papadopoulos et al., 1989b).
1.4.2(3) Serotonergic input

The serotonin (5-HT) innervation of the rat cerebral cortex originates in the midbrain raphe region (Dahlstrom and Fuxe, 1964). Later studies using HRP tracing or double-labelling techniques have found this projection to be topographically organized (Waterhouse et al., 1986). Several studies have shown that not all of the neurons in the raphe nuclei utilize serotonin and that adjacent neurons in this nucleus may use different transmitters in their terminations; however, the majority are serotonergic (Kohler and Steinbusch, 1982; Kosofsky and Molliver, 1987). Immunocytochemical and high pressure liquid chromatography measurements have shown that serotonergic axons form a rich fiber network throughout all cortical layers, these studies also illustrate differences in laminar innervation in different cortical regions (Steinbusch et al., 1978; Lidov et al., 1980; Steinbusch, 1981).

1.4.2(4) Cholinergic input

In the rat cerebral cortex there are two sources of cholinergic afferents, these include a large projection from the basal forebrain as well as a smaller number of intrinsic cortical neurons (Eckenstein et al., 1988; Lysakowski et al., 1989). Most of the neurons in the basal forebrain are cholinergic (Mesulam et al., 1983b) and these project to a number of regions such as the cingulate and retrosplenial.
cortex, entorhinal and olfactory cortex, and most other cortical areas (Eckenstein et al., 1988). The cholinergic innervation seems relatively uniform throughout the cortex although the olfactory and entorhinal cortices receive a higher density of innervation. The laminar pattern of distribution of the cholinergic input appears to be mainly due to the projection from the basal forebrain since lesions of these projections causes the lamination pattern to disappear. In the rodent cerebral cortex, the lamination organization is characterized by relatively high density of cholinergic terminals in layers I to III, low density in layer IV, a high density in layer V, and a variable density in layer VI (Eckenstein et al., 1988; Lysakowski et al., 1989).

1.4.3. Development of Afferent Systems

Newly-generated postmitotic neurons, which migrate from the ventricular zone towards the pial surface, encounter the ingrowing corticopetal axons which are composed of mainly monoaminergic fibers from the midbrain and pons; these form fiber tracts in the primordial plexiform layer. The monoaminergic afferents invade the cortex very early in development. The serotonin-containing axons are among the first to enter the cortical plate in the somatosensory cortex (Rhoades et al., 1990), while NA-containing fibers invade the primordial plexiform layer and the developing intermediate zone at embryonic day 17 (E17) in the rat (Seiger and Olson,
1973; Levitt and Moore, 1978); these finally penetrate the
cortical plate at E18 (Coyle and Molliver, 1977; Kristt,
1979). Dopaminergic terminals can first be detected in the
cortical subplate by E17 and in the cortex at postnatal day 1
(P1) to P3 (Lindvall et al., 1974; Berger et al., 1976). Like
the dopaminergic afferents, thalamocortical afferents enter
the cortical plate at P1 to P4 (Wise and Jones, 1978). Prior
to entering the cortical plate, the axons wait in the subplate
where they form temporary synapses on interstitial neurons
(Molliver et al., 1973; Konig et al., 1975; Friauf et al.,
1990). However, recent reports using techniques which show
axonal processes in more detail, suggest that thalamic fibers
arrive in the cortex at an early age and arborize within the
forming cortical layers without waiting in the subplate

1.4.4. Efferent Projections

The projection neurons of the cerebral cortex are
generally pyramidal neurons which are arranged in laminae,
depending on their axonal termination sites. Because pyramidal
neurons form extensive intracortical collaterals, there are no
layers devoted exclusively to output. Pyramidal neurons of
layers II and III form the corticocortical efferents which
contain both ipsilaterally and contralaterally projecting
fibers. Generally, the pyramidal neurons of layer V mainly
project to a number of subcortical areas and layer VI
pyramidal cells send their axons to the thalamic nuclei.
1.5. Development

1.5.1. Neurogenesis

The neurons and glia of the rat cerebral cortex, as in all mammalian species, originate from a narrow band of germinal cells which line the lateral ventricles in the rostral telencephalon (Sidman et al., 1959; Sidman and Rakic, 1973). By embryonic day 12 (E12), this proliferative region, termed the ventricular zone (Boulder Committee, 1970), rapidly increases in size. The cells are produced and mature in the rat in a gradient where the anterior ventro-lateral cortical areas develop first, followed by the posterior dorso-medial cortical areas. Neurepithelial cells in this initial proliferative region utilize interkinetic nuclear migration to divide exclusively at the area surrounding the lateral ventricle (Sidman et al., 1959). A possible explanation for this movement of the nucleus is that it may allow regionally differing cytoplasmic factors to enter the nucleus, therefore stimulating differential gene activity in these dividing cells (Sauer, 1936, 1937).

Several studies have reported the presence of rudimentary junctions between the mitotic cells lining the ventricle, these are readily apparent when viewed with the electron microscope and/or with freeze fracture. Most of these junctions are zonulae adherens (intermediate junctions), although some authors also report the presence of gap junctions and the remnants of tight junctions (Shoukimas and
Hinds, 1978). Consistent with the early work of Sauer (1936, 1937), the study of Shoukimas and Hinds (1978), which utilized electron microscopic analysis of serial sections, confirmed that cells in the ventricular zone alter their location depending on their cytokinetic state. They found some neuroepithelial cells extended processes through the entire length of the cerebral wall, contacting both the pial surface and also the lateral ventricular surface. Other cells contacted only the lateral ventricular surface, while others contacted neither the basal or ventricular surface, but were located in various positions within the proliferative region. Shoukimas and Hinds (1978) also provided evidence that some of the ventricular cells have short processes extending from the soma for a few micrometers.

Later in development, starting at E16, a second proliferative zone appears above the ventricular region, the so-called, subventricular zone. It is characterized by the appearance of mitotic cells which have the ability to divide away from the lateral ventricle; this is in contrast to the early progenitor cells which undergo interkinetic nuclear migration to divide exclusively at the ventricle (Smart and Sturrock, 1979; Raedler et. al., 1980; Rakic, 1981). Another difference between the two areas is in the orientation of the constituent cells. Cells in the ventricular zone usually have a columnar-radial orientation, while cells in the subventricular zone show variable orientation.

Shortly after the ventricular zone begins its formation, another zone can be discerned. This area has been termed the
marginal zone and contains processes from ventricular layer cells and a few cell nuclei (Boulder Committee, 1970). The Golgi studies of Marin-Padilla on the cat neocortex (1971, 1978, 1983) examined this area in more detail. He proposed that there are two phases in the development of the mammalian cerebral cortex. The first is the development of the primordial plexiform layer (PPL) which is later split into two zones, layer I (marginal zone) and layer VII (subplate). Marin-Padilla has hypothesized that the PPL represents the phylogenetically older cortical region, reminiscent of the reptilian cortex. The second phase involves the development of the mammalian cortical plate, or layers II to VI, which develops between layer I and the subplate. Studies which utilized $[^3]H$-thymidine administered at the very beginning of neurogenesis, labelled cells in layer I and the subplate first, clearly suggesting that these cells are generated before most neurons of the cortical plate (Raedler and Raedler, 1978; Rickman et. al., 1977). The PPL receives an early ingrowth of cortical afferents which are thought to emanate from brainstem monoaminergic nuclei (Seiger and Olson, 1973; Molliver and Kristt, 1975).

A unique feature of layer I in the mammalian neocortex, are the Cajal-Retzius cells, first revealed by Golgi studies during the last century (Cajal, 1891; Retzius, 1891). Autoradiographic studies suggest that the Cajal-Retzius cells differentiate early in development (Raedler and Raedler, 1978; Rickman et. al., 1977) and electron microscopic analysis shows that these cells have the first mature synaptic contacts in
the developing cortex (Shoukimas and Hinds, 1978). The somata of Cajal-Retzius neurons are usually horizontally oriented and contain long processes which run tangentially; most of these processes look dendritic rather than axonal. During the period that the brainstem afferents invade the cortex, the first synapses, most of which are asymmetrical, appear on the dendrites of Cajal-Retzius neurons (Stensaas, 1967), however, the exact source of these synaptic terminations has not yet been determined. There is some suggestion that these early born neurons may play a role in organizing the development of the cortex (Marin-Padilla, 1971, 1978) although their eventual fate remains a source of controversy. Some investigators utilizing $[^{3}H]$-thymidine, suggest the Cajal-Retzius cells die in the postnatal period (Luskin and Shatz, 1985b), while others utilizing electron microscopic analysis indicate these cells transform into classical nonpyramidal neurons of the adult cortex (Edmunds and Parnavelas, 1982; Parnavelas and Edmunds, 1983). In contrast, the Golgi studies of Marin-Padilla (1972) suggest that the Cajal-Retzius cells do not die or transform but survive in the adult cortex. Marin-Padilla maintains that these cells are not readily apparent because their number are so diluted due to the prodigious growth of the cortex. As yet, however, this question is still unresolved.

The subplate, immediately beneath the cortical plate, also contains some of the oldest postmitotic neurons in the cortex (Kostovic and Rakic, 1980, 1990; Luskin and Shatz, 1985a,b). By the time the cortical plate begins development,
the subplate neurons are already mature and many are already receiving synapses (Chun and Shatz, 1988); immunohistochemical studies indicate that these cells are also expressing neuron-specific antigens and peptides (Chun et al., 1987). Golgi studies suggest that even in early stages of cortical development, subplate neurons have axons which extend to layer I, although their final termination sites are unknown (Stensaas, 1967; Marin-Padilla, 1971). In addition to the migrating neurons and radial glial processes, the subplate contains a multitude of afferent axonal fibers from many diverse sources (Rakic, 1983; Crandall and Caviness, 1984; Kalsbeek et al., 1987). These include acetylcholine-containing axons from the basal telencephalon, dopaminergic axons from the ventral tegmental area, noradrenergic fibers from the locus coeruleus, serotonin-containing fibers from the raphe nuclei, as well as fibers from the thalamus. Most of these afferents arrive at the presumptive cerebral cortex either before the cortical plate begins to develop or when the lower layers are forming (approximately E14-E18). Some of these afferents wait in the subplate or layer I before invading the developing cortex while others invade upon arrival to the cortex, at birth, while some invade postnatally. There is some evidence that the subplate neurons assist the thalamocortical afferents in finding their proper cortical target area (Lund and Mustari, 1977; Rakic, 1977; Shatz and Luskin, 1986; McConnell et al., 1989). These studies suggest that there may be an interplay between the subplate neurons and the thalamocortical axons, although the nature of the interaction
is unknown. One suggestion is that the subplate influences the extracellular matrix in some way to allow the proper axons to enter (Shatz and Luskin, 1986). Other studies indicate that the subplate neurons pioneer the first axon pathway to the thalamus which help to guide the thalamocortical axons to their proper target (McConnell et al., 1989). Related studies in the cat using intracellular recordings from subplate neurons suggest that patterns of activity in the subplate, generated by synapses from the thalamic afferents, may set up reciprocal circuits which aid the formation of the mature functional circuits (Friauf et al., 1990).

Later in development the intermediate zone can be perceived. It is characterized by a lack of mitotic cells, although it does contain postmitotic neurons which can be of variable orientation. The intermediate zone consists of many traversing fibers, formed from incoming axonal fibers and the axons of newly generated cortical neurons; in addition, it also contains migrating neurons and glia on their way to the putative cortical plate (Smart and Sturrock, 1979; Caviness et. al., 1981; Caviness, 1982). The upper portion, directly beneath the marginal zone, contains more horizontally oriented cells, while in the lower portion, above the subventricular zone, the cells are more radially oriented. It is also reported that many cells sprout processes while in the intermediate zone, especially radial processes (Shoukimas and Hinds, 1978).

1.5.2. Development of the cortical plate
The autoradiographic studies of Angevine and Sidman (1961) were the first to propose that the cortical layers in the mouse were produced in an "inside-out" gradient, that is, that the earliest born cells reside in the deeper layers of the cortex, while the cells produced later in neurogenesis migrate through the deeper layers to settle in the more superficial laminae. Later, this gradient was also shown to be true in the rat by the studies of Berry and Rogers (1965). However, an exception to this generalization are the previously discussed neurons of layers I and the subplate which are generated earlier than the cortical plate neurons (Raedler and Raedler, 1978; Rickman et al., 1977).

As demonstrated by autoradiography combined with immunocytochemistry, this "inside-out" trend is followed for many cortical neuronal types including most peptide-containing neurons (Cavanagh and Parnavelas, 1988, 1989), as well as pyramidal and nonpyramidal neurons (Miller, 1985). These studies utilized pulse labelled [\(^3\)H]-thymidine to demonstrate that GABAergic and non-GABAergic neurons are generated concurrently.

Once a neuroblast completes its final mitotic division, it migrates away from the ventricular area toward the developing cortical plate. Studies in the monkey cortex suggest that neuroblasts utilize surface contact with radial glial fibers which span the entire thickness of the cerebral wall (Rakic, 1971, 1972). Migrating neurons appear bipolar in shape with a leading process directed upward toward the pial surface and a trailing process directed downward toward the
ventricle (Rakic, 1972; Peters and Feldman, 1973; Shoukimas and Hinds, 1978). At present, it is not certain whether all cells follow a strictly radial pathway as Rakic has suggested or whether migratory pathways are more diverse. However, studies of Misson and colleagues (1991) using immunocytochemistry with the RC2 antibody which is selective to radial gila cells, as well as β-galactosidase staining, show that migrating neurons follow radial glial fibers; in medial and dorsal areas migration tends to be radial, while in lateral cortical areas, tangential dispersion is encountered more frequently.

1.5.3. Differentiation

Once a newly-generated neuron completes its final division and begins its migration to the cortical plate, it initiates the development of its morphological and transmitter phenotype. Although early Golgi studies suggested that differentiation was only initiated once migration was completed (Berry and Rogers, 1965), more recent studies show that projection neurons can extend their axons and axon collaterals during the period of migration (Schreyer and Jones, 1982; Valentino and Jones, 1982). However, this is not true of local circuit neurons which appear to delay axogenesis and synaptogenesis until they have reached their final cortical position (Miller, 1986b).

First postnatal week
Apparent during the first postnatal week is a feature common to all cortical areas, the "inside-out" developmental gradient for cortical neurons. By birth, the earliest born neurons reside in the presumptive layers V and VI and are already beginning to differentiate while later born neurons, destined for more superficial layers, are in the process of migrating through the lower layers to reach the layers above. The cortex contains cell bodies and processes of migrating and postmigratory neurons, glia, and glioblasts. At birth, and for the next few days, the cells of the developing cortex appear tightly packed and immature; under the electron microscope all neurons exhibit elongated perikarya with little cytoplasm containing few loosely packed organelles (Parnavelas and Lieberman, 1979). At this stage, some neurons can be distinguished from glia by their paler nuclei (Parnavelas and Lieberman, 1979). A few presumptive pyramidal neurons with a developing apical dendrite can be identified in the deep cortical layers. By the end of the first postnatal week, migration is completed and the neurons of the supragranular layers have begun to differentiate. Also evident at this time is the distinctive lamination typical of the cortex (Parnavelas et al., 1978).

Cells begin to develop their dendritic patterns during the first postnatal week; growth cones can also be seen along dendritic shafts or at the tips of dendrites at this stage (Miller and Peters, 1981; Miller, 1988). By postnatal day 4 (P4), spines first appear on the dendrites and spine density increases significantly towards the end of the week (Miller,
In the infragranular layers, which are most developed at this time, the thick apical dendrite of pyramidal neurons is readily apparent since it derives from the leading process of the migrating neuron. In addition, the basal dendritic skirt also begins its formation (Peters and Feldman, 1973; Miller, 1981). Pyramidal neurons are also distinctive in their abundant cytoplasm which contains rough endoplasmic reticulum, ribosomes, mitochondria and Golgi apparatus.

During the first postnatal week, nonpyramidal neurons always appear less differentiated than pyramidal neurons. They can first be distinguished with certainty by P5 and P6 in the infragranular layers, identified by their more electron-dense appearance and asymmetrical axosomatic synapses (Parnavelas and Lieberman, 1979; Miller, 1988). Under the electron microscope, nonpyramidal neurons appear darker than pyramidal cells and they often display an eccentrically oriented and deeply invaginated nucleus. During this time they may exhibit thin dendrites and a short axon (Parnavelas et al., 1978; Miller, 1986a).

Although a few synapses can be detected at E16 in the rat cerebral cortex (Konig et al., 1975), by birth, further synapses can be found in layer I and the infragranular layers, the majority of which are asymmetrical (Blue and Parnavelas, 1983a,b). At this stage, synapses are immature with poorly defined pre- and postsynaptic specializations and few vesicles in their presynaptic terminals. By the end of this week, however, there is a considerable increase of both asymmetrical and symmetrical synapses in layers I, IV and V (Blue and
Parnavelas, 1983b). The first axosomatic synapses can be detected at P3 which are always symmetrical; they greatly increase in number by the end of the first postnatal week (Miller and Peters, 1981). Sources of the asymmetrical synapses onto cortical neurons include axon collaterals of cortical pyramidal cells (Parnavelas et al., 1977b), callosal afferents (Miller and Vogt, 1984), thalamic afferents (Lund and Mustari, 1977), as well as noradrenergic afferents from the locus coeruleus (Molliver and Kristt, 1975). The main source of symmetrical synapses are thought to be axonal terminations from nonpyramidal neurons (Peters and Fairen, 1978; Ribak, 1978).

Second postnatal week

During the second postnatal week, cortical neurons undergo major advances in maturity. Perikaryal size expands as the cytoplasmic volume and organelles increase. Extensive arrays of endoplasmic reticulum and numerous neurofilaments, mitochondria, and microtubules are apparent, especially in cells in the infragranular layers (Miller, 1981). Apical dendrites and axons of pyramidal neurons expand in length and thickness, as do their basal dendrites (Parnavelas and Lieberman, 79). Both apical and basal dendritic branching becomes more elaborate and complex, and spine density increases progressively (Miller, 1981). At the beginning of the second week, it is still difficult at times to definitively identify nonpyramidal neurons utilizing electron
microscopy; however, some can be recognized at this time in the deeper cortical layers. Many of these nonpyramidal cells, some with immature cytoplasmic characteristics, receive numerous axosomatic synapses (Parnavelas and Lieberman, 1979).

By the end of the second week, most neurons can be identified as either pyramidal or nonpyramidal utilizing ultrastructural characteristics. During this period, which coincides with eye-opening in the rat (P14) and extending into the first part of the fourth week, nonpyramidal neurons display a major increase in the amount of cytoplasmic organelles, as if they are in a highly active synthetic state (Parnavelas and Lieberman, 1979). Also by the end of this week, there are no distinct differences in maturity between pyramidal and nonpyramidal neurons, nor are there differences between neurons in the infragranular and supragranular layers, however, smaller cells in all layers appear less differentiated than larger neurons (Parnavelas and Lieberman, 1979).

Third and fourth postnatal week

By the third postnatal week, pyramidal and nonpyramidal neurons of all layers appear mature in terms of perikaryal size and cytological features. Nonpyramidal neurons at this stage display conspicuous organelle-rich cytoplasm with elaborate arrays of endoplasmic reticulum and a prominent Golgi apparatus. By the end of this week all neurons appear to have the adult complement of axosomatic synapses; and by the
fourth postnatal week, pyramidal and nonpyramidal neurons of all layers are virtually indistinguishable from those of an adult.

1.5.4. Determination of Cell Fate

All neurons and glia of the cerebral cortex are ultimately derived from the precursor cells of the early proliferative regions. It is still uncertain, however, whether these proliferative regions are composed of a homogeneous population of precursor cells with equal developmental potential, or whether the precursors are a heterogeneous population of cells which diverge very early in development.

Early studies suggested the precursors were a homogenous population since ventricular cells looked nearly identical from both an electron microscopic viewpoint (Hinds and Ruffet, 1971) and an autoradiographic perspective (Sidman et al., 1959; Fujita, 1963). However, a growing body of evidence, utilizing a variety of techniques, suggests that mature cortical cells may be fated while their precursors are still proliferating in the germinal zone. Much of this evidence stems from the finding of several biochemical markers within the telencephalic germinal zone.

1.5.4(a) Biochemical Markers

Studies by Levitt et al. (1981) and Barbe and Levitt (1991, 1992) on the early expression of limbic-system-associated membrane protein (LAMP), a neuronal cell surface
glycoprotein found on both cortical and subcortical neurons, have been important in identifying the heterogeneity and the early restricted developmental fate of cortical precursors. These studies showed that LAMP-expression occurred in neurons destined for limbic areas even when these cells were transplanted to sensimotor cortex, however, this occurred only in tissue transplanted at E14 or later; if transplanted at E12, the donor cortex failed to develop limbic characteristics (Barbe and Levitt, 1991, 1992). Their tissue culture studies which involved the dissection and growth of E12 rat limbic and nonlimbic cerebral wall tissue grown in culture, showed that after four days in culture, most of the progenitors from presumptive limbic cortex expressed LAMP upon differentiation. In contrast, very few of the nonlimbic cortical cells expressed LAMP (Ferri and Levitt, 1993). The authors suggest that most cortical progenitors are already fated to a specific phenotype while they are still mitotically active in the ventricular zone.

Other research which illustrated the biochemical heterogeneity of ventricular precursor populations, were studies in the chick brain and spinal cord which found antibodies to the neuronal marker neurofilament protein (NF) in a subpopulation of ventricular mitotic cells while other cells failed to express this protein (Bennett and DiLullo, 1985a,b). Another biochemical marker, designated PC3.1 that binds to a 29-kDa polypeptide, has been isolated in subpopulation of cells from lateral by not dorsolateral neocortex in the rat from embryonic day 13 and later (Arimatsu
et al., 1993). Other studies suggesting precursor heterogeneity derive from DiI-injected mouse forebrain cells which show that cortical precursors do not cross the border between the cortical ventricular zone and the adjacent basal telencephalic ventricular zone, this implies that very early in development, molecular and/or cellular signals exist which establish these borders in the early germinal zones (Fishell et al., 1993). These studies, as well as the research by Levitt et al. (1981) and Barbe and Levitt (1991, 1992) suggest that fate-specific markers can be expressed very early in development and can be modified by the environment, but only up to a certain stage of development, implying that neocortical progenitors are characterized by selective sensitivity to genetic and epigenetic factors and then progressive restriction of cell fate.

Other lines of evidence for a heterogenous population of ventricular cells stems from the finding that some proliferating cells in the ventricular zones have differing cell cycles. Analyses of cell cycle times utilizing $[^3H]$thymidine as an S-phase marker, showed the existence of progenitor populations with different cell cycle kinetics, some with faster cycling times therefore producing more progeny, and others with slower cycling times (Waechter and Jaensch, 1972; Schultz et al., 1974; Schultze and Korr, 1981). More recent detailed studies of these populations, again utilizing $[^3H]$thymidine, suggested that the cortical ventricular zone can be segregated into different bands; in early embryogenesis these different cycling cells are divided
into vertical bands while later in development the bands are horizontal (Reznikov et al., 1984; Johnston and van der Kooy, 1989; Altman and Bayer, 1990a,b; Acklin and van der Kooy, 93).

1.5.4(b) Transplant Studies

Transplant studies, which have sometimes yielded conflicting results, have nonetheless, afforded some interesting insights into the determination of individual cell fate. Studies where ventricular precursors from the occipital proliferative zone of E30 or E31 donor ferrets were labeled with \(^{3}H\)-thymidine then transplanted to the proliferative zone of newborn host ferrets (heterotopic transplants), suggest that cortical neurons become committed to their final laminar fate sometime during or after the DNA-synthesis (S-) phase of their final mitotic division (McConnell, 1988a, 1989; McConnell and Kaznowski, 1991). The authors suggest that cues in the embryonic environment trigger a cell or its progeny to generate either a deep layer neuron or a superficial layer neuron. In contrast, other transplant studies suggest that cortical lamination is largely determined genetically but that innervation of the cortex is influenced by the surrounding environment (Chang et al., 1986). These studies involved transplanting frontal or occipital E15 rat cerebral cortex to occipito-parietal cortex of newborn rats. The transplants always developed normal laminar patterns regardless of the orientation they were placed into the host brain. However, the donor tissue received thalamocortical and callosal afferents appropriate for the host tissue only, this
suggests that immature cortical tissue may not contain the information necessary to specify these cortical regions.

Other insights from transplant studies concern the efferent projections of cortical neurons. These studies indicate that the determination of efferent projections is decided relatively late in development since neurons transplanted to a new cortical region are capable of radically changing their pattern of connections (Stanfield and O'Leary, 1985; O'Leary and Stanfield, 1989; Schlagger and O'Leary, 1989). For example, when presumptive visual cortex is transplanted to motor areas, the layer V neurons retain the spinal connection they would normally have lost (Stanfield and O'Leary, 1985; O'Leary and Stanfield, 1989); also, if motor cortex is transplanted to occipital cortex the layer V neurons lose their connections to the spinal cord and conserve the connection to the superior colliculus (O'Leary and Stanfield, 1989). In addition, if visual cortex is transplanted to somatosensory regions, the transplanted tissue can develop normal-looking barrel fields which are unique to somatosensory cortex (Schlagger and O'Leary, 1989). However, other types of research dispute these findings. Disruption of neocortical development with ionizing radiation before, during, or after the production of neurons destined for layer V, results in clusters of these cells located in abnormal positions; even so, regardless of their final location, these cells still develop their normal corticospinal projections (Jensen and Killackey, 1984). Therefore, in contrast to the previous studies, the authors suggest that the initial projections of
corticospinal neurons are determined early in development, prior to migration.

However, an important consideration in transplant studies is whether the connections formed by the transplanted neurons are actually functional. Recent research using retrograde fluorescent tracers and electrical stimulation have addressed this issue and have shown that the grafts appear to be nonfunctional when transplanted to a host of a different age than the donor (heterotopic transplant) (Castro et al., 1991). The transplanted neurons show an absence of response to electrical stimulation, demonstrating that the transplants were not integrated within the host's sensory pathway. A possible explanation for this result was that the heterotopic transplants appeared to receive fewer afferent inputs than that found on cells transplanted to age-matched hosts (homotopic transplants) (Castro et al., 1991). Therefore, while a transplanted cell may have formed projections typical for that cortical area and may appear to have integrated into the host tissue, it may, in fact, not be recognized as normal by the surrounding cells and afferents which results in the observed impaired functional ability.

1.5.4(c) Lineage Studies

Given these conflicting results, the recently developed technique of retrovirally-mediated gene transfer has been very useful in determining lineage relationships and has, therefore, been applied to the study of determination of cell fate. These experiments involve the introduction of a readily
detectable marker, usually the gene for β-galactosidase from Escherichia coli, into progenitor cells of the cortical germinal zone by infection with a retrovirus (Sanes et al., 1986; Price et al., 1987). Some of this research has examined the cortical germinal zone by infecting early ventricular cells then analysing the brain shortly afterwards, 48 hours to several days later (Acklin and Van der Kooy, 1993). However, most lineage studies have concentrated on survival times much later, usually two weeks or a few months, postnatally. Several recent studies have focused on whether the lineages for neurons and glia diverge early in development. It is well established in rodent non-cortical areas of the central nervous system that progenitors are capable of giving rise to both neurons and glia, as well having the ability to populate many, if not all, lamina; this is true for such areas as the mammalian retina (Turner and Cepko, 1987), the chick optic tectum (Gray et al., 1988; Gray and Sanes, 1989), and the chick spinal cord (Leber et al., 1989).

However, lineage studies in the rodent cerebral cortex are not in complete agreement. Some studies which analysed embryonically-infected rats argue that lineage restrictions exist early in development (Luskin et al., 1988; Barfield et al., 1990; Grove et al., 1993; Mione et al., 1994). Other studies which utilized similar injection times, found that some progenitors are capable of producing a variety of cell types during much of neurogenesis (Price and Thurlow, 1988; Walsh and Cepko, 1988).

Another question concerns the generation of pyramidal
and nonpyramidal neurons in the cerebral cortex. Some studies, which identified cells on the basis of light microscopic appearance, have suggested that clusters of clonally-related cells can contain both pyramidal and nonpyramidal neurons (Price and Thurlow, 1988; Walsh and Cepko, 1988); while other studies have argued separate lineages for these two cortical neuronal classes (Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994). Two major problems with many of these studies, which may partially account for the conflicting results, is that animals of different ages have been used in this research, and that some studies have used light microscopic appearance to determine cell phenotype. Correlative investigations by Peters and Kara (1985a,b) have shown that light microscope appearance is an inaccurate method of cell identification, especially for pyramidal and nonpyramidal neurons.

To finally resolve these issues, this study has utilized retrovirally-mediated gene transfer, electron microscopy, and postembedding immunocytochemistry for GABA, Glutamate and Aspartate to clearly identify and label pyramidal and nonpyramidal neurons. In addition, brains were first analysed in adulthood at 1 to 3 months postnatally, and in similar experiments, at two weeks postnatally. The results of these studies should provide a more reliable analysis of cell lineage of pyramidal and nonpyramidal neurons.
2. MATERIALS AND METHODS

2.1. Production of Virus

The BAG vector used in this study (Sanes et al., 1986; Price et al., 1987) is a replication defective retrovirus which can be used as a heritable marker for a cell and its progeny. Several viral genes were excised and replaced by the E.coli Lac-Z gene which encodes for β-galactosidase and can be visualized histochemically. This defective construct was then transfected into fibroblast producer cells (Psi 2) which contain the viral genes necessary to complete replication and budding. The virions secreted into the culture medium were collected by filtration and centrifugation. The virus producing cells, purchased from ATCC (Rockville, MD) and subcloned to generate a higher titer of viral particles, were donated by Dr. M. B. Luskin (Emory University).

The producer cells containing the transfected BAG virus were thawed to room temperature and cultured in 250cm² flasks then allowed to grow until confluent. The media (Dulbecco’s Modified Eagle’s Medium with 4.5g/l glucose) was then removed and the cells were washed in Hanks balanced salt solution (HBS). The HBS was removed and 1 ml of 0.25% trypsin was added, then flasks were put in the incubator for approximately 3 mins. The flasks were gently agitated, and when the cells were in suspension 9 mls of media was added. The cells were then diluted and then plated into fresh flasks containing
media. When the cells were a day past confluence the media was discarded and replaced with half the original volume of fresh media. The following day the supernatant was harvested and centrifuged at 3000 rpm for 3-5 minutes. The supernatant was then filtered through a 0.45μm filter and pooled in a sterile flask. Polybrene was added to produce a final concentration of 8μg/ml before dividing into 1ml aliquots. The tubes were then frozen and stored at -70°C until ready for use.

2.2. Helper Assay and Viral Titration

The helper assay was performed to confirm that the virus remained incapable of reinfection. In all tests performed, the virus was found to be replication defective. Uninfected 3T3 cells were split 1 to 10 (that is, 1 ml of cells in 9 mls of media) into 25cm² flasks the day before they were to be infected. The day of infection the media was removed and 1 ml of virus stock was added to the flasks. Enough polybrene was added to make the concentration 8 μl/ml. Flasks were then incubated for 1 to 3 hours to allow the virus to infect the 3T3 cells, and then 3 mls of media were added to dilute the polybrene to 2 μg/ml. When the cells were confluent (2-3 days), the flasks were split 1 to 10, retaining one flask for each assay and 2 μl/ml polybrene was added to each flask. Once the infected cells became confluent, the media was removed and replaced with half the volume of fresh media. That same day, uninfected 3T3 cells were split 1 to 10, keeping one flask for each assay. The following day the supernatant was
harvested from the confluent cells and filtered through a 0.45 μm filter before polybrene was added to produce a concentration of 8 μl/ml. The uninfected 3T3 cells were then infected with 1 ml of the filtered supernatant and incubated for 1 to 3 hrs. The polybrene was then diluted to 2 μg/ml by adding 3 ml of media, and the cells were allowed to grow until confluent. Once confluent, usually after 2-3 days, the cells were washed in phosphate buffer (PB), then fixed with 2% paraformaldehyde and 0.4% gluteraldehyde in 0.1 M PB followed by a wash in PB. The cells were then stained with X-gal (Sigma), the substrate for β-galactosidase, overnight at room temperature. The following morning, the X-gal was removed and replaced with 70% ethanol. Flasks were then viewed under the inverted microscope for blue cells. The presence of any blue cells would indicate the virus was no longer replication defective and therefore invalid.

The viral titration consisted of the following procedure. After fixation with 2% paraformaldehyde and 0.4% glutaraldehyde in 0.1 M PB (pH 7.2) the number of stained cells was counted in the field of view (using 100x magnification), yielding the number of cells per 1 mm². The number of cells per 1 mm² was then multiplied by 2500, the surface area in mm² of the flask. Since the volume of the viral concentrate (virus and media) was known, usually 10 ml, the concentration of the virus could be determined by multiplying the number of infections by the surface area to yield the number of infectious virions in 10 ml. The viral concentration used in these studies was approximately 3 x 10⁶
viral particles per ml.

2.3. Surgery and Injection of Retrovirus

The aim of the procedure was to inject embryos of specific age (E14 to E20), the period of cortical neurogenesis in the rat, with the replication defective BAG virus. The embryos were then left to be born normally and continue their development until two weeks postnatally (P13-P14) or to adulthood (P40-P94). The animals were perfused, their brains serially sectioned and processed for electron microscopy, immunocytochemistry, or both.

Pregnant Sprague-Dawley albino rats at various stages of gestation, E14-E20 (the day at which a plug was found being day 1) were anaesthetized with halothane and the uterus exposed. A fiber optic light source was used to transilluminate the head and telencephalic ventricle of the embryos. Approximately 1 µl of the viral mixture containing 0.5 µg polybrene and 0.5 µg of fast green dye, was injected into the telencephalic ventricle of each embryo through a 30 gauge needle attached to a Hamilton microsyringe. The virus was kept on ice until the moment it was injected. The mother's abdominal wall was then sutured and the pups were left to be born normally and continue their development until two weeks postnatally (P13-P14) or to adulthood (P40-P94). The animals were then perfused, the brains serially sectioned and processed for electron microscopy and/or immunocytochemistry.
2.4. Tissue Preparation

Rats of various ages which had previously received injections of retrovirus, were deeply anaesthetized with ether. A midline incision was made and the heart exposed. A hypodermic needle (the size varied with the age of the animal) was inserted into the left cardiac ventricle and the animal was perfused with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde (pH 7.2-7.4) for approximately 15 mins. The brain was then extracted from the skull and placed in chilled fixative without glutaraldehyde for up to but not exceeding half an hour. It was then cut into 2 hemispheres (left and right), and each hemisphere was coronally sectioned with a Vibratome at 100 μm. Each cut section was serially placed in a 12 well tissue culture dish (Linbro) filled with 0.1 M PB. When cutting was complete, the buffer was extracted and 500 μl of X-gal (5-bromo-4-chloro-3-indol-B-D-galactoside) was added to each well. The dishes were then covered with foil and placed on a shaker for 2-4 hours, then left undisturbed overnight at room temperature.

The following morning, the X-gal was pipetted away and replaced with 2 rinses of fresh phosphate buffer. The sections in each well were viewed with an Olympus dissecting microscope to check for infected cells. X-gal reacts with the β-galactosidase, which is the enzymatic product of the Lac-Z gene inserted into the virus, to produce a compound that is blue in bright field microscopy and electron dense in the electron microscope. Hemispheres found to contain infected
cells were then processed for electron microscopy.

The sections were rinsed twice in fresh 0.1 M PB, allowing a 5 minute interval between rinses. After the final rinse the buffer was extracted and 500 μl of 1% osmium in 0.1 M PB and distilled water, was added to the wells for 30 minutes on a rotator. The osmium was then removed and the sections were rinsed twice in 0.1 M sodium acetate, with an interval of 5 minutes per rinse. Next, a solution of 1% aqueous uranyl acetate was added for 30 minutes on a rotator. Again, the sections received 2 rinses of the 0.1M sodium acetate for 5 minutes each rinse. They were then dehydrated in increasing concentrations of alcohol: 25% for 2 minutes, 50% for 2 minutes, 70% for 10 minutes, 90% for 20 minutes (2X), and finally 100% for 30 minutes (3X), covered and on a rotator.

Sections were gently transferred to individual aluminium cups filled with fresh Araldite, with each tissue culture well to a different cup, therefore retaining the serial order. These were left overnight, and the following morning the Araldite was discarded and replaced with fresh Araldite. The sections were left for at least 1 hour before embedding on microscope slides, but in some cases, up to 2 hours. Squares of acetate film were placed on glass slides with a drop of Araldite in each corner to hold the sheet in place. Sections were then placed on the slides (6 sections per slide) in serial order. A larger acetate sheet, slightly overlapping the sides of the glass slide, was placed on top of the sections. Then, another glass slide was placed on top of this, followed
by 2 slides acting as weights. These were then placed in a 60 °C oven for approximately 48 hrs.

2.5. Establishing Clusters of Cells and Defining a Clone

Camera lucida drawings using a Zeiss Universal microscope were made of all sections containing stained cells, with the position of each cell marked. These were then viewed section by section on a light box, superimposing each section on the previous one. From this procedure, clusters of closely spaced cells would emerge. Brains with too many labelled clusters (9 clusters or more) were discarded. These clusters of cells were drawn using computer aided serial reconstruction with Neurotrace software (InterAction Co., Cambridge MA). The program provides an accurate synthesis of consecutive sections. A section including the stained cells and major blood vessels was drawn on the computer using a 4X objective lens. With the Neurotrace program it was then possible to superimpose this first drawing onto the subsequent section of the group using blood vessels, pia and white matter to align the sections accurately. This procedure continued, until all sections in the group were included. The computer then produced a final image containing all the drawings and the dispersion of the cells in the group was then measured.

Isolated clusters of cells separated from any other stained cell by at least 500 μm were considered clonally-related. This meant that a group of cells must have been separated from any other cell by at least 500 μm in the
rostral-caudal plane (that is, 5 sections rostral and caudal of the clonal group). In addition, the cells in the group must have been within 500 μm of each other in the lateral dimension.

Different groups of investigators (Price and Thurlow, 1988; Walsh and Cepko, 1988) have defined clusters of clonally related neurons in different ways. Our definition is more restricted in spatial terms than those used by other researchers. In doing so, we believe there is a greater chance of having a cluster of clonally related cells derived from a single progenitor. However, there is always a possibility of excluding a stray cell(s) from a clone, for this reason, we use the term "cluster of clonally-related cells" rather than the term "clone".

Clusters of cells were then analysed in the following way. Every labelled cell was assigned to a cortical area according to Zilles (1985), and to a layer. A few cells could be identified by light microscopic appearance alone (approximately 10%), however, the vast majority required immunocytochemical analysis (90%) to definitively identify phenotype. In this case, immunocytochemistry was performed on cells of each cluster using GABA, glutamate, and/or aspartate anti-sera; ultrathin sections were taken of some of the cells to confirm phenotype. The sections (60-80 nm), were collected on copper grids and stained with 1% uranyl acetate and lead citrate, for 20 mins each. Sections were then viewed under a Philips 300 or 301 electron microscope.
2.6. Immunocytochemistry

Semi-thin sections (0.5 µm thick) of cells in a cluster of clonally related neurons were collected on gelatinized slides. The Araldite resin was removed by a 1:1:1 mixture of absolute alcohol, propylene oxide, and NaOH pellets, leaving the slides in the mixture for 10 min. Slides were then rinsed in absolute alcohol for (2x5 min) then in distilled water for (2x5 min). Sections were then treated with 2% sodium metaperiodate in distilled water for 15 min. They were then rinsed first with distilled water (2x5 min), then with 0.1 M PBS with 0.9% sodium chloride for 5 min. Next, sections were preincubated with 10% normal goat serum (NGS) in PBS containing 0.2% triton-X and 0.01% sodium azide for approximately 2 hrs. Slides were then rinsed in PBS then incubated with the primary antiserum: polyclonal anti-GABA (Sigma; 1:2000 dilution), anti-aspartate or anti-glutamate (Arnell; 1:2500 dilution) in PBS containing 0.2% triton X, and 3-5% NGS, overnight in a moist chamber at room temperature. In the morning slides were rinsed in PBS (3x5 min) then incubated with a 1:200 dilution of biotin-conjugated goat anti-rabbit IgG (Vector Lab) in PBS without triton-X for 2-3 hours. Slides were then rinsed in PBS (3x5 min) then incubated for 2-3 hrs with the ABC complex (Vector) in PBS which had been prepared at least 30 min in advance. Slides were then rinsed in PBS for 5 mins then preincubated in 0.05% diaminobenzidine (DAB) in 0.1 M TRIS buffer (pH 7.2 -7.4) for 10 mins. Sections were then incubated for 15 mins in 0.5% DAB in TRIS and 0.03% H₂O₂ plus 0.03%
Nickel ammonium sulphate facultative. Slides were then rinsed in TRIS buffer for 5 min, then in distilled water (5 min), followed by dehydration and clearing in alcohol and xylene. Slides were left in 50% alcohol for 5 mins (1X), 70% for 5 mins (1X), 90% for 5 mins (1X), 100% for 5 mins (2X), and xylene for 2 mins (2X). They were then wiped dry and mounted in DPX.
3. RESULTS

One hundred and five clusters were examined in the present study using postembedding immunocytochemistry and light and electron microscopy to determine cellular phenotype. Ventricular progenitors were marked with a retroviral vector and the resulting clonally related neurons were examined in adulthood and at two weeks postnatally. The phenotype of every cell in the clusters of clonally related neurons was assessed with either immunocytochemistry (approximately 90% of cells) or light microscopic appearance (approximately 10% of cells). In some cases, electron microscopy was used to confirm phenotype (approximately 20% of cells). Results from the adult brains showed extensive homogeneity of phenotype in clonally related neurons, although the brains injected at E18 showed a few mixed clusters. In contrast, the two week old brains contained many mixed clusters, composed of both pyramidal and nonpyramidal neurons. Finally, a small number of clusters of clonally related cells contained both neurons and glia, these were found in the two week and adult brains.

3.1. Pyramidal Neurons

Fifty one pyramidal clusters of clonally-related neurons, from both adult and two week animals, were examined in this study. Under the light microscope, most β-
galactosidase labelled pyramidal neurons appeared lightly stained with the reaction product forming a pale blue outline surrounding the nucleus (figure 2). In some cases the proximal dendrites were stained although the majority of pyramidal cells showed either no dendritic staining or several spots of product in the proximal apical or basal dendrites (figure 8). Under the electron microscope the reaction product was shown to be associated with the nuclear membrane and sometimes appeared as "puffs" surrounding the nucleus (figures 4, 9). Generally, there was little staining in the nucleoplasm although the surrounding cytoplasm usually contained the β-galactosidase reaction product; this cytoplasmic staining was commonly associated with specific organelles, especially the granular endoplasmic reticulum but also the Golgi apparatus (figure 16). Under the electron microscope all pyramidal neurons displayed exclusively symmetrical axosomatic synapses (figure 3). Pyramidal neurons were either Glu-positive or Asp-positive, but were never GABA immunopositive (figures 28-31). The results show that from animals injected between E14 to E16, clusters of clonally related pyramidal neurons were distributed in all cortical layers, that is from layers II to layer VI; however, in rats injected in the latter stages of neurogenesis, clonal neurons were predominately present in the supragranular layers (figure 54). The size of pyramidal clusters decreased with the age of the embryo at the time of injection. For example, in animals injected at E14, clusters contained an average of 6.3 neurons, but by E19, clusters contained on average 2.8 pyramidal neurons (table 11; figure
3.2. Nonpyramidal Neurons

Thirty six nonpyramidal clusters from both adult and two week old animals were examined in this study. Nonpyramidal neurons generally showed much greater β-galactosidase staining than pyramidal neurons; in most cases, both the nucleus and cytoplasm were filled with reaction product (figure 6). Under the electron microscope, nonpyramidal neurons showed an indented nucleus and an abundance of cytoplasmic organelles characteristic of this cell type (figures 7,11,14,21); as well as the presence of both symmetrical and asymmetrical axosomatic synapses (figures 18-20). Immunocytochemical characterization revealed nonpyramidal neurons to be GABA immunoreactive, rarely immunoreactive for Asp (in less than 5% of cases), but never immunoreactive for Glu (figures 36-40). Results show that following early injections (E14 and E15), nonpyramidal clusters were generally found in the infragranular layers, by E16 clusters were more concentrated in layer IV, while the later injections (E17 to E19), showed clusters predominately in the supragranular layers (figure 54). In addition, cells in a particular nonpyramidal cluster would usually be located in one, or sometimes in two layers; this is in contrast to the pyramidal clusters which up until E16 in corticogenesis, would be spread throughout most cortical layers. Nonpyramidal cluster sizes were also generally smaller than the pyramidal clusters. The average
number of neurons in a nonpyramidal group at E14 was 2.8 (compared to a E14 pyramidal size of 6.3), this was reduced to 2.0 by E19 -the pyramidal size by E19 was 2.8 (table 11; figure 55).

3.3. Mixed Clusters

Eighteen mixed clusters of clonally-related cells were examined in the present study, including clusters from both the adult and two week old brains. In the animals perfused in adulthood, one, E14-injected cluster of clonally related neurons was composed of neurons and glia (table 1), and four, E18-injected clusters were composed of both pyramidal and nonpyramidal neurons (table 6). In the animals perfused at two weeks postnatally, one, E15-injected cluster was composed of neurons and glia, two, E15-injected clusters contained both pyramidal and nonpyramidal neurons (table 8), and ten, E16-injected clusters contained both pyramidal and nonpyramidal neurons (table 10).

3.4. Astrocytes

Astrocytes were found in most of the brains studied, including in our earliest injections at E14. Generally, these cells appeared much smaller than neurons and they varied in staining with the β-galactosidase reaction product. Since neurons were the focus of this study, glial elements were not examined in detail.
In all neurotrace plots as well as light and electron micrographs, the pial surface is towards the top of the page.

**Figure 1** Neurotrace plot of two clonally related β-gal positive pyramidal neurons from an animal injected with virus at E16 and perfused P39. This cluster is from the lateral somatosensory cortex, layer IV.
Figures 2-4  Light and electron micrographs of cell A in figure 1.

Figure 2 Light micrograph of cell A pictured in figure 1. Note the rim of β-galactosidase staining surrounding the nucleus. 400X.

Figure 3 High magnification electron micrograph of a symmetrical axosomatic synapse from figure 4. 42640X.

Figure 4 Electron micrograph of cell A. This cell exhibits sparse β-galactosidase staining, although staining can be detected in the "puffs" surrounding the nucleus (large arrows). Small arrows indicate symmetrical synapses. 8580X.
Figure 5 Neurotrace plot of two clonally related β-gal positive nonpyramidal neurons from an animal injected with virus at E16 and perfused at P39. This cluster is from the motor cortex, layer IV. These cells were all found to be GABA immunoreactive.
Figure 6 Light micrograph of cell B from figure 5. Note the heavy β-galactosidase staining. 450X.

Figure 7 Electron micrograph of cell B. This cell shows ultrastructural features typical of nonpyramidal neurons, including an indented nucleus, and a rich complement of cytoplasmic organelles. Note the reaction product in the nucleus. This cell was found to be GABA immunoreactive. 13200X.
Figure 8-9  Photographs of two neurons from a three cell cluster of β-gal positive pyramidal neurons in the dorsal visual cortex, layer III. These cells illustrate that clonally related pyramidal neurons often have similar β-gal staining patterns.

Figure 8  The first neuron from a three cell cluster displays morphological features typical of pyramidal cells such as a large pale nucleus and a prominent apical dendrite. Note reaction product in the apical dendrit and "puffs" surrounding the nuclear envelope (indicated by arrows). 8580X.
Figure 9 The second cell from a three cell cluster illustrated in figure 8. This pyramidal neuron shares a similar β-gal staining pattern as the cell in figure 8, both are lightly stained, with the reaction product concentrated on the nuclear envelope and little staining on the cytoplasmic organelles. 12720X.
Figures 10-14 Photographs of two cells from a four cell cluster of β-gal positive nonpyramidal neurons from an animal injected with virus at E16 and perfused P39. This cluster is from the motor cortex, layer V. All cells were found to be GABA immunoreactive.

Figure 10 Light micrograph of cell pictured in figure 11. 450X.

Figure 11 Electron micrograph of first cell from a four cell cluster of clonally related neurons. This neuron displays features typical of nonpyramidal neurons, including an indented nucleus and a rich complement of cytoplasmic organelles. The staining was very heavy in this neuron and included both the nucleus and the cytoplasm. Arrows indicate heavy staining in organelles. The cell was found to be GABA immunoreactive. 10890X.
Figure 12 High magnification electron micrograph of an asymmetrical axosomatic synapse, characteristic of nonpyramidal neurons, from the cell in figure 14 taken from a subsequent section. 70000X.

Figure 13 Light micrograph of cell in figure 14. 450X

Figure 14 The second cell from a four cell cluster of clonally related neurons. This nonpyramidal neuron shows a similar β-gal staining pattern as the cell in figure 11. In both cells the nucleus and cytoplasm are heavily labelled with the reaction product. Arrows indicate heavily stained organelles, thick arrows indicate parallel rows of stained cisternae of granular endoplasmic reticulum, characteristic of some nonpyramidal neurons. This cell was found to be GABA immunoreactive. 10890X.
Figure 15 Camera lucida composite drawing of three clonally related β-gal positive pyramidal neurons from an animal injected with virus at E16 and perfused P40. This cluster is from the motor cortex, layer II/III. All three cells were found to be GABA negative. Immunostaining results are shown for cells A and B in figures 22-25.
Figure 16 Electron micrograph of cell A depicted in figure 15. The cell shows morphological features typical of pyramidal neurons such as a round nucleus and an apical dendrite. The β-gal staining pattern was comparatively dark for a pyramidal neuron. Arrows indicate the reaction product staining both cytoplasmic organelles as well as the proximal and distal areas of the apical dendrite. 8580X.
Figure 17 Camera lucida composite drawing of a cluster of two clonally related β-gal positive nonpyramidal neurons from an animal injected with virus at E16 and perfused P35. This cluster is from the motor cortex, layers IV and VI. Both cells were found to be GABA immunoreactive. Electron micrographs of these cells are shown in figures 18-21. Immunostaining results of cell A are shown in figures 26-27.
Figure 18 High magnification electron micrograph of an asymmetrical synapse from cell A, depicted in figures 17 and 20. Nonpyramidal neurons, and not pyramidal neurons, receive asymmetrical axosomatic synapses. Arrow in figure 20 indicates the position of this synapse. 52640X.

Figure 19 High magnification electron micrograph of a symmetrical axosomatic synapse from cell in figure 20 taken from a subsequent section. Both pyramidal and nonpyramidal neurons receive symmetrical axosomatic synapses. 52640X.

Figure 20 Electron micrograph of cell A depicted in figure 17. The cell was found to be GABA immunoreactive. Arrow indicates position of an asymmetrical axosomatic synapse seen at a higher magnification in figure 18. 10400X.
Figure 21 Cell B depicted in figure 17, found to be GABA immunoreactive. This nonpyramidal neuron exhibited extremely heavy β-gal staining which concealed the nuclear envelope and cytoplasmic organelles. 8580X.
Figures 22-27 Light micrographs of immunostaining results of the pyramidal cluster depicted in figure 15, and the nonpyramidal cluster depicted in figure 17. 600X.

Figure 22 Unstained semithin section containing cell A in figure 15 (a pyramidal cluster) illustrating the position of β-gal labelled cell A (indicated by arrowhead) in relation to neighbouring blood vessels (indicated by arrows) and an unlabelled neuron nearby (indicated as 1).

Figure 23 GABA immunostaining of the subsequent semithin section of cell A, indicated by arrowhead. This cell was found to be GABA negative, although the nearby cell (cell 1) was found to be GABA positive.

Figure 24 Unstained semithin section containing cell B in figure 15 (a pyramidal cluster) illustrating the position of β-gal labelled cell B (indicated by arrowhead) in relation to neighbouring blood vessels (indicated by arrows).

Figure 25 GABA immunostaining of the subsequent semithin section of cell B, indicated by arrowhead. This cell was found to be GABA negative.

Figure 26 Unstained semithin section containing cell A in figure 17 (a nonpyramidal cluster) illustrating the position of β-gal labelled cell A (arrowhead) in relation to blood vessels (arrows).

Figure 27 GABA immunostaining of the subsequent semithin section of cell A, indicated by arrowhead. This cell was found to be GABA immunoreactive.
Figures 28-35 Light micrographs of immunostaining results of a two cell pyramidal cluster of clonally related β-gal positive neurons. The animal was injected with virus at E15 and perfused P14. Cell A in this cluster is depicted in figures 28-31, while cell B is depicted in figures 32-35. In all photographs, arrowheads indicate the β-gal labelled cell and asterisks indicate blood vessels. 650X.

Figure 28 Unstained semithin section of cell A from the pyramidal cluster described above. Arrowhead indicates labelled cell, 1 and 2 indicate nearby unlabelled cells.

Figure 29 GABA immunostaining of a subsequent semithin section of cell A depicted in figure 28. This cell was found to be GABA negative.

Figure 30 Glutamate immunostaining of a subsequent semithin section of cell A depicted in figure 28. This cell was found to be Glutamate positive.

Figure 31 Aspartate immunostaining of a subsequent semithin section of cell A depicted in figure 28. This cell was found to be Aspartate positive.
Figures 32-35 Immunostaining results of cell B from the pyramidal cluster described in figures 28-31. Arrowheads indicate the β-gal positive cell and asterisks indicate blood vessels. 650X.

Figure 32 Unstained semithin section of cell B from the pyramidal cluster described in figures 28-31. 1, 2, and 3 indicate nearby cells.

Figure 33 GABA immunostaining of a subsequent semithin section of cell B. This cell was found to be GABA negative.

Figure 34 Glutamate immunostaining of a subsequent semithin section of cell B. This cell was found to be Glutamate positive.

Figure 35 Aspartate immunostaining of a subsequent semithin section of cell B. This cell was found to be Aspartate positive.
Figures 36-40 Light micrographs of immunostaining results of a two cell nonpyramidal cluster of β-gal positive clonally related neurons. The animal was injected with virus at E16 and perfused P56. Figures 36-37 refer to cell A, figures 38-40 refer to cell B. In all photographs arrowheads indicate the β-gal labelled cell and asterisks indicate blood vessels. 650X. 

**Figure 36** Unstained semithin section of cell A from the nonpyramidal cluster described above. 
**Figure 37** GABA immunostaining of a subsequent semithin section of cell A. This cell was found to be GABA positive. 

**Figure 38** Unstained semithin section of cell B from the nonpyramidal cluster described above. 

**Figure 39** GABA immunostaining of a subsequent semithin section of cell B. This cell was found to be GABA positive. 

**Figure 40** Glutamate immunostaining of a subsequent semithin section of cell B. This cell was found to be Glutamate negative.
Figure 41 Light micrographs of immunostaining results of a two cell mixed cluster of β-gal positive neurons. The animal was injected with virus at E16 and perfused P14. In all photographs arrows indicate β-gal labelled cells; and stars, circles, and triangles indicate different blood vessels. 650X.

A-1: unstained semithin section of the first cell in this cluster, arrowhead indicates a nearby β-gal unlabelled cell. A-2: GABA immunostaining of a subsequent semithin section of cell depicted by arrows in A-1. This cell was found to be GABA immunoreactive while the neighbouring cell is GABA negative. A-3: Glutamate immunostaining of a subsequent section. The β-gal labelled cell was found to be Glu negative while the neighbouring cell was Glu positive.

B-1: Unstained semithin section of the second β-gal labelled cell in a mixed cluster.

B-2: GABA immunostaining shows this cell to be GABA negative.

B-3: Glutamate immunostaining shows this cell to be Glu immunoreactive.
3.5. Adult Animals

3.5(a) Injection E14

A total of 9 clusters were studied for this age which included 4 pyramidal clusters containing a total of 25 cells; 4 nonpyramidal clusters which contained a total of 11 cells; and one mixed group containing 4 pyramidal neurons and many astrocytes (table 1). The pyramidal clusters of clonally-related cells contained 4 to 10 neurons with an average of 6.3 neurons per group. These neurons were usually scattered throughout all layers, (II to VI), although in one group all cells were in layer IV. The average radial spread for clusters of clonally related pyramidal cells injected at E14 was 1063μm. Nonpyramidal clusters usually contained 2 cells although one group contained 3, therefore the average number of cells per nonpyramidal group was 2.2. Unlike the pyramidal clusters which usually spanned the depth of the cortex, nonpyramidal groups remained concentrated in one or two layers, especially layers IV to VI. The only mixed clone of this group of animals exceeded the 500μm limit in the rostral-caudal plane (it measured 600μm) but was included because it was located several millimeters from other labelled cells. The group contained 4 pyramidal neurons and a large number of astrocytes (over 20) scattered throughout all layers (II to VI). The radial spread of this cluster was 700μm.
3.5(b) Injection E15

Ten clusters of clonally-related cells were examined from animals injected with retrovirus at E15. They included 8 groups of pyramidal neurons, containing a total of 47 cells, and 2 clusters of nonpyramidal neurons containing a total of 5 cells (table 2). The pyramidal clusters contained 2 to 9 neurons, however, half of these (4 out of 8) were composed of 7 cells. The average number of cells for pyramidal clusters was 5.9. These cells were scattered in layers II through V. The average radial spread per pyramidal cluster was 670µm. The nonpyramidal clusters included one with 2 cells and another with 3 neurons. In the former, both cells were present in layers IV to V, while in the latter, cells were located in layers IV.

3.5(c) Injection E16

Twenty five clusters of clonally-related cells were examined in the rats injected with retrovirus at E16. Twelve of the groups were shown to contain only pyramidal neurons and included a total of 48 cells (table 3). The pyramidal clusters were made up of 2 to 8 cells, the average was 4 cells per group. One third of the clusters were concentrated exclusively in layer IV, while other clusters were scattered in layers III to VI. The average radial spread was 270µm. Interestingly, one group of clonally-related cells containing 2 neurons had a typically nonpyramidal appearance under the electron
microscope, exhibiting an abundance of cytoplasmic organelles and dark staining with the β-galactosidase reaction product. However, immunocytochemical staining showed it to be GABA negative. It was, therefore, considered as a pyramidal cluster although this must be viewed with some uncertainty. It should be mentioned that this was the only incidence where the electron microscopic appearance did not exactly match immunocytochemical results.

A total of 13 nonpyramidal, clonally-related groups were found containing a total of 31 cells (table 4). Most groups contained only 2 cells (9 out of 13 clusters) although 3 groups were composed of 3 cells, and one of 4 cells. The average number of cells per group was 2.4. By far the vast majority of groups were concentrated either exclusively in layer IV, or in layer IV and in one of the adjacent layers (III or V). The average radial spread per cluster was 290μm.

3.5(d) Injection E17

Eleven clusters of clonally related cells were examined in the rats injected with retrovirus at E17. (table 5). This included 9 clusters of pyramidal neurons and 2 clusters of nonpyramidal neurons. The pyramidal clusters contained a total of 25 cells with 2 to 4 cells per cluster. The average number of cells per cluster was 2.9. Cells were mainly concentrated in cortical layers II to III. The average radial spread for E17 injected pyramidal clonally-related clusters was 166μm. Two nonpyramidal groups were studied, each composed of 2 cells.
which were concentrated in layers II and III.

3.5(e) Injection E18

Eleven clusters of clonally related cells were examined in the rats injected with retrovirus at E18. These consisted of 3 clusters composed of pyramidal neurons, 4 clusters of nonpyramidal neurons, and 4 mixed clusters containing both pyramidal and nonpyramidal neurons (table 6). The 3 pyramidal clusters included a total of 9 cells, with 2 to 4 neurons per cluster. The average number of cells per group was 3, which occupied layers II to III. The average radial spread for this age was 133μm. Four nonpyramidal clusters were examined, with a total of 8 cells. Each nonpyramidal group contained 2 neurons. Both nonpyramidal clusters were in layers II and III with an average radial spread of 36μm.

The rats injected with retrovirus at E18 and perfused as adults also contained 4 mixed clusters containing a total of 11 cells. Three of the groups were composed of 3 cells while the final group was composed of 2 cells, this made the average number of cells per group 2.8. Unlike the nonpyramidal clonally-related cells which almost always occupied a single layer or two adjacent layers, the mixed clusters were usually spread over 3 layers (II to IV) or in one 2-cell cluster, layers II and VI. There was no apparent pattern in cell type in the mixed groups, two groups contained 2 pyramidal neurons and 1 nonpyramidal neuron; one group contained 1 pyramidal neuron and 2 nonpyramidal neurons; and one cluster was
composed of 1 pyramidal and 1 nonpyramidal neuron.

3.5(f) Injection E19

Seven clusters of clonally related cells were examined in the rats injected with retrovirus at E19. Five of the groups were composed of pyramidal neurons which included a total of 14 cells (table 7). The pyramidal clusters contained 2 to 4 neurons per group; the average was 2.8. Pyramidal clusters were found in layer II, or in layers II and III. The average radial spread per cluster was 100\(\mu\)m. Two clusters of nonpyramidal neurons were examined, each containing 2 neurons. One group was found in layers II to III while the other was in layer VI.

3.5(g) Injection E20

No neuronal clusters of two or more cells were found for this age of injection. Instead, brains contained some scattered isolated neurons but no clusters. However, many glial cells were found, most of which appeared astrocytic.

3.6. Two Week Old Animals

Thirty four clusters of clonally related neurons were examined at two weeks postnatally. Some clusters were found to contain exclusively pyramidal neurons, some exclusively nonpyramidal neurons, other clusters contained a mixture of
both pyramidal and nonpyramidal neurons, and in two of the clusters, one of the cells was reactive for both GABA and glutamate.

3.6(a) Injection E15

Animals injected at E15 with retrovirus and perfused postnatally at two weeks after birth produced a variety of clusters of clonally-related cells (table 8). Two clusters of pyramidal neurons, 2 clusters of nonpyramidal neurons, and 3 clusters of mixed cells were found for this age of injection. The pyramidal clusters contained a total of 7 cells, on average 3.5 cells per group which were scattered in layers II to V. The nonpyramidal clusters each contained 2 cells which were distributed in layers II to VI. Finally, the 3 mixed clusters contained a total of 12 neurons. One mixed group was composed of 3 pyramidal neurons and a number of astrocytes in layers II to IV. This group was over 500μm in dimension but was included because it was located several millimeters from other labelled cells. Another mixed cluster contained 3 cells, two nonpyramidal neurons and one pyramidal neuron which were located in layers II to IV. This group was also over 500μm in dimension but was included because it too was isolated from other labelled cells. The fourth mixed cluster contained a total of 6 cells, 5 pyramidal neurons and 1 nonpyramidal neuron which were located in layers II to VI.

3.6(b) Injection E16
Twenty seven clusters of clonally related cells were examined in animals injected with retrovirus at E16 and perfused two weeks postnatally. The E16 injections produced three types of clusters, those that contained pyramidal neurons, those that contained nonpyramidal neurons, and those that contained a mixture of pyramidal and nonpyramidal cell types. The pyramidal clusters contained a total of 27 cells with an average of 3.4 cells per group (table 9). The majority of cells were found in layers II and III, but some were present in layers IV and V. The nonpyramidal clusters contained a total of 16 cells with an average of 2.3 neurons per cluster (table 9). Similar to the pyramidal groups, the nonpyramidal clusters were found mainly in layers II and III, although cells were also found in layers V and VI. This age group also included 10 mixed clusters containing a total of 26 neurons (table 10). The average number of cells per group was 2.6, these cells were found throughout the cortex in layers II to VI. The vast majority of the clusters (6) contained 1 pyramidal neuron and 1 nonpyramidal neuron. Two clusters contained 2 pyramidal neurons and 1 nonpyramidal neuron; one cluster contained 2 pyramidal neurons and 2 nonpyramidal neurons; and one cluster contained 3 nonpyramidal neurons and 1 pyramidal neuron. In addition to the other groups, two clusters were found to contain bi-reactive neurons. Both of these groups contained a neuron which was both GABA and Glu immunoreactive.
<table>
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<th>Layer</th>
<th>Cell identification</th>
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<tr>
<td>E14-1</td>
<td>PYRAMIDAL</td>
<td>4</td>
<td>IV</td>
<td>4 GABA- 4 GLU+</td>
</tr>
<tr>
<td>E14-2</td>
<td>PYRAMIDAL</td>
<td>10</td>
<td>II-VI</td>
<td>2 GABA-, 8 LM:pyr *Conf. EM</td>
</tr>
<tr>
<td>E14-3</td>
<td>PYRAMIDAL</td>
<td>5</td>
<td>II-V</td>
<td>5 GABA- 5 Glu+</td>
</tr>
<tr>
<td>E14-4</td>
<td>PYRAMIDAL</td>
<td>6</td>
<td>II-VI</td>
<td>5 GABA- 5 Glu+, 1 EM:pyr</td>
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<tr>
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<td>IV-V</td>
<td>2 GABA+ 2 Asp- 1 Glu+, 1 Glu-</td>
</tr>
<tr>
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<td>2</td>
<td>V</td>
<td>2 GABA+ 2 Glu- 2 Asp-</td>
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<td>V</td>
<td>3 GABA+ 3 Glu- 3 Asp-</td>
</tr>
<tr>
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<td>2</td>
<td>VI</td>
<td>2 GABA+</td>
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<tr>
<td>E14-9</td>
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<td>4 Pyr</td>
<td>II-VI</td>
<td>All LM/ *over 500μm</td>
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<tr>
<td></td>
<td></td>
<td>+ astro</td>
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<td>II-V</td>
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<td>III-V</td>
<td>7 EM:pyr</td>
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<td>II</td>
<td>3 GABA- 3 Glu+ 1 Asp+ 2 Asp-</td>
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<td>7 GABA-</td>
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<td>II-V</td>
<td>7 GABA- 7 Glu+ 6 Asp- 1 Asp+</td>
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<tr>
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<td>II-IV</td>
<td>5 GABA- 5 GLU+</td>
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<tr>
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<td>7</td>
<td>II-V</td>
<td>6 GABA- 6 ASP+, 1 EM:pyr * Over 500μ</td>
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<tr>
<td>E15-8</td>
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<td>IV-V</td>
<td>2 GABA- 2 GLU+ 1 Asp+ 1 Asp-</td>
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<p>| E15-9 | NonPYR | 3 | IV | 3 GABA+ |
| E15-10| NonPYR | 2 | IV-V | 2 GABA+ |</p>
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<th>Layer</th>
<th>Cell identification</th>
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<td>IV</td>
<td>3 EM:pyr</td>
</tr>
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<td>PYRAMIDAL</td>
<td>6</td>
<td>II-IV</td>
<td>6 EM:pyr</td>
</tr>
<tr>
<td>E16-3</td>
<td>PYRAMIDAL</td>
<td>4</td>
<td>IV-VI</td>
<td>2 EM:pyr, 2 LM:pyr</td>
</tr>
<tr>
<td>E16-4</td>
<td>PYRAMIDAL</td>
<td>3</td>
<td>IV</td>
<td>3 GABA-</td>
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<tr>
<td>E16-5</td>
<td>PYRAMIDAL</td>
<td>2</td>
<td>IV</td>
<td>1 EM:pyr, 1 LM:pyr</td>
</tr>
<tr>
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<td>2</td>
<td>III</td>
<td>2 GABA-</td>
</tr>
<tr>
<td>E16-7</td>
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<td>2</td>
<td>VI</td>
<td>2 GABA- 2 Glu+ 2 Asp+</td>
</tr>
<tr>
<td>E16-8</td>
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<td>7</td>
<td>IV-VI</td>
<td>7 GABA- 7 Glu+ 7 Asp+</td>
</tr>
<tr>
<td>E16-9</td>
<td>PYRAMIDAL</td>
<td>3</td>
<td>V-VI</td>
<td>3 GABA- 3 Glu+</td>
</tr>
<tr>
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<td>5 ASP+ Glu+, 3 Asp+ 3 Glu-</td>
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<td>E16-11</td>
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<td>2 Glu+</td>
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<td>III-V</td>
<td>6 Glu+ 2 Asp+, 4 Asp-</td>
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<td>MEAN</td>
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### TABLE 4

Clusters of Nonpyramidal Neurons

Injected E16-Perfused Adulthood

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<td>II-IV</td>
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<td>IV</td>
<td>2 GABA+</td>
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<tr>
<td>E16-15</td>
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<td>2</td>
<td>IV+VI</td>
<td>2 GABA+</td>
</tr>
<tr>
<td>E16-16</td>
<td>NonPyr</td>
<td>4</td>
<td>IV+V</td>
<td>4 GABA+</td>
</tr>
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<td>NonPyr</td>
<td>3</td>
<td>IV</td>
<td>3 GABA+</td>
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<tr>
<td>E16-18</td>
<td>NonPyr</td>
<td>3</td>
<td>IV-V</td>
<td>3 GABA+ *Over 500μm</td>
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<td>NonPyr</td>
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<td>IV</td>
<td>2 GABA+</td>
</tr>
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<td>E16-20</td>
<td>NonPyr</td>
<td>2</td>
<td>IV</td>
<td>2 GABA+</td>
</tr>
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<td>E16-21</td>
<td>NonPyr</td>
<td>2</td>
<td>III-IV</td>
<td>2 GABA+</td>
</tr>
<tr>
<td>E16-22</td>
<td>NonPyr</td>
<td>2</td>
<td>IV-V</td>
<td>2 GABA+</td>
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<tr>
<td>E16-23</td>
<td>NonPyr</td>
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<td>III-IV</td>
<td>2 GABA+</td>
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<tr>
<td>E16-24</td>
<td>NonPyr</td>
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<td>II-III</td>
<td>2 GABA+</td>
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<tr>
<td>E16-25</td>
<td>NonPyr</td>
<td>2</td>
<td>II-III</td>
<td>2 GABA+</td>
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TOTAL NonPyr 31

MEAN NonPyr 2.4
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<th>Cell identification</th>
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<tr>
<td>E17-1</td>
<td>PYRAMIDAL</td>
<td>2</td>
<td>III</td>
<td>2 Asp+ 2 Glu-</td>
</tr>
<tr>
<td>E17-2</td>
<td>PYRAMIDAL</td>
<td>4</td>
<td>II-VI</td>
<td>4 Glu+ 2 Asp+ 2 Asp-</td>
</tr>
<tr>
<td>E17-3</td>
<td>PYRAMIDAL</td>
<td>2</td>
<td>IV</td>
<td>2 Glu+ 2 Asp+</td>
</tr>
<tr>
<td>E17-4</td>
<td>PYRAMIDAL</td>
<td>3</td>
<td>II</td>
<td>3 LM:pyr</td>
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<tr>
<td>E17-5</td>
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<td>4</td>
<td>II-III</td>
<td>4 Glu+ 2 Asp+ 2 Asp-</td>
</tr>
<tr>
<td>E17-6</td>
<td>PYRAMIDAL</td>
<td>2</td>
<td>II-III</td>
<td>1 Glu+, 1 LM:pyr</td>
</tr>
<tr>
<td>E17-7</td>
<td>PYRAMIDAL</td>
<td>3</td>
<td>IV</td>
<td>3 GABA-</td>
</tr>
<tr>
<td>E17-8</td>
<td>PYRAMIDAL</td>
<td>3</td>
<td>III</td>
<td>3 GABA- *over500μm</td>
</tr>
<tr>
<td>E17-9</td>
<td>PYRAMIDAL</td>
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<td>II-III</td>
<td>2 GABA- 2 Glu+</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>PYRAMIDAL</td>
<td>25</td>
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</tr>
<tr>
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<td>PYRAMIDAL</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>E17-10</td>
<td>NonPYR</td>
<td>2</td>
<td>II-III</td>
<td>2 GABA+</td>
</tr>
<tr>
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<td>2</td>
<td>II</td>
<td>2 GABA+</td>
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**TABLE 6**
Clusters injected E18-Perfused Adulthood

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<th>CLONE</th>
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<th>No. cells</th>
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<th>Cell identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>E18-1</td>
<td>PYRAMIDAL</td>
<td>3</td>
<td>III-IV</td>
<td>3 LM:pyr</td>
</tr>
<tr>
<td>E18-2</td>
<td>PYRAMIDAL</td>
<td>4</td>
<td>II-III</td>
<td>1 GABA- 3 LM:pyr</td>
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<tr>
<td>E18-3</td>
<td>PYRAMIDAL</td>
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<td>II-III</td>
<td>2 LM:pyr</td>
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</tbody>
</table>

**TOTAL PYRAMIDAL** 9
**MEAN PYRAMIDAL** 3

| E18-4 | NonPYR   | 2         | II-III| 2 LM:nonPYR        |
| E18-5 | NonPYR   | 2         | II-III| 2 GABA+            |
| E18-6 | NonPYR   | 2         | II-III| 2 LM:nonPYR        |
| E18-7 | NonPYR   | 2         | II-III| 2 LM:nonPYR        |

**TOTAL NonPyr** 8
**MEAN NonPyr** 2.0

| E18-8 | MIX       | 3         | II-III| 2 GABA-, 1 GABA+   |
| E18-9 | MIX       | 3         | II-IV | 1 GABA+, 2 LM:pyr  |
| E18-10|MIX        | 3         | II-IV | 2 GABA+, 1 GABA-   |
| E18-11|MIX        | 2         | II+VI | 1 GABA+, 1 GABA-   |

**TOTAL Mix** 11
**MEAN Mix** 2.8
<table>
<thead>
<tr>
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<th>Layer</th>
<th>Cell identification</th>
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<tbody>
<tr>
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<td>PYRAMIDAL</td>
<td>4</td>
<td>II-III</td>
<td>4 Glu-Asp+</td>
</tr>
<tr>
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<td>PYRAMIDAL</td>
<td>3</td>
<td>II-III</td>
<td>3 LM: pyr</td>
</tr>
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<td>E19-3</td>
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<td>II</td>
<td>2 LM: pyr</td>
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<tr>
<td>E19-4</td>
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<td>3</td>
<td>II</td>
<td>3 LM: pyr</td>
</tr>
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<td>PYRAMIDAL</td>
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<td>II-III</td>
<td>2 LM: pyr</td>
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</table>

<p>| E19-6  | NonPyr    | 2         | II-III| 2 LM: nonpyr        |
| E19-7  | NonPyr    | 2         | VI    | 2 GABA+             |</p>
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<td>II-IV</td>
<td>4 LM:pyr, 1 GABA-</td>
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<tr>
<td>-2</td>
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<td>II</td>
<td>2 GABA+ 2 Glu-</td>
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<td>3 LM:pyr, many astrocy * over 500μm</td>
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<td>-6</td>
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<td>II-IV</td>
<td>2 GABA+ 2 Glu- 2 Asp- 1 GABA- 1 Glu+ 1 Asp+ *over 500μm</td>
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<td>II-VI</td>
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<td>V</td>
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<td>II-IV</td>
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</tr>
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<p>| E16-26      | Bi-reactive | 4 | II-V | 1 GABA+ Glu+ |
|            |             |   |      | 3 GABA- Glu+ |
| -27        | Bi-reactive | 2 | V-VI | 1 GABA+ Glu+ |
|            |             |   |      | 1 GABA+ Glu- |</p>
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<th>Clusters from Animals Perfused 2-Week</th>
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Figures 42-53 Neurotrace plots of clusters of clonally related cells from animals injected with virus at various stages of gestation. In all plots the scale bar equals 1mm.

Figure 42 Plot of 6 clonally related β-gal positive pyramidal neurons from the dorsomedial motor cortex, layers II–VI. The animal was injected with virus at E14 and perfused P47. At this age, cells in pyramidal clusters are scattered throughout all layers of the cortex.
**Figure 43** Plot of 3 clonally related nonpyramidal neurons from the somatosensory cortex, layer VI. The animal was injected with virus at E14 and perfused P47. In contrast to pyramidal neurons, nonpyramidal cells at this age tend to be found in the infragranular layers.
Figure 44 Plot of 6 clonally related pyramidal neurons from the dorsal somatosensory cortex, layers II-VI. The animal was injected with virus at E15 and perfused P14. Cells are still scattered throughout all cortical layers.
Figure 45 Plot of 2 clonally related nonpyramidal neurons from the frontoparietal somatosensory cortex, layers IV and VI. The animal was injected with virus at E15 and perfused P13. At E15, clonally related nonpyramidal neurons still tend to be scattered in the lower layers, although in some cases, a neuron may migrate to an upper layer.
Figure 46 Plot of 6 clonally related pyramidal neurons from the somatosensory cortex, layers II-V. The animal was injected with virus at E16 and perfused P13. At E16, clonally related pyramidal neurons still tend to be scattered through many layers.
Figure 47 Plot of 3 clonally related nonpyramidal neurons from the visual cortex, layer II-IV. The animal was injected with virus at E16 and perfused P39. At E16-E17, clonally related nonpyramidal neurons tend to be concentrated in the supragranular layers.
Figure 48 Plot of 4 clonally related pyramidal neurons from the dorsal somatosensory cortex, layer II. The animal was injected with virus at E17 and perfused P35. By this later stage of development, clonally related pyramidal neurons tend to be found in the supragranular layers, as are the nonpyramidal neurons.
Figure 49 Plot of 2 clonally related nonpyramidal neurons from the visual cortex, layer II/III. The animal was injected with virus at E17 and perfused P27.
Figure 50 Plot of 3 clonally related pyramidal neurons from the frontoparietal somatosensory cortex, layers III-IV. The animal was injected with virus E18 and perfused P55.
Figure 51 Plot of 2 clonally related nonpyramidal neurons from the lateral somatosensory cortex, layer II. The animal was injected with virus at E18 and perfused P56.
Figure 52 Plot of 3 clonally related pyramidal neurons from the frontoparietal somatosensory cortex, layer II. The animal was injected with virus at E19 and perfused P94.
Figure 53 Plot of 2 clonally related nonpyramidal neurons from the lateral somatosensory cortex, layers II/III.
**Figure 54** Diagram illustrating the differing lamination pattern of clonally related pyramidal and nonpyramidal neurons. Clusters of pyramidal neurons resulting from viral injection at E14 to E16 populate virtually all layers while clusters of nonpyramidal neurons tend to be concentrated in the infragranular layers. However, in animals injected with virus at E17 or later, both pyramidal and nonpyramidal neurons are usually found in the supragranular layers.
LAMINATION PATTERN OF PYRAMIDAL AND NON PYRAMIDAL NEURONS

INJECTION DATE

P = PYRAMIDAL    NP = NON-PYRAMIDAL
Figure 55 Diagram illustrating the differing cluster size of clonally related pyramidal and nonpyramidal neurons. Average pyramidal cluster size typically exceeds nonpyramidal cluster size throughout development, although this difference gradually decreases over time. The average number of cells comprising a nonpyramidal cluster tends to remain steady during development, usually containing 2-3 neurons.
NUMBER OF CELLS PER INJECTION DATE

NUMBER OF CELLS

E14  E15  E16  E17  E18  E19

INJECTION DATE
PYR.  NON-PYR.
4. DISCUSSION

The present study marked progenitor cells of the rat telencephalic ventricular zone with a retroviral vector during the period of cortical neurogenesis. Resulting clusters of clonally related cells were then examined in adulthood and then also at two weeks postnatally. The clonally related cells were analysed with immunocytochemistry, and light and electron microscopy to determine their phenotype. The results confirmed homogeneity of clonally-related cells in adult animals (Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994), however, the clusters of neurons derived from the animals perfused at two weeks postnatally showed significant heterogeneity of phenotype. Although many clusters in these immature brains contained only one cell type, a substantial number of clusters of clonally-related cells contained both pyramidal and nonpyramidal neurons. In addition, the results illustrated the difference in the genesis of the two types of cortical neuron; pyramidal and nonpyramidal neurons have their own unique patterns of lamination as well as differing in number of cells per cluster.

4.1. Defining Clusters of Clonally Related Neurons

We defined our clusters of clonally related cells as a discrete group of β-gal positive cells separated from any other labelled cells by at least 500μm in the rostral/caudal
plane. In addition, all the cells in the cluster must have been within 500μm of each other in the lateral plane.

4.1.1. Possibility of Widespread Migration

The recent studies of Walsh and Cepko (1992, 1993) which utilized a library of viral constructs, have cast doubt on the definition of clonally related neurons in some lineage studies. In these experiments, a library of 100 different viral constructs, each containing its own individual DNA-insert tag, were injected into the ventricles of E14, E15 and E17 rats; animals were then sacrificed 3, 6, 9 or 10 days later and the brains stained for β-galactosidase. Pieces of tissue containing just one cell were analysed using polymerase chain reaction (PCR) and the individual viral constructs were identified on the basis of size of the PCR product and the restriction enzyme digest pattern. The surprising finding was that while some of the cells in a presumed clone remained in a tight cluster (within 1.5mm of each other), a large number of the cells in a clone were non-clustered, with the cells scattered 5mm or more apart. This report of widespread migration of some members of a clone led to the identification of two possible sources of error in lineage studies, first, "splitting error" (Walsh and Cepko, 1992) where two spatially distinct clusters of retrovirally labelled cells are actually members of the same clone and second, "lumping errors" (Walsh and Cepko, 1992) where one spatially distinct cluster of retrovirally labelled cells actually contains two clones.
Since the publication of this work, however, a number of questions have arisen concerning these results. These include criticisms for the general experimental design of the studies (Kirkwood et al., 1992; Grove et al., 1993; Luskin et al., 1993) as well for the DNA amplification procedure used on β-galactosidase positive cells (Halliday and Cepko, 1992; Walsh and Cepko, 1992, 1993). However, the most important cause for concern is the possibility of multiple infection, that is, the possibility that the same viral tag could have infected twice in the same brain. Computer simulations have estimated the probability of this occurring to be 3% in a brain with 3 tags, however, in a brain labelled with 11 tags, the probability increases to 50% (Walsh and Cepko, 1992). If multiple infections were occurring, it would make clones appear larger and more widespread than they actually were. Another problem is the authors' assumption that the initial cocktail of viral constructs contained an equal representation of all the tags. Studies on the distribution of tags recovered from labelled brains showed that in 235 of the clones, only 79 of the original construct tags appeared (Walsh and Cepko, 1992); this suggests that all virions may not have been equally represented in the original viral titres for injection. This would have an important implication for this study; if the initial cocktail of viral particles were not equally represented, the chance of finding brains with multiple infections by the same tag would be increased. Studies where the titre of each tag is measured before injection will be needed to resolve this question.
Other studies have emphasized the radial, and not the widespread nature of clonal migration. These include studies by Nakatsuji (Nakatsuji et al., 1991) where stem cell lines in chimeric mice expressed radial patches of β-galactosidase stained neurons, suggesting that migration is predominately radial in the rodent cortex. Experiments by Tan and Breen (1993) show similar results. These studies used transgenic mice for marking progenitor cells with β-galactosidase, then using X-chromosome inactivation to turn off the gene in half of the cells. Results in adult neocortex showed that radial bands dominated, suggesting that radial migration is predominant, although some tangential dispersion was also evident. Studies by Fishell et al. (1993) which fluorescently marked mouse telencephalic ventricular precursors and followed their migration over short time periods, found that most cells do not move far from the radial plane of migration. It seems probable, therefore, that Walsh and Cepko have overestimated the occurrence of tangential migration and that radial migration is the primary mode of clonal dispersion in the cortex.

It is certain, however, that tangential dispersion does play at least a minor role in cortical migration. Tangential migration is thought to be a passive displacement of cells caused by the developing lateral cerebral cortex (Moore and Price, 1992). At early stages of cortical development, the ventricular zone is directly beneath the cortical plate so the first neurons migrate in a nearly strictly radial pathway. As the lateral cortex develops, the ventricular zone and the
lateral cortical plate become progressively separated and distorted (Smart and McSherry, 1982); this may explain why in medial and dorsal cortical areas migration appears to be predominately radial, while in lateral cortical areas some tangential migration is evident (Austin and Cepko, 1990; Misson et al., 1991). Of course, other factors may be guiding this dispersion such as cell adhesion molecules or gradients of neurotrophic factors, although this would not explain why these processes were more active in the lateral cortex rather than the medial cortex. It seems more probable, therefore, that in cases where "splitting error" does occur, the loss of a neuron from a cluster of clonally related cells is more likely to be a random loss, and thus of slightly less significance than a neuron lost by more non-random factors.

4.1.2. Statistical Studies

A criticism of lineage studies in general, has been whether each cluster of clonally related cells is a complete clone, that is, whether all the progeny of the infected ventricular progenitor are represented in the cluster of clonally-related cells. The other side of the question is whether all cells in a presumed clone actually stem from the same originally infected progenitor cell or whether cells from another progenitors have migrated into a preexisting clone. The possibility of "lumping error" (Walsh and Cepko, 1992), defined as including a "stray" labelled cell from a different progenitor in a single cluster, has been addressed in our
study by including only hemispheres containing a minimum (one to eight) widely spaced clusters of β-galactosidase positive cells. Statistical studies have shown that in brains with low numbers of infections, and therefore with a low number of clusters, the likelihood of lumping error greatly decreases (Grove et al., 1992). Statistical tests have also been utilized to evaluate the probability of randomly superimposing progeny of two or more infected precursors into a single clone. These studies have been performed using a binomial distribution in both the hippocampus (Grove et al., 1992) and the cerebral cortex (Luskin et al., 1993; Mione, et al., 1994). For example, in a recently published study by Mione et al. (1994) in our laboratory, the probability of not superimposing randomly dispersed β-galactosidase positive cells into preexisting clusters of clonally related cells was assessed by applying the following formula from (Mione, et al., 1994):

\[ P = 1 - [(V_t - V_{xc})/V_t]^n \]

where \( P \) was the probability of superimposition on \( n \) randomly dispersed cells and \( x \) preexisting clusters, \( V_t \) was the volume of the cerebral cortex in one hemisphere of an adult rat, \( V_{xc} \) was the volume of cortex occupied by \( x \) clusters of β-galactosidase positive neurons. Cortical volume in one hemisphere was taken to be 125mm\(^3\), which is half the volume of the entire cortex as calculated by Korbo et al., (1990). The average volume of each cluster was calculated to be 0.1308\(^3\). To evaluate \( n \), the study considered that 50% of all β-galactosidase labelled neurons within a cluster were actually
unrelated to the other neurons within the cluster and had randomly migrated there; this amount was a generous overestimation of the proportion of randomly dispersed cells since, in studies by Walsh and Cepko (1992), 25% of all clones were estimated to be not clustered. Using the criteria described above, the study found that in 45% of experiments the probability of superimposing randomly dispersed neurons and preexisting clusters to be less than 1%; in 36% of experiments the probability was less than 5%; in only 2 out of 22 experiments was the probability over 10%. In concurrence with other studies, the probability of superimposition rose with the number of clusters and with the number of cells per cluster. Therefore, by keeping the viral titres and the number of clusters low in each hemisphere, the chance of superimposing randomly distributed cells and true clusters of clonally related cells is minimal, especially in hemispheres with only a few clusters (see Mione et al., 1994).

4.2. Brains Analysed at Two Weeks Postnatally

One of the findings of the present study was that the brains analysed at two weeks postnatally had a substantial number of mixed clusters compared with the brains analysed in adulthood. The mature brains, analysed at one to three months postnatally, contained few mixed clusters. In the adult animals, which included brains infected between E14 to E19, sixty eight of the clusters of clonally-related cells contained cells of only one phenotype, either all pyramidal or
all nonpyramidal neurons. However, there were two exceptions. The first was a cluster injected with retrovirus at E14 which contained several pyramidal neurons and a number of astrocytes. The second type of mixed cluster were the four, E18-injected clusters, which contained both pyramidal and nonpyramidal neurons. These four clusters were interesting because of their rarity, only four mixed clusters in comparison to the 68 single-cell type clusters. It should be noted, however, that these 4 clusters were all from the same brain and the mixing could possibly be due to an abnormality of that particular brain, although superficially, no abnormalities were apparent. Further research on E18-infected adult animals is necessary to verify this finding.

4.2.1. Possible explanations of results

The animals perfused two weeks postnatally showed a substantial number of mixed clusters. Out of a total of 25 clusters of clonally related cells injected at E16, 15 of these were composed of a single cell type and 10 were composed of a mixture of pyramidal and nonpyramidal neurons, therefore, nearly half of the clusters were mixed for this age. The same trend could be seen in the E15-injected two week old animals. While 4 clusters were composed of a single cell type, two clusters were composed of both pyramidal and nonpyramidal neurons, and one group contained neurons and glia. The two most likely explanations are, first, that there are at least two types of cortical progenitor in the rodent, one committed
to producing only one type of neuron, either exclusively pyramidal neurons or exclusively nonpyramidal neurons; another progenitor could be multipotent producing a variety of cortical neuronal phenotypes. The second possibility is that only multipotent progenitors exist, but that the uniformity seen in some clusters is derived from cell death or cell transformation. These possibilities are considered.

A possible explanation for the homogeneous clusters observed in the two week old animals is that there may be a subpopulation of fated progenitors in rat cortical development. In the animals injected early in corticogenesis on E15 and E16, the majority of clusters of clonally-related cells contained a single cell type; 19 clusters were composed of all pyramidal or all nonpyramidal neurons, while 13 of these were mixed. If clonally-derived cells were all multipotent one would expect to see more mixed clusters at this early stage of development. Instead, over half were homogeneous, which may indicate that a subset of progenitors in the rat ventricular zone may be fated to produce only one neuronal phenotype.

4.2.2. Tissue culture experiments

Tissue culture experiments have proven useful in testing the validity of the fated progenitor hypothesis. Studies by Buse (1990) involved culturing neuroblasts from the cerebral cortices of embryonic mice (E10-E16) in one of three culture conditions. Some were cultured as whole mount preparations
which were cultured in plasma clot and serum containing medium; in these preparations the cell-cell connections were preserved. Other neuroblasts were grown in suspension cultures where cell contacts were initially interrupted by the dissociation procedure, however, contacts were re-established when the cells re-aggregated. In the final group, neuroblasts were cultured without cell to cell interactions in single-cell cultures. The potential to differentiate into GABAergic neurons was then studied for the three different conditions. Results showed that all three culture preparations produced GABAergic neurons, in some preparations from tissue as early as embryonic day 11.5. These results indicate that, at least in a subpopulation of cells, the GABAergic phenotype is expressed irrespective of whether cell to cell contacts are present. Other studies using GABA immunohistochemistry on dissociated cell culture in serum-free, conditioned media attained similar results (Stichel and Muller, 1991). This study utilized E15 rat cerebral cortex and found GABA-like immunoreactivity in a subpopulation of neurons from the first day of culture. In addition, during the first week the number of GABAergic neurons reached mature values of 10.5 to 12.6% of all neurons; this is a similar value of GABAergic neurons in vivo. The tissue culture results suggest that at least some neuroblasts in the rodent cerebral cortex are not dependent on cell-to-cell contacts and follow an intrinsic genetic program for the induction of the GABAergic phenotype.

4.2.3. Possibility of multipotential progenitors
On the other hand, the 13 mixed clusters of two week old animals also suggest that a multipotential progenitor may exist which is capable of giving rise to clusters of phenotypically heterogenous neurons. Other studies from a variety of regions in the central nervous system also support this finding. In the vertebrate retina, lineage studies have shown that an infected progenitor is capable of producing a wide variety of different combinations of retinal cell types (Turner and Cepko, 1987; Turner et al., 1990). The same is true in the chick optic tectum, where a single progenitor is capable of producing neurons as well as several types of glia (Gray et al., 1988; Galileo et al., 1990; Gray and Sanes, 1992). However, studies on neuronal lineages in the cerebral cortex have not been conclusive. Some authors have identified neurons purely on the basis of light microscope appearance and have reported that a single progenitor is capable of producing both pyramidal and nonpyramidal neurons (Price and Thurlow, 1988; Walsh and Cepko, 1988). The validity of this approach has been criticized because of correlative light and electron microscopic studies which showed it is virtually impossible to discriminate pyramidal and nonpyramidal neurons by light microscope alone (Peters and Kara, 1985a,b). Other neuronal lineage studies using electron microscopic ultrastructural analysis have found homogeneity for pyramidal and nonpyramidal neurons in clusters of clonally related cells (Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994). However, these studies analysed the brains in adulthood (1 to 3 months postnatally), so other factors may have influenced clonal
development. Therefore, the present study which has analysed the brains early in development (2 weeks postnatally) as well as in adulthood, and has also definitively identified neurons both from an ultrastructural and immunocytochemical standpoint, has supplied the most convincing evidence for multipotent progenitors.

An important question is whether progenitors are fated by their intrinsic genetic program to produce either homogeneous or heterogeneous clusters, or are the progeny of some of these cells initially uncommitted and their fate decided by other extracellular/epigenetic factors? At present, this question in cortical development has been extremely difficult to resolve. This is because cell phenotype is probably determined from both intrinsic cell programs and also from environmental signals in the ventricle and/or in the extracellular matrix (Banker and Waxman, 1988; Lasek, 1988; McConnell, 1988). Also the interaction between the intrinsic program inherent in a cell and outside environmental influences are difficult to separate since neither can be visualized without the other.

Nevertheless, some research has implied that both mechanisms may be in operation. For example, many studies analysing the development of nonpyramidal neurons have reported the delayed and prolonged maturation of GABA immunoreactivity in the cortex (Miller, 1986b, 1988; Lauder et al., 1986). Although the first GABAergic cells in the cortical plate can be detected in the embryonic and perinatal period, it is not until later postnatal stages that peak densities of
GABAergic expression occur. Recent studies have demonstrated an elaborate GABAergic system at prenatal stages of development, however, the maturation of GABA immunoreactive elements in postnatal development was not addressed in these studies; therefore, later stages of GABAergic expression were missed (Van Eden et al., 1989; Cobas et al., 1991). The delayed maturation of nonpyramidal neurons has also been reported in Golgi and electron microscopic analyses (Parnavelas and Lieberman, 1979; Miller and Peters, 1981; Blue and Parnavelas, 1983a,b; Miller, 1986a, 1988). Recent GABA-immunoreactive studies in the mouse neocortex (Del Rio et al., 1992) have found two phases of GABAergic expression in the cortical plate. In the first phase, certain cells express GABA shortly after they leave the proliferative zone, the second phase of GABAergic expression occurs in the later postnatal stages. The authors interpretation is that there are at least two progenitor populations of GABAergic neurons. One subset of these progenitors are committed to GABAergic expression very early in development (2-3 days after becoming postmitotic); these cells are probably more reliant on genetic factors due to the immaturity of the cortical plate and the lack of synaptic contacts at that time (Konig et al., 1975; Rickman, et al., 1977). The second population of neurons which expresses GABA at later developmental periods suggests they need inducing factors other than those determining GABAergic expression in the early immunoreactive populations (Del Rio et al., 1992). The authors have noted that the second wave of GABAergic expression occurs after the cortical plate
decondenses and coincides with the entry of some cortical afferents (Wise and Jones, 1976, 1978; Catalano et al., 1991). Whether there are two populations of GABAergic progenitors, one relying on intrinsic genetic factors while the other is dependent on extrinsic epigenetic influences is a question which requires much further research, however, it remains an interesting possibility.

There are a variety of possible factors which may influence neuronal phenotypic expression and differentiation. A number of proteins, neurotransmitters, and/or hormones may be involved in these processes. For example, in the rat optic nerve two peptide growth factors are implicated in glial differentiation; these include compounds similar to ciliary neurotrophic factor (CNTF) and platelet-derived growth factor (PDGF) (Raff, 1989). Transmitters such as glutamate (Mattson et al., 1988) and serotonin (Wallace and Lauder, 1983) may also be involved in differentiation. In addition, it has also been found that many proteins are up or down regulated during rat corticogenesis (Geschwind and Hockfield, 1989). An example is the POU class of homeobox genes, where some members are expressed in the superficial cortical layers (Brn-2) while others are expressed in the deeper layers (Tst-1) (He et al., 1989).

4.3. Brains Analysed in Adulthood

An important question is how does this difference between homo- or heterogeneity of clonally related cells in immature
and adult brains arise? One possible explanation is that cells from different progenitors may be transiently grouped together during migration when they are observed in the two week old brains. This, however, is highly unlikely since migration in the rat cortex is virtually complete by the end of the first postnatal week, therefore, the neurons observed in the two week old brains are post-migratory and are in their final position.

4.3.1. Gap junction coupling

Since the brains analysed at two weeks postnatally contained many mixed clusters while those analysed in adulthood did not, it seems probable, therefore, that a mechanism must be operating during development to make clusters of clonally related cells uniform in cell type. What this mechanism may be and why uniformity in clusters is necessary in adulthood is highly speculative. The studies of Katz and colleagues (Yuste et al., 1992; Peinardo et al., 1993), may be instructive on this issue. Initial research using optical recordings of brain slices labelled with the fluorescent calcium indicator fura-2, visualized discrete domains, sometimes spanning several layers, of spontaneously coactive neurons in the rat neocortex (Yuste et al., 1992). Later studies using the intracellular tracer Neurobiotin injected into single neurons of living slices of rat neocortex, confirmed that the neurons within each domain were coupled by gap junctions (Peinardo et al., 1993). In these
studies, single neurons were injected between postnatal days 5-16 resulting in labelled clusters of neurons surrounding the injected cell. However, injections performed in the presence of halothane, a gap junction blocker, abolished the spread of tracer to nearby cells, therefore implying that gap junctions mediate the observed coupling. The gap junctions occurred mainly through dendrites and was not observed in injections performed after postnatal day 16. In addition, the coupling appeared to be highly specific and non-random. For example, labelled clusters contained either all neurons or all glia, never a mixture of both. In addition, not every injection resulted in labelling a cluster of neurons, approximately 30% of injections resulted in staining of the injected neuron and no others, therefore, at any given time, a significant number of neurons remains in an uncoupled state. The most striking example of specificity came from the injections of smooth nonpyramidal neurons which differed from clusters following injection of pyramidal cells. First, the smooth nonpyramidal clusters contained on average only 6.3 cells, whereas the pyramidal cell clusters contained an average of 27.6 cells. In addition, injection into nonpyramidal neurons resulted in labelling of other nonpyramidal neurons only, while injection into pyramidal neurons appeared to label only other pyramidal neurons. Other studies have also noted intermediate-type junctions which form close apposition of dendritic membranes of neighbouring cells, these form as early as 24 hours after cortical plate formation (Hirst et al., 1990). These results suggest that from very early in development, transient local
coupling by gap junctions link neurons into highly specific, nonrandom clusters. It is conceivable that groups of clonally-related neurons are joined through these transient dendodendritic gap junctions, perhaps because they share certain cell-surface markers; these linked clusters may somehow be involved in mediating the uniformity of cell type seen in the immature and mature brains, although why this uniformity is necessary is not known. Studies utilizing retroviral labelling of progenitors combined with intracellular tracing may, in the future, help resolve this question.

4.3.2. Change of cell phenotype

One method whereby a mixed cluster could eventually become uniform is if some of the cells in the mixed groups alter their phenotype. It is possible that in some of the mixed groups, which are usually made up of only two or three cells, one of the cells could transform to make the cluster uniform in phenotype. This may be possible in the case of pyramidal and nonpyramidal neurons which differ in their ability to convert glutamate into GABA; this is due to the presence of the enzyme glutamic acid decarboxylase (GAD). The most likely sequence would be for a pyramidal neuron to transform into a nonpyramidal neuron, perhaps by switching on the gene encoding GAD; of course, whether this would occur due to genetic or to environmental cues is highly speculative. There have been a few examples in the literature where, as a
result of a single gene expression, a cell changes its phenotype (Lasser et al., 1986; Weintraub et al., 1991); although at present, there is little direct evidence for this theory. The more general hypothesis, that some nonpyramidal neurons may originate from pyramidal neurons has had several proponents, including the Golgi studies of Marin-Padilla (1992) and the work of Vercelli et al., (1992). In addition, recent research in the monkey cerebral cortex utilizing immunocytochemistry and electron microscopy has found that during the early stages of the intermediate fetal period, all GABA-immunolabeled neurons in the upper cortical plate share a pyramidal-like appearance (Schwartz and Meinecke, 1992). It is possible that the two bireactive neurons found in the two week old animals in the present study, are in such a transitory state, perhaps evolving from a pyramidal neuron into a nonpyramidal neuron. Preliminary studies in our laboratory suggest that the number of bireactive neurons is higher in early postnatal life then gradually decreases during the third and fourth postnatal week (Lavdas et al., in preparation). This may indicate that the bireactive cells, which initially were immunopositive for both GABA and glutamate, eventually change phenotype and utilize only one transmitter, however, further research is required to confirm this finding.

4.3.3. Cell death

Cell death is another method whereby a mixed cluster of
clonally related neurons could become uniform in cell phenotype. Naturally occurring cell death has now been well established as a common phenomenon during normal development in the rat cerebral cortex (Ferrer et al., 1990). Studies by Acklin and van der Kooy (1993), suggest cell death begins from the earliest stages of development, while progenitors are still in the ventricular and subventricular zones. Cell death can also be observed during the perinatal period and it reaches a peak by the end of the first postnatal week; cell death then gradually decreases, and by adulthood it can no longer be observed (Ferrer et al., 1990).

A question is whether one or some of the cells in a mixed cluster die, leaving the clone uniform in cell type. Unfortunately, no research at present has specifically addressed this question, although there is indirect evidence that this may be occurring. Recent studies show that during the period of postnatal day seven to adulthood, there is a significant increase in the percentage of single β-galactosidase positive cells in the rat neocortex (Mione et al., 1994); this increase in single cells could indicate that some members of a clonally related group have died, leaving only one remaining cell. Whether this death is preprogrammed into some of the cell(s) of a mixed cluster, or whether it occurs as a result of extrinsic factors such as afferent input, has yet to be determined. Future lineage experiments analysing cell death in clonally related neurons from the earliest developmental stages should help to resolve these questions.
4.4. Clusters of pyramidal and nonpyramidal neurons differ in number of cells and laminar location

A finding of the present study was the vast differences between the pyramidal and nonpyramidal neuronal clusters. In the animals injected at E14, pyramidal clusters contained an average of 6.3 neurons while nonpyramidal clusters contained an average of 2.8 neurons. By E19, pyramidal clusters contained only 2.8 neurons while nonpyramidal clusters contained an average of 2.0 neurons. These results show that during corticogenesis the size of pyramidal clusters was usually double that of the nonpyramidal clusters and that nonpyramidal clusters were usually composed of 2 or 3 neurons. In addition, laminar spread was very different for the two neuronal types. During the early stages of corticogenesis, E14 to E16, pyramidal clusters were found in all cortical layers (layers II to VI); however, nonpyramidal clusters were localized only in layers IV to VI. By the later injections, E17 to E19, both pyramidal and nonpyramidal clusters were mainly confined to the supragranular layers.

An explanation of these results is difficult with present technology, however, it seems probable that pyramidal and nonpyramidal neurons may be generated by different mechanisms. The two most likely possibilities are that either different progenitors give rise to similar numbers of neurons but in the nonpyramidal clusters neurons die or migrate away. The second possibility is that pyramidal and nonpyramidal progenitors produce different numbers of neurons. This differential
production could be accomplished by progenitors undergoing a different number of mitotic divisions, therefore, nonpyramidal progenitors divide only once or twice to produce the 2 to 3 cell clusters found in the present study, while the progenitors which will give rise to pyramidal clusters, continue to divide much longer producing many more progeny. This explanation would mean, however, that during corticogenesis one would expect to find mitotically dormant precursors in the ventricular areas which will later give rise to the two to three cell nonpyramidal clusters. As yet, there is no evidence for this, in fact, present research indicates the opposite. This is based on studies in the rodent utilizing autoradiographic labelling of ventricular precursors (Waechter and Jaensch, 1972; Takahashi et al., 1993). These studies suggest that nearly 100% of the E17 cortical germinal zone cells are heavily labelled after 14 hours of cumulative $[^3]$H-thymidine injections (Waechter and Jaensch, 1972); therefore, since nearly all ventricular precursors appear to be mitotically active, it is unlikely that dormant precursors exist in the rodent ventricular areas. This possibility, however, can not be ruled out since several studies, based on $[^3]$H-thymidine labelling of proliferating cells, suggest that the cortical germinal zone progenitors can be divided into compartments of progenitors with different cell cycle kinetics (Waechter and Jaensch, 1972; Reznikov et al., 1984; Johnston and van der Kooy, 1989; Altman and Bayer, 1990a,b). This may mean that some progenitors cycle so slowly that they produce only a few daughter cells while other, more mitotically active
precursors, produce many more progeny.

It is conceivable, however, that the first possibility is correct, that is progenitors give rise to similar numbers of neurons but that in the nonpyramidal clusters some cells die. Evidence for this comes from double-labelling studies in the rat combining retroviral- and simultaneous $[^3]$H-thymidine labelling of dividing E17 ventricular cells, animals were then perfused 48 hours later (Acklin and van der Kooy, 1993). In these experiments, the retroviral marker was used in minute dilutions which resulted in only one retrovirally labelled clone per brain hemisphere. The study found a substantial amount of cell death in the cortical germinal zone; in fact, 100% of clones in the ventricular zone and in parts of the subventricular zone showed some cell death, while only 30% of clones in the lower portion of the subventricular zone exhibited it. This result was surprising since most descriptions of cell death in the rodent central nervous system have been reported in the perinatal and postnatal periods (Mensah, 1982; Cowan et al., 1984), although some studies have observed degenerating cells in the mouse telencephalic wall (Smart and Smart, 1982; Smart and McSherry, 1982). Since cell death appears limited to certain bands in the developing germinal zones, it may function as a mechanism to regulate the number of different cell types produced, (Smart and Smart, 1982; Smart and McSherry, 1982), perhaps via feedback signals from postmitotic cells as in the larval frog retina (Reh, 1987). Unfortunately, in the Acklin and van der Kooy study (1993) it was not possible to determine neuronal
phenotype of clonally-related ventricular cells at such an early stage of development, although in the future it may be possible to determine whether nonpyramidal clusters exhibit more cell death than pyramidal clusters.

In conclusion, the present research suggests two possible modes of cortical neurogenesis. One possibility is that the rodent ventricular zone is composed of several types of neuronal progenitor cells, those which are committed to producing only one type of neuron, either all pyramidal neurons or all nonpyramidal neurons, and another type of progenitor which produces both neuronal phenotypes. The second possibility is that progenitors are multipotent and are therefore capable of producing both neuronal phenotypes, but that during development, some of the cell(s) in a cluster die or transform to produce a clone uniform in cell type. Further research analysing brains even earlier in development will be necessary to resolve this. However, a scheme with two different types of progenitor may be more likely as it could add great adaptability to the development of the cortex. The committed progenitors would ensure that the proper ratio of output neurons and interneurons is maintained, while the multipotential progenitors add flexibility since the final phenotype of the cluster could be manipulated by individual developmental circumstances.

In addition, the discrepancy between the immature and adult brains was striking; the two week old brains exhibited many mixed clusters, while the adult brains consistently
exhibited cells of uniform phenotype. This suggests there must be an important mechanism, or mechanisms, which convert the mixed clusters into homogeneous clusters of clonally related neurons. At present, it is unknown what these the mechanisms may be or how they operate, although two possibilities include cell death and transformation of phenotype. In addition, the question still remains why it is necessary for clusters to be uniform in phenotype in the adult rat cortex. Further research must be undertaken to answer these important questions in cortical development.
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