DEVELOPMENT OF THE CEREBRAL CORTEX:

Emx GENES AND INTERNEURON MIGRATION

By

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To my Grandmother
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TABLE OF CONTENTS

Acknowledgements .................................................................................................. 3
Publications arising from this work ......................................................................... 4
Table of contents ..................................................................................................... 5
Abstract .................................................................................................................... 6
Abbreviations ........................................................................................................... 8
Chapter One- Introduction........................................................................................ 9
Chapter Two- Expression of Emx1 in the developing and mature neocortex....... 52
Chapter Three- Migration of interneurons from the ganglionic eminence into the developing neocortex................................................................. 91
Chapter Four- Emx2 in the development of the neocortex...................................... 132
Chapter Five- General discussion............................................................................. 191
References ............................................................................................................. 198
ABSTRACT

The homeobox genes *Emx1* and *Emx2* are mouse homologues of the *Drosophila empty spiracles* gene, which has been shown to be important in the development of the *Drosophila* nervous system. Previous studies have demonstrated the expression of *Emx1* and *Emx2* in overlapping regions of the developing forebrain, specifically in the neocortex and hippocampus. *Emx2* is also expressed in parts of the ventral telencephalon, including the proliferative ventricular zone of the ganglionic eminence and amygdala. Recent evidence has shown that the vast majority of cortical interneurons are generated in the ganglionic eminence and migrate tangentially into the neocortex. The migration of these cortical interneurons is dependent upon the homeobox genes *Dlx1* and *Dlx2*. The expression domain of *Emx1* and *Emx2* suggests a role in the development and regional patterning of the neocortex, and that *Emx2* may also be involved in the generation and migration of cortical interneurons derived from the ganglionic eminence of the ventral telencephalon.

Using a variety of techniques, including organotypic slice culture, in-situ hybridisation, and immunocytochemistry at the light and electron microscope levels, the present study examines the expression and function of *Emx1* and *Emx2* in the developing neocortex. In addition, using organotypic slice cultures, we investigated the tangential migration of neurons from the ganglionic eminence to the neocortex, and examined possible guidance cues involved in this mode of migration.

The new findings of the present study can be summarised as follows: 1) Expression of *Emx1* begins very early in embryonic life and continues through all stages of development to adulthood. This homeobox gene is expressed by cells containing the neurotransmitter glutamate, a marker of pyramidal neurons, and is absent from the majority
of GABA-containing interneurons. Thus, the homeobox gene $Emx1$ can be reliably used as a marker of the pyramidal cell lineage. 2) $Emx2$ appears to be important in the migration of cortical interneurons from their site of origin in the ganglionic eminence. Lack of $Emx2$ results in disruptions in the tangential migration of these neurons. This defect appears to be time-dependent, and is more severe during the early stages of development. 3) The migration of interneurons from the ganglionic eminence to the neocortex follows strict migratory pathways, and utilises the fibres of the corticofugal system. The neural cell adhesion molecule TAG-1 mediates the interaction between the migrating neurons and the cortical efferent fibres.

Studies during the last decade have documented the importance of a number of homeobox gene transcription factors in the development of the central nervous system. The present study provides new information about the expression of the $Emx$ homeobox gene family in the developing rodent neocortex, and examines some functional roles for these genes in this system.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
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<tr>
<td>CP</td>
<td>Cortical plate</td>
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<tr>
<td>cDNA</td>
<td>cloned deoxyribose nucleic acid</td>
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<tr>
<td>DAB</td>
<td>3, 3’ diaminobenzidine</td>
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<td>DIG</td>
<td>Digoxygenin</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>E</td>
<td>Embryonic day</td>
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<td>IR</td>
<td>Immunoreactive</td>
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<td>IZ</td>
<td>Intermediate zone</td>
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<tr>
<td>LGE</td>
<td>Lateral ganglionic eminence</td>
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<td>Medial ganglionic eminence</td>
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<td>Messenger ribonucleic acid</td>
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<td>MZ</td>
<td>Marginal zone</td>
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<td>P</td>
<td>Postnatal day</td>
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<td>probability</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SP</td>
<td>Subplate</td>
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<td>SVZ</td>
<td>Subventricular zone</td>
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<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
CHAPTER ONE

List of Contents

1.1. Introduction ................................................................................................................ 10

1.2. Structure of the Cerebral Cortex ............................................................................. 11

1.2.1. Cytology of the rat cerebral cortex ................................................................... 11

1.2.2. Cytoarchitectonics in the rat cerebral cortex ...................................................... 16

1.2.3. Connections of the cerebral cortex ..................................................................... 19

1.3 Development of the Rat Neocortex ........................................................................ 21

1.3.1. Early development of the nervous system ....................................................... 21

1.3.2. Laminar organisation of the developing telencephalic vesicles ....................... 22

1.3.3. Heterogeneity of the ventricular zone ............................................................... 28

1.3.4. Radial migration of cortical neurons ................................................................. 30

1.3.5. Tangential migration of neurons ........................................................................ 35

1.3.6. Specification of cortical areas; Protomap or Protocortex? .............................. 38

1.4. Homeobox genes in the developing telencephalon ................................................. 41

1.4.1. Regulation of gene transcription: Transcription factors ................................... 41

1.4.2. Homeodomain transcription factors .................................................................. 42

1.4.3. Genetic control of cortical development ............................................................ 43

1.4.4. Emx genes in the developing telencephalon ..................................................... 48

1.5. Aims of the study ....................................................................................................... 49
1.1. Introduction

The neocortex is by far the largest part of the mammalian brain, and in the rat contains an estimated 34 million neurons (Uylings et al., 1990). It is also evolutionary the most recent addition to the cerebral cortex, taking its place alongside the older archicortex and paleocortex. What is even more remarkable is that research on fossil records has demonstrated that the hominid brain has trebled in size over the last 3 million years, and that the increase in size has been largely due to the expansion of the neocortex and its connections. These evolutionary changes in the size of the neocortex can also be seen when phylogenetic differences in modern mammalian brains are studied. In reptiles, there is a basic primordia of cerebral cortex, but these structures are often described as being allocortex, a term used to describe the archicortex together with paleocortex. It is only in mammals that the first true neocortex can be seen and, thus, the neocortex is regarded as a structure unique to mammalian species. The size of the neocortex in relation to body mass varies greatly amongst the different mammalian groups. A comparison between the brain of the rat and that of the cat clearly shows an increase in the size of the neocortex in proportion to other brain structures in the cat. Likewise, when the cat brain is compared to that of a primate, the increase in neocortical size in the primate brain is clearly evident. Although these changes might come about due to increases in body mass, it is more likely that they occur through increased specialisation in the neocortex. This is shown more clearly when the human brain is compared to that of a primate. The human brain is three times the size of an equivalent weight primate (Passingham, 1982), which clearly shows that the increase in neocortical size does not arise solely due to increases in body mass. Comparisons of the human and primate brain show that the increase in size does not occur in all parts of the brain, but instead occur mainly in cortical structures, particularly the cerebellum and neocortex. The expansion in the neocortex is uneven, with some areas expanding more
in relation to other areas. For example, compared to primates, the association and premotor areas in the human brain have expanded in relation to sensory areas. This suggests that increases in neocortical volume take place due to increases in specialisation of particular areas of the neocortex.

1.2. Structure of the Cerebral Cortex

1.2.1. Cytology of the rat cerebral cortex

In the rat, the neocortex consists of a sheet of cells measuring 1.2-1.5 mm (Bayer and Altman, 1991) in thickness. All parts of the neocortex share the same basic structure, with the neurons arranged into six layers or laminae. This is a feature unique to the neocortex and distinguishes it from the phylogenetically older archi- and paleocortex that have variable laminar structures. The layering is produced by differences in the size and shape of cells, and in their packing density within each of the six layers (Gilbert, 1983).

Each layer contains a complement of the two main classes of neurons found in the cortex, the pyramidal cells (projection neurons) and the nonpyramidal cells (interneurons). Pyramidal neurons, which constitute about two-thirds of the neurons in the cortex, utilise the excitatory amino acids L-glutamate or L-aspartate as neurotransmitters (Fagg and Foster, 1983; Dori et al., 1992). Nonpyramidal cells utilise GABA and wield an inhibitory effect on cortical neurons (Fagg and Foster, 1983). The other major cell types found in the cortex are the macroglia, with astrocytes present in all layers of the cortex, whereas oligodendrocytes are mostly found in the lower layers (Parnavelas et al., 1983).

Pyramidal cells

The major neuronal cell type found in the neocortex are the pyramidal cells. They constitute about two-thirds of the neurons in the neocortex (Parnavelas et al.,
1977; Rockel et al., 1980; Winfield et al., 1980), and are located in layers II through VI. The pyramidal cells are also known as projection neurons as they are the major type of output neuron in the neocortex, and form connections with other cortical areas, and with subcortical structures such as the thalamus, basal ganglia, brain stem, and spinal cord (Parnavelas et al., 1989). They utilise the amino acid L-glutamate as a neurotransmitter and exert an excitatory action on the areas that they project to (Fagg and Foster, 1983; Dori et al., 1992).

Pyramidal cells can take on a variety of different shapes, the most common being a conical cell body. Typically, they have a prominent dendrite that originates from the apex of the conical soma, and extends upwards towards the pial surface. This so-called apical dendrite gives rise to numerous oblique and horizontal branches and, in most cases, terminates in layers I and II. At the base of the soma, two or more basal dendrites extend obliquely or horizontally, their branches forming the basal dendritic field. As well as the basal dendrites, an axon extends from the base of the soma, or in some cases, from the proximal part of a basal dendrite. This axon projects towards the white matter, giving rise on the way to numerous collaterals. These features are characteristic of pyramidal cells that populate layers III and V. Pyramidal cells in layer II differ slightly in that they have more rounded or ovoid cell bodies with shorter apical dendrites. In some cases, when the cell is positioned near the top of layer II, it may lack a prominent apical dendrite, but may have two or three obliquely orientated ascending dendrites that branch near the soma (Peters and Kara, 1985). Other pyramidal cells found in layer V and VI have 'apical' dendrites that extend horizontally or obliquely, or are inverted, such that the apical dendrite is orientated towards the white matter (Parnavelas et al., 1977).

In general, pyramidal cells can be identified by several characteristic morphological features; a round to oval or conical cell body, a high cytoplasmic/nuclear
ratio, a round nucleus, a prominent apical dendrite that extends up towards the pial surface, and only symmetrical synapses on their cell bodies. Another characteristic feature of the pyramidal cell type are the small projections, or spines, that cover their dendrites, hence the term spiny neurons.

Symmetrical synapses, also known as Gray's type II synapses, are often GABAergic and, therefore, inhibitory. In contrast, asymmetrical synapses, or Gray's type I, are usually glutamatergic and therefore excitatory (Gray, 1959; Colonnier, 1968; Peters and Jones, 1984). Electron microscopic studies have shown that in type I synapses the cleft is approximately 30 nm, the presynaptic active site zone is 1-2 \( \mu m^2 \) in area, and have prominent dense projections, the presumed site of neurotransmitter release. The dense region on the presynaptic membrane is extensive, and amorphous basement material is visible in the cleft. In type II synapses the cleft is 20 nm across, the active zone is smaller, the presynaptic membrane specialisations and dense projections are less obvious, and the cleft contains little or no basement membrane (De Felipe and Farinas, 1992).

Nonpyramidal cells

Nonpyramidal cells, which comprise 15-30% of the neurons in the cortex, have an inhibitory effect on neurons of the cortex, and are known as cortical interneurons as they have locally branching axons that do not reach the white matter. They utilise the amino acid GABA as their main neurotransmitter, but some also produce one or more neuropeptides (Parnavelas et al., 1989). Nonpyramidal cells can also be classified according to their calcium-binding protein content. The three main calcium-binding proteins found in the neocortex are calbindin-D28K (CB), parvalbumin (PV), and calretinin (CR). In the cerebral cortex, almost all of CB-, PV-, CR-immunoreactive cells are nonpyramidal, although some pyramidal cells also express these proteins (Demeulemeester et al., 1991; reviewed in DeFilipe, 1997; and Hof et al., 1999). In the
cat visual cortex, almost all PV-positive, and about 80% of CB-positive cells, are GABAergic. About 37% of GABAergic neurons contain PV and 18% contain CB (Demeulemeester et al., 1991). Calretinin-positive cells are found in all cortical layers (Hof et al., 1999), including layer I where it is expressed in Cajal-Retzius cells (Weisenhorn et al., 1993; del Rio et al., 1995).

In contrast to the pyramidal cells, the dendrites of nonpyramidal neurons are largely free of spines and are therefore often referred to as smooth neurons. Nonpyramidal cells are heteromorphic, as many different forms have been described. They vary in size and shape of the cell body, and also in their dendritic morphology. The various dendritic patterns exhibited by nonpyramidal cells have been classified as multipolar, bipolar, or bitufted (Feldman and Peters, 1978).

The most common type of nonpyramidal cell is the multipolar cell, which comprises approximately 60% of cortical interneurons. These cells are found in all layers of the cortex, and have a spherical, ovoid or irregular soma, with dendrites emanating from various parts of the soma and extending in different directions giving a stellate appearance (Feldman and Peters, 1978).

Bipolar cells are present mainly in layers II-IV (Feldman and Peters, 1978). They usually have a vertically elongated cell soma, with one dendrite emerging out from each of the poles, hence the term bipolar. The dendrites usually extend a considerable distance from the soma, and axons usually arise from one of the dendrites and extend in a predominantly vertical orientation (Feldman and Peters, 1978; Peters and Kimerer, 1981; Peters and Kara, 1985b).

Bitufted cells comprise about 20% of nonpyramidal cells, and are found mainly in layers IV and V. Like the bipolar cells, they have a vertically elongated soma, with one or more dendrites emanating from each pole. The dendrites are shorter and branch
repeatedly to form two almost symmetrical tufts. Their axons extend from the soma or from one of the primary dendrites (Feldman and Peters, 1978).

In addition to these types of nonpyramidal cell, other layer specific cell types have been described in the rat cerebral cortex. The Cajal-Retzius cells of layer I are a transient population of cells that appear early during corticogenesis, but are not present in the mature cortex. These cells express the calcium-binding protein calretinin (Weisenhorn et al., 1993; del Rio et al., 1995) and secrete the extracellular matrix molecule Reelin (Ogawa et al., 1995). Cajal-Retzius cells that express Reelin are required for the proper formation of the cortical lamination (Ogawa et al., 1995), and in their absence, the cortical layers form in an outside-in sequence (Caviness and Sidman, 1973). The Cajal-Retzius cells eventually degenerate or are transformed into other types of layer I nonpyramidal cells during neonatal development (Edmunds and Parnavelas, 1982).

Glial cells

Although the rat cerebral cortex contains an estimated 34 million neurons, they are not the major cell type found in the cortex. There are around twice as many glial cells as there are neurons in the rat cerebral cortex (Parnavelas et al., 1983). All glial cells have processes that conduct potentials. They play important roles in the maintenance and functioning of the neurons around which they are located. Glial cells can be classified into two major classes, the microglia and the macroglia. In addition to these, there are also some specialised forms of glial cell such as those that line the surface of cavities in the CNS, known as ependyma cells. Other specialised glial cell types include the Muller cells of the retina, the Bergman cells of the cerebellum, and the pituicytes of the posterior pituitary gland.

Microglia, so-called because of their small size, may constitute 5-20% of glial cells (Parnavelas et al., 1983). They are considered to be of mesodermal origin,
invading the nervous system during embryonic development, where they undergo morphological changes to resemble the microglia found in the adult CNS. Microglia are phagocytes that are activated in response to injury, infection, or disease (Rio Hortega, 1932). Macroglia can be further classified into astrocytes and oligodendrocytes.

Astrocytes are the most numerous of the glial cells (Parnavelas et al., 1983). They can have many short or long processes, and on this basis can be divided into two kinds, the protoplasmic astrocytes and the fibrous astrocytes (Peters et al., 1991). Some astrocytes send processes that make contact with the surface of capillaries, where the terminus of the process expands to form ‘endfeet’. Astrocytes also make and maintain intimate contact with neurons, suggesting that they may exchange substances between them. One important function of astrocytes is to remove or degrade neurotransmitters from the interstitial space, preventing the build up of these transmitters extracellularly. Astrocytes also play a role in regulating the potassium (K+) ion concentration in the extracellular space, important because neuronal excitability is strongly influences by extracellular K+ ion concentration (Peters et al., 1991).

Oligodendrocytes are small cells with relatively few processes. The cell body is often positioned close to neuronal perikaryon, and function by providing axons with a myelin sheath that greatly enhances the conduction of nerve impulses. They form this sheath by wrapping their membranous processes concentrically around the axon in a tight spiral. One oligodendrocyte may provide myelination for more than one neuron (Peters et al., 1991).

1.2.2. Cytoarchitectonics in the rat cerebral cortex

One distinguishing feature of the neocortex is the arrangement of neurons in six layers. However, the thickness and structure of the various layers is not homogenous, and can vary between different areas of the neocortex. The study of these differences, known as cytoarchitectonics, has enabled the subdivision of the cortex into
cytoarchitectonie areas. In the early part of the previous century, many scientists used these cytoarchitectural differences to subdivide the cortex into different areas based on differences in size, shape, and arrangement of neuronal cells. Perhaps the best known of these cytoarchitectonic maps was produced by Brodmann, and is still widely used today. Although the division of the cortex into cytoarchitectonic areas was based initially on anatomical differences, it has since been shown that these areas also differ in their function. In addition to this, cytoarchitectonic areas are unique not only in their structure and function, but also in the afferent and efferent connections, and physiological properties of its cells.

Figure 1 illustrates the laminar organisation of the neocortex into six distinct layers. The outermost layer of the cortex, layer I, or molecular layer, is a neuron sparse, but fibre-rich, layer that contains the horizontally orientated Cajal-Retzius neurons (CR), which are unique to this layer. It also contains many afferent fibres from subcortical areas, and the apical dendrites of pyramidal cells located in deeper layers. Layer II, the external granular layer, is comprised of small, densely packed cells. Layer III, the external pyramidal layer, contains larger, medium-sized pyramidal cells that provide much of the output to other cortical areas. Layer IV, the internal granular layer, is similar to layer II, containing small, densely packed cells. This layer is rich in non-pyramidal cells, and receives most of the afferent connections from the thalamus. Layer V, the internal pyramidal layer, consists of loosely packed, large pyramidal cells. These cells are mainly responsible for subcortical projections to the basal ganglia, brain stem, and spinal cord. Layer VI, the multiform layer, contains many small, spindle shaped cells, and is mainly responsible for sending efferent axons back to the thalamus (Parnavelas et al., 1989).
A characteristic feature of the mammalian neocortex is the arrangement of neurons into six layers. Each layer contains a complement of pyramidal and nonpyramidal although the thickness, structure, and composition of each layer can vary. The outermost layer of the cortex, layer I, or molecular layer, is a neuron sparse, but fibre-rich, layer that contains the horizontally orientated Cajal-Retzius neurons (CR), which are unique to this layer. It also contains many afferent fibres from subcortical areas, and the apical dendrites of pyramidal cells located in deeper layers. Layer II, the external granular layer, is comprised of small, densely packed cells. Layer III, the external pyramidal layer, contains larger, medium-sized pyramidal cells that provide much of the output to other cortical areas. Layer IV, the internal granular layer, is similar to layer II, containing small, densely packed cells. This layer is rich in non-pyramidal cells, and receives most of the afferent connections from the thalamus. Layer V, the internal pyramidal layer, consists of loosely packed, large pyramidal cells. These cells are mainly responsible for subcortical projections to the basal ganglia, brain stem, and spinal cord. Layer VI, the multiform layer, contains many small, spindle shaped cells, and is mainly responsible for sending efferent axons back to the thalamus.
1.2.3. Connections of the cerebral cortex

Cortico-cortical connections

The neurons of the cerebral cortex send projections to other neurons within the cerebral cortex, creating a vast network of fibre connections. The simplest connections are those made by neurons in close proximity to each other, such as those made by the nonpyramidal cells. These neurons do not project out of the cortex, but instead have locally branching axons that act on other cortical neurons within a small area, and are known as the cortical interneurons. Three main types of interneurons can be distinguished based on the route and patterning of their axons; one has an axon that forms numerous branches close to the perikaryon, and thus influences neighbouring neurons in the same layer; a second type has an axon that extends vertically towards the pia or towards the white matter, enabling it to communicate with neurons in several layers; a third type sends its axon in a horizontal direction parallel to the cortical surface, thus influencing cells located some distance away but in the same cortical layer (Feldman and Peters, 1978).

Association fibres are responsible for connecting various parts of the cortex to each other, and can vary in length from short fibres that connect minor regions within one area (U fibres), to longer fibres that connect neighbouring areas (such as those connecting functionally related areas in different lobes). Most association fibres are reciprocated, and each cortical area will establish connections to many others, and will also receive fibres from several other areas, thus creating an extensive network of connections that allow the various parts of the cortex to co-operate in performing complex tasks (Parnavelas et al., 1989).

There is also extensive communication between the two cerebral hemispheres, mediated by the commissural fibres, which are mostly found in the corpus callosum, a thick plate of white matter that link the two hemispheres across the midline. The
commissural fibres connect corresponding areas in the two hemispheres, and can vary in density between different regions, with some areas having little or no commissural connections. The variation in the number of commissural connections is related to the degree of co-operation between the two hemispheres (Parnavelas et al., 1989).

**Thalamocortical connections**

The thalamus sends projections to all cortical areas, supplying most layers of the neocortex with afferents. The thalamic input is highly ordered, with each part of the cortex receiving fibres primarily from one specific thalamic nucleus (Parnavelas et al., 1989). Each thalamic nucleus also has the potential to project to more than one cortical area. Some thalamic nuclei act as a relay in the pathway from sensory receptors to specific parts of the cortex. For example, the lateral geniculate body receives afferents from the retina, and relays the information to the striate area (Clark, 1933; Waller, 1934; Lashley, 1934, 1941). Other thalamic nuclei relay information from other parts of the brain, such as from the cerebellum and the basal ganglia to the motor and premotor areas.

**Corticothalamic connections**

The thalamocortical and corticothalamic connections are reciprocal, that is, the thalamic nuclei receive afferents from the cortical areas to which they send their efferents. Corticothalamic projections mainly originate from the neurons located in layer VI, with most other layers sending their projections to other subcortical structures (reviewed in Peters, 1985). Corticothalamic fibres can influence the behaviour of cells in the thalamus to which they project; the properties of the relay cells in the lateral geniculate nucleus are in part determined by signals received from the striate area.

**Subcortical connections**

In addition to thalamocortical connections, the cerebral cortex also receives afferents from a number of subcortical cell groups. These areas provide sparse
projections to large parts of the cortex but without any distinct topographical pattern. Many areas provide afferents to the cortex, each using a particular transmitter. These include the **raphe nuclei** (serotonin), the **locus coeruleus** (norepinephrine), dopaminergic cell groups in the mesencephalon, the **basal nucleus** (acetylcholine), and the **tuberomammillary nucleus** of the hypothalamus (histamine) (Parnavelas *et al.*, 1989; Bayer and Altman, 1991).

1.3 Development of the Rat Neocortex

1.3.1. Early development of the nervous system

The development of the nervous system begins with the formation of the neural plate, a thickening of tissue in the prospective cranial end of the developing embryo. This thickening is caused by the growth of ectoderm cells that form a columnar neuroepithelium, and differentiation proceeds in a rostral to caudal fashion. At E10 to E11, a longitudinal folding occurs, forming the neural groove, which subsequently closes at E12 to form the neural tube. The neural tube is lined with primitive neuroepithelial cells that will proliferate and develop into neurons and glial cells. Three vesicles begin to form at the cranial end of the neural tube, termed the prosencephalon, mesencephalon, and rhombencephalon. These vesicles then transform into five developing brain structures, the two telencephalic vesicles, diencephalon, mesencephalon (midbrain), metencephalon (pons & cerebellum), and myelencephalon (brainstem) (Uylings *et al.*, 1990).

The telencephalon, a pair of vesicles that form on either side of the prosencephalon, develops to become the cerebral cortex, basal ganglia, and olfactory bulbs.
1.3.2. Laminar organisation of the developing telencephalic vesicles

Ventricular zone

The generation of neuronal and glial populations is an important event that takes place during the development of the cerebral cortex. Cells destined to become neurons and glial cells are generated from proliferating neuroepithelial cells that line the telencephalic vesicles (Bayer and Altman, 1991). This region is called the ventricular zone (VZ), and contains columnar neuroepithelial cells that are arranged radially to the ventricular surface. The VZ contains actively proliferating cells that undergo a phenomenon known as interkinetic nuclear migration (see section 1.3.3.) during the cell generation cycle (Sauer, 1935). This led Sauer to describe the VZ as a "pseudostratified" germinal matrix, although more recent studies have shown that it changes into a stratified matrix at later stages of development.

Marginal zone

The MZ is a layer of cells located just beneath the pial surface early in cortical development (Boulder Committee, 1970). The horizontally orientated Cajal-Retzius cells, the earliest neurons to differentiate in the developing cortex (Rickmann et al., 1977), populate this zone. The MZ is formed by the splitting of the preplate (PP), also known as the primordial plexiform layer (PPL), into the MZ and the subplate (SP). The PP is a transient layer that appears shortly before the accumulation of later born neurons accumulates in the cortical plate (CP), and in the process, separating into the MZ and SP (Marin-Padilla, 1971, 1978; Luskin and Shatz, 1985).

The Cajal-Retzius cells of the MZ and the polymorphous cells of the SP are amongst the earliest population of cells to be generated in the VZ. Recent evidence has shown that a sub population of Cajal-Retzius cells arises in the medial ganglionic eminence (MGE) of the ventral telencephalon. These subcortically generated cells
follow a tangential migratory route to take up positions within the MZ (Lavdas et al., 1999).

**Subplate**

The SP is formed when the PP is split by the arrival of the CP neurons. It is a cell rich zone located just beneath the CP, populated predominantly by polymorphous cells. These cells, along with the Cajal-Retzius cells of layer I, are the oldest neurons in the cortex (Luskin and Shatz, 1985, Chun et al., 1987). The SP neurons are thought to play an important role in the development of thalamocortical connections (Luskin and Shatz, 1985; Ghosh et al., 1990). Studies in a number of mammalian species have shown that thalamocortical axons arrive in the subplate, wait there for several weeks, before finally invading the CP (Shatz and Luskin, 1986; Ghosh and Shatz, 1992). The SP neurons are also amongst the first cells to send axons back to the thalamus (Auladell et al., 2000). Despite this, very few SP neurons appear to persist through to adult life, those that do are found scattered sparsely within the white matter (Luskin and Shatz, 1985). The majority of SP neurons appear to be eliminated by programme cell death in early postnatal life (Chun and Shatz, 1989; Valverde et al., 1995).

**Subventricular zone**

The subventricular zone (SVZ) is a second proliferative region that is located just above the VZ (Kershmann, 1938). The SVZ can be easily distinguished from the VZ by the random arrangement of cells, both radially and tangentially. Another important difference is that cells dividing in the SVZ do not undergo interkinetic nuclear migration, but instead appear to divide in situ (Bayer and Altman, 1991). The SVZ is thought to be a main source of glial cells, both prenatally and postnatally (Smart, 1961). Its role in the generation of glial cells in early postnatal development is well known, and it has been speculated that the SVZ also replenishes glial cells in adult life (Morshead et al., 1994). More recently, a subpopulation of adult SVZ cells has been
shown to generate neurons that are destined for the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1993).

**Intermediate zone**

As the development of the cortex progresses, a new zone begins to form above the SVZ. This intermediate zone (IZ) (Boulder Committee, 1970) contains an assortment of variably orientated cells and a rich matrix of fibres. The fibres comprise the early developing cortical afferents and efferents. Two portions of the IZ can be distinguished; an upper part that contains mostly horizontally orientated cells, and a lower portion that has mostly vertically (radially) orientated cells. It is generally thought that a fair proportion of cells in the IZ are neurons that migrate through this region on their way to the CP (Gadisseux *et al.*, 1990). The IZ increases in size as corticogenesis progresses before shrinking, and eventually becomes the cortical white matter.

**Cortical plate**

The CP is formed when newly generated postmitotic neurons migrate outwards from the cortical VZ, past the SVZ and IZ, and settle in a position within the preplate, thereby splitting it into the superficial MZ and the deep SP (Luskin and Shatz, 1985). The thickness of the CP increases during the development of the cortex as more cells are generated and migrate to take up positions within it. The cells of the CP eventually become the neurons of layers II through VI.

Autoradiographic studies, in which cells are marked with tritiated thymidine during cell division, has shown that the earliest generated neurons are deposited in the lower layers of the CP. Newly generated neurons migrate past the earlier ones, and take up positions above them. This continues until the last set of neurons take up their positions in the most superficial layer of the CP, just below the MZ. Thus, the layers of the neocortex are formed in an “inside-out” manner, with the exception of layer I
(Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic et al., 1974; Raedler et al., 1980).
Fig. 1.2. Stages of neocortical development.

Before the onset of neurogenesis, the telencephalic wall consists only of proliferating neuroepithelial cells which forms the ventricular zone; (B) as neurogenesis begins, the marginal zone, which initially contains the cytoplasmic processes of ventricular zone cells, appears at the pial surface; (C) the intermediate zone forms between the marginal zone and ventricular zone as postmitotic neurons begin to migrate away from the ventricular surface; (D) during the mid-stages of cortical neurogenesis, the subventricular zone appears between the ventricular zone and the intermediate zone. This zone contains radially and tangentially orientated cells, in addition to numerous mitotic cells. At this stage, the cortical plate is also visible, formed by the migration of postmitotic neurons along radial glial fibres which settle in an ‘inside-out’ manner below the marginal zone. (E) During the late stages of neurogenesis, the ventricular zone is reduced in size, whilst the subventricular zone, which gives rise to glioblasts, expands. The intermediate zone is cell sparse and contains a rich matrix of cortical afferents and efferents.

V- ventricular zone, M- marginal zone, I- intermediate zone, S- subventricular zone, CP- cortical plate. (Boulder Committee, 1970).
Fig. 1.3. Light micrograph of a semithin section through E17 rat forebrain illustrating the developing layers of the cortex. LV-Lateral Ventricular, VZ-Ventricular zone, SVZ-Subventricular zone, IZ-Intermediate zone, SP-Subplate, CP-Cortical Plate, MZ-Marginal zone, PS-Pial surface (Boulder Committee, 1970). Scale Bar, 10 µm.
1.3.3. Heterogeneity of the ventricular zone

For over a century the question of whether neurons and glial cells share a common progenitor, or whether they arise from separate progenitors has been the subject of intense investigation. In 1889, His described two cell types within the VZ, those that he called *Keimzellen* (germinal cells), which he believed were progenitors of neurons, and *Epithelzellen* (Spongioblasts), which he thought were neuroglia progenitors. His described the *Kiernzellen* as cells that round up during mitosis at the ventricular surface, and the *Epithelzellen* were cells that extends processes to both the ventricular and pial surfaces. However, Schaper (1897) argued that the *Kiernzellen* were actually epithelial cells that were undergoing cell division. Schaper also postulated that the neuroepithelium cells were “indifferent” and could give rise to both neurons and glia. A study by Sauer (1935) supported Schaper’s notion that the *Kiernzellen* and *Epithelzellen* were cells of the same type. Sauer noted that germinal cells in the neural tube were anchored to the inner and outer surfaces of the neuroepithelium by thin cytoplasmic processes. He hypothesised that during cell division the nuclei of the neuroepithelial cells would undergo a to-and-fro movement within the cytoplasm, and referred to this as interkinetic nuclear migration. He showed that during the cell cycle, the nuclei of the dividing cell would undergo interkinetic movement at different stages of the cycle. During M-phase, the nuclei are located at the ventricular surface, and begin to ascend to the outer margin of the epithelium during G1. At the outer margin, S-phase is initiated, before the nuclei descend towards the ventricle during S-phase and G2 (Takahashi et al., 1993). Sauer also described the neuroepithelium as a pseudostratified matrix rather than a stratified one, and concluded that only one cell type was present in the neuroepithelium.

Sauer’s observations that the spongioblasts were interkinetic stages, and the germinal cells the mitotic stages of the same cell were later confirmed in
autoradiographic studies (Sidman et al., 1959; Sauer and Walker, 1961), and by both scanning (Seymour and Berry, 1975) and transmission (Hinds and Ruffet, 1971) electron microscopy. Early evidence from autoradiographic studies indicated that glia were produced only after the production of neurons had ceased. This, combined with the homogenous appearance of the VZ, led to the assumption that neurons and glia shared a common progenitor. Fujita (1964) proposed that stems cells produced only neuroblasts during early development, and once the full complement of neurons had been generated, the VZ cells lost their mitotic activity and differentiated into glial and ependymal cells.

These views were challenged in light of new evidence from immunohistochemistry and electron microscopy studies carried out in the developing cortex of the monkey. Glial elements, detected using an antibody to glial fibrillary acidic protein (GFAP), were shown to be present in the developing cortex at the time when neurons destined for the CP were being generated (Levitt and Rakic, 1980; Levitt et al., 1981). The coexistence of glial and neuronal cells in the developing cortex indicates that either both cell types arise from a single undifferentiated stem cell, as proposed by Schaper, or that two stem cell populations, one neuronal and one glial, existed in the VZ, as suggested by His. Using an antibody to GFAP, Levitt et al. (1981), demonstrated that GFAP-positive and -negative cells were both present, intermingled within the VZ and SVZ during the peak of neurogenesis, and that both cell types were recognizable at all stages of the cell cycle. Similar results were obtained using antibodies to neurofilament protein (NF), a neuron specific marker, in the VZ of the chick brain and spinal cord (Bennett and DiLullo, 1985a,b). These results indicate the presence of two separate cell populations in the VZ, and that the mitotic cells expressing NF or GFAP may be committed to the neuronal or glial lineage. However, these studies do not show whether there may be a stage during which unlabelled VZ cells may be indifferent, or whether expression of their cell specific marker has yet to begin.
Studies using recombinant retroviral vectors to mark cell lineage have shown early divergence of neurons and glia. Retroviral infection of E16 rat progenitors resulted in cell clusters composed of exclusively oligodendrocytes, astrocytes, pyramidal cells, or nonpyramidal cells (Parnavelas et al., 1991; Luskin et al., 1993). These results indicate that neurons and glia derive from separate cell lineages, and support the existence of distinct neuronal and glial progenitor cells in the VZ. However, since our understanding of the migration patterns of glial cells and their progenitors is limited, it remains possible that distant clusters of neurons and glia could be derived from a common progenitor cell (McConnell, 1995).

1.3.4. Radial migration of cortical neurons

An important event in cortical development is the migration of newly generated neurons from the proliferative VZ to their correct laminar position. The migration of cortical neurons is thought to involve surface contact with radial fibres of glial cells that span the entire thickness of the developing cortex (Rakic, 1972, 1988). The bipolar radial glial cells have somata located within the cortical VZ and extend long radial processes towards the pial surface (Rakic, 1972; Gadisseux et al., 1990; Misson et al., 1991a,b). They are the dominant astroglial cell type observed during the period of corticogenesis, and subsequently transform into protoplasmic astrocytes in the mature cortex (Cameron and Rakic, 1991). Serial-section electron microscopy of the fetal monkey neocortex has shown that the entire length of a migrating neuron is in close apposition to radial glial fibres (Rakic, 1972). Reconstructions of serial sections reveal that the leading processes of the migrating neurons are complex, and the cells often extend pseudopodia onto more than one radial fibre, giving the impression that migration is an active process involving the extension and retraction of the leading process (Rakic et al., 1974).
The molecular mechanism of neuronal migration along radial glial fibres is not well understood. Migration of newly generated neurons is presumed to involve a number of steps: recognition of a migratory pathway by the young neurons, displacement of the cell body along this pathway, and cessation of migration and detachment from the radial glial fibre. Differential cell adhesions, or the 'selective affinity of postmitotic cells for specific surfaces in their environment', as proposed by Rakic (1985), may account for all three processes. He also suggested that neuron-glial cell adhesion molecules (Edelman, 1983) could underlie the initial recognition of radial glia by young neurons. One such molecule is Astrotactin, a neural glycoprotein that can induce the transformation of glia into radial glia in vitro, and is found at points of contact between migrating neurons and radial glial fibres (Fishell and Hatten, 1991). Another class of molecules thought to be involved in neuronal migration along radial glial fibres is the glial growth factor (GGF) or neuregulin. Anton et al. (1997) demonstrated that GGF is expressed by migrating neurons and promoted their migration along radial glial fibres. In addition, they showed that GGF promotes the maintenance and elongation of radial glial cells, essential for guiding migrating neurons in the developing cortex.

A migrating neuron would require a mechanism for movement and navigation along its migratory pathway, through the processes of other cells that are in its path, particularly when the extracellular spaces become small. Differential cell adhesion and chemotactic substances (Letourneau, 1975) may account for cell motility and guidance that propels the neuron towards the cortical plate. Rakic (1985) has postulated that the release of proteolytic enzymes, such as plasminogen and plasminogen activator (Moonen et al., 1982) at the growing tip of the leading process could aid in tissue penetration, thus facilitating neuronal navigation through other cells and processes.
An event that is important for correct laminar formation is the cessation of migration by a neuron. At some point along its migratory path, a neuron must detach itself from the radial glial fibres, thereby ending its migration. This may be mediated by changes in cell adhesion or the introduction of a completely different signal that may initiate detachment and differentiation (Rakic, 1985). Recent studies have demonstrated the presence of membrane proteins localised to the plasmalemmal junction between migrating neurons and radial glial fibres that may be important in regulating cell migration (Cameron and Rakic, 1994; Anton et al., 1996). Perturbation studies, using antibodies against these membrane proteins, resulted in the withdrawal of leading processes, changes in microtubular organisation, and detachment of neurons from their radial glial substrates (Anton et al., 1996).

Recent evidence suggests that Cajal-Retzius cells are important for normal inside-out lamination of the neocortex (Ogawa et al., 1995; Mallamaci et al., 2000a). The mouse mutant reeler has provided a valuable insight into the role of Cajal-Retzius cells in neuronal migration. The reeler mouse, first described by Falconer in 1951, has abnormalities affecting the cerebral cortex, hippocampus, and cerebellum. The characteristic inside-out formation of the cortical layers is disturbed, with later born neurons accumulating beneath earlier generated neurons ('outside-in') (Caviness and Sidman, 1973). Reeler mice carry a mutation in which the reelin gene is deleted. In-situ hybridisation localised reelin mRNA to Cajal-Retzius cells of the MZ. Similarly, an antibody (CR-50) raised by immunising reeler mice with wildtype homogenates immunostained CR cells (Ogawa et al., 1995). Reelin is a glycoprotein that is synthesised and released by Cajal-Retzius cells in the MZ, and appears to play a key role in regulating radial migration in the cortex. A recent study by Dulabon et al. (2000) demonstrates that reelin can arrest neuronal migration and induce detachment from radial glial fibres. This function is carried out by interactions between reelin and α3β1
integrin, a receptor previously shown to mediate neuronal adhesion to radial glial fibres (Anton et al., 1999). This correlates with previous studies that have demonstrated an overly adhesive phenotype of early born neurons and persistent apposition of migrating neurons with radial glial fibres in the reeler cortex (Hoffarth et al., 1995; Pinto-Lord et al., 1982). Thus, mutations in reeler cortex appear to be caused by the inability of migrating neurons to stop their migration and detach from the radial glial scaffold.
Fig. 1.4. Serial electronscopic reconstruction of a migrating neuron juxtaposed to a radial glial fibre in the intermediate zone of the developing cerebral neocortex of the fetal monkey. A-migrating neuron, LP-leading process, TP-trailing process, RF-radial glial fibre.
1.3.5. Tangential migration of neurons

Up until the early 1990s, it was generally thought that cortical neurons were generated within the proliferative cortical VZ and subsequently migrated radially towards the pial surface to take up positions within the CP. Rakic (1988) proposed that neuronal migration in the cortex followed a strict radial pathway that preserved a 'protomap' (see section 1.3.6) of presumptive cortical areas imprinted into the cells of the cortical VZ. However, many recent studies (O'Rourke et al., 1992, 1995, 1997; Fishell et al., 1993; De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999) have shown that cortical neurons can migrate tangentially within the developing neocortex, and that this mode of migration is not negligible.

Lineage studies with retroviral markers have shown that the progeny of a single progenitor cell may have a wide tangential dispersion within the cortex (Walsh and Cepko, 1992). Other lineage studies have provided evidence suggesting separate progenitors for pyramidal and nonpyramidal neurons (Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994). Studies combining BrdU with retroviral markers (Mione et al., 1997) indicate that pyramidal cells maintain a close spatial relationship with clonal relatives, presumably through radial migration. In contrast, nonpyramidal cells were found as isolated or pairs of clonally related cells, and their low BrdU content suggested that they were parts of much larger clones. The dispersion pattern of these isolated, but clonally related, cells indicate a mode of tangential migration in the developing neocortex.

Direct observation of migrating cells in living slice preparations has demonstrated that although the majority of cells migrate along a radial pathway, a small fraction of cells in the IZ migrate along a tangential route (O'Rourke et al., 1992, 1995). In this study, up to 30% of cells were seen to move in a nonradial direction. Some cells were observed to change direction during the course of migration, making right-angle
turns from radial to tangential or vice-versa. In a further study, O'Rourke et al. (1995) examined the migratory routes taken by cells in vivo. They demonstrated that tangential migration took place at all levels of the developing cerebral wall, and many of the tangentially migrating cells were young neurons.

Tangential migration of cells has also been described in the cortical VZ (Fishell et al., 1993; O'Rourke et al., 1997). In the earlier study, Fishell et al. (1993) observed rounded cells that extended short lamellar moving in a 'random walk' throughout the cortical VZ of mouse. These cells displayed morphological characteristics of progenitor cells, expressed antigen markers for VZ cells including nestin and RC2, and incorporated BrdU (Fishell et al., 1993), indicating that they were neural progenitors. The dispersion of these neural progenitors in a random walk appeared to be restricted between cortical and basal telencephalic VZs, as labelled cells in the boundary region appeared to move in a rostral or caudal direction, and did not cross the border. In the study by O'Rourke et al. (1997) on ferret cortex, focal Dil injections into the cortical VZ labelled cells that moved over long distances within the VZ and SVZ. However, these cells were shown to be postmitotic as indicated by the expression of the neuron-specific marker TuJ1 (Lee et al., 1990), and did not incorporate BrdU. In contrast to the study by Fishell et al. (1993), cortical progenitors in the ferret cortex did not appear to move in a tangential direction, although daughter cells were observed to elongate or move in a radial direction.

The use of transgenic mice carrying the lacZ marker has also provided evidence for widespread tangential migration in the neocortex (Tan and Breen, 1993; Tan et al., 1995, 1998). Using transgenic mice in which roughly half of the brain cells are marked by lacZ (X-chromosome inactivation), Tan and Breen (1993) demonstrated that the neocortex is not assembled by a single mechanism of cell dispersion. Cell dispersion in the neocortex was predominantly radial, as shown by alternating blue and white stripes.
of labelled and unlabelled cells. The majority of cells within each stripe was of the same colour, suggesting that the stripes were created by radial migration. The radial stripes were not homogenous in colour indicating that some tangential migration had also occurred, with cells dispersed in this manner ignoring clonal boundaries (Tan and Breen, 1993; Tan et al., 1995). In a further refinement of this technique, highly unbalanced chimeric mice were constructed which enables a small number of progenitors to be marked in each cerebral hemisphere (Tan et al., 1998). The results of this study confirmed that both radial and tangential migration occurred, and that progenitors were phenotypically committed at an early stage. Columns of radially dispersed cells were predominantly glutamatergic, whilst GABAergic cells appeared scattered throughout the cerebral wall, in agreement with previous studies (Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994).

The question of tangential migration within the neocortex has received new impetus from recent investigations that have revealed a new source of cortical neurons. Several independent studies have shown that some cortical neurons appear to be generated in a subcortical location and subsequently migrate tangentially into the developing neocortex (De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Wichterle et al., 1999; Lavdas et al., 1999; Zhu et al., 1999). This new population of tangentially migrating neurons would appear to be distinct from those observed by O'Rourke et al. (1992) as, in this study, labelled cells originated from the cortical VZ. However, these subcortically generated cells could account for some of the tangentially migrating cells described in their subsequent study using intact brains (O'Rourke et al., 1995). Similarly, these cells may also contribute to the tangentially dispersed cells described by Tan and co-workers in X-inactivated transgenic mice (Tan and Breen 1993; Tan et al., 1995) and unbalanced chimeric mice (Tan et al., 1998).
The first direct evidence for the subcortical origin of cortical neurons came from tracing studies in rat embryos (De Carlos et al., 1996). Focal injections of DiI were made into the lateral ganglionic eminence (LGE) of living embryos and cultured in vitro. Although the majority of cells from the LGE appeared to migrate radially towards the ventrolateral surface of the telencephalic vesicle, a few cells migrated tangentially into the preplate. More recent studies have shown that cells originating from the LGE not only migrate to the preplate/marginal zone, but also into the intermediate zone (Anderson et al., 1997; Tamamaki et al., 1997; Zhu et al., 1999). A study by Anderson et al. (1997) showed that cells migrating tangentially from the LGE to the neocortex contained GABA, the neurochemical signature of nonpyramidal cells, and the calcium-binding protein calbindin. A significant reduction in the number of GABA- or calbindin-expressing cells was observed in slices that were transected at the cortical/subcortical junction. The molecular mechanisms that regulate the migration of cortical neurons from a subcortical site of generation to the developing neocortex are now the subject of intense investigation. Recent studies have indicated that the homeobox-containing genes, Dlx1 and Dlx2, play important roles in specifying the phenotype of these migrating neurons and controlling their migration (discussed in detail in section 1.4.3).

1.3.6. Specification of cortical areas; Protomap or Protocortex?

How are cortical areas specified? According to the radial unit hypothesis proposed by Rakic (1988), the progenitor cells of the cortical VZ provide a ‘protomap’ of the prospective cytoarchitectonic areas. This protomap is then translated to the developing cortex by means of radial migration of young neurons to produce arrays of ontogenetic columns. Thus, the radial unit consists of cells that originate from progenitor cells that are positioned at the same spot in the VZ, migrate along a common radial pathway, and settle within the same column. This enables information contained
within the VZ to be passed on to the developing cortex, with the X and Y axis of cell position determined by the location of the progenitor cell, and the Z axis along the depth of the cortex provided by the time of its birth.

The protomap model postulates that cortical neurons are committed to areal identities at an early stage, before they migrate outwards to form the cortical plate (Rakic, 1988). This model suggests that the positional and identity information required by newly generated neurons is intrinsic, and is passed onto daughter progeny by the progenitor cell. However, this model does not fully account for the behaviour of neurons that migrate along non-radial paths, and those that have subcortical origins. Several lines of research have also called into question the idea that cortical neurons are committed at an early stage.

Transplantation studies have shown that the regional location of a piece of developing neocortex can exert an influence on the acquisition of many area-specific properties. Transplantation of visual cortical neurons to the sensorimotor region resulted in the transplanted cells taking on the physiological properties appropriate to their new surroundings. They extended and retained axons to the spinal cord, a subcortical target appropriate for the sensorimotor cortex (Stanfield and O’Leary, 1985; O’Leary and Stanfield, 1989). Reciprocal transplants of sensorimotor neurons into visual cortex resulted in similar observations in which the sensorimotor neurons initially extend, then lose axons to the spinal cord, but retain axons to the superior colliculus, the subcortical target of the visual cortex. The heterotopic transplants also established callosal and thalamic afferents and efferents appropriate for their new location (O’Leary and Stanfield, 1989). Heterotopic transplants, in addition to forming the appropriate axonal connections, can also take on the cytoarchitectural appearance of the host environment. Pieces of occipital cortex transplanted into the presumptive barrel-field of primary somatosensory cortex can develop barrel fields when innervated by thalamic afferents.
(Schlaggar and O'Leary, 1991). These studies suggest that different regions of the neocortex have the capability to acquire area-specific features associated with other cortical areas, and that extrinsic environmental influences may play a key role in this.

The protocortex model (O'Leary, 1989) postulates that cortical areas are not determined at the time of neurogenesis, but rather are acquired through interactions with epigenetic influences. This model suggests that the cortical VZ generates neurons that are not committed to a particular areal fate, but instead gives rise to a uniform, laminated cortex. This 'unspecified' neocortex then has the potential to develop specific features appropriate for a particular cortical area in response to thalamic afferents. This model is supported by evidence that manipulation of thalamic input early in development can cause changes in cortical organisation (O'Leary, 1989). For example, the cortical barrel fields of rodents develop through interactions with thalamic afferents, but fail to form if the input is removed at birth (Woolsey and Wann, 1976).

Cortical area specification may in fact occur through mechanisms that combine aspects of both the protomap and protocortex models. Recent evidence has shown that early neocortical regionalisation can occur in the absence of thalamic afferents (Miyashita-Lin et al., 1999) and that the developing cortex may contain intrinsic positional information (Gitton et al., 1999). Studies of mutant mice, in which the thalamocortical afferents fail to develop (Miyashita-Lin et al., 1999; Nakagawa et al., 1999), have revealed the intrinsic nature of cortical regionalisation. Despite the lack of thalamic input, these mice appear to develop a normal cortical arealisation. Transplantation studies, using the H-2Z1 enhancer trap transgenic mouse line in which the lacZ reporter is expressed in a subset of layer IV neurons in the somatosensory cortex, have demonstrated the intrinsic properties of cortical regionalisation (Cohen-Tannoudji et al., 1994; Gitton et al., 1999). Pieces of embryonic H-2Z1 cortex
transplanted into newborn hosts maintain their presumptive expression of lacZ in their new environments (Cohen-Tannoudji et al., 1994).

Therefore, it seems that neither the protomap or protocortex models can account for the mechanism of cortical regionalisation, but instead it may be a combination of both. These experiments suggest that regional specification is at least in part controlled by mechanisms that are intrinsic to the developing cortex. Regional specification may rely upon a number of different factors: (a) developmental timing, (b) spatial distribution within the developing neuroepithelium, and (c) intrinsic capacity of the cell to respond to epigenetic cues. Thus, early cortical specification may involve the acquisition of areal identity information at an early stage before radial migration takes place (protomap model) which specifies positional information, and is further refined by epigenetic influences such as thalamocortical afferents (protocortex model).

1.4. Homeobox genes in the developing telencephalon

1.4.1. Regulation of gene transcription: Transcription factors

Transcription factors are regulatory proteins that are involved in the transcription of information encoded by the genomic DNA into mRNA, the first step in the synthesis of new proteins. To perform this function, transcription factors must be able to recognise, and bind to, specific sequences of DNA that are located 'upstream' of the target gene. These specific DNA sequences will often be located in enhancer, promoter, and other regulatory elements that influence the transcription of the target gene. Transcription factors can act to promote or suppress the transcription of a particular gene. In addition to binding to the specific DNA sequence, transcription factors often bind to additional components of the transcriptional machinery.

Transcription factors contain DNA binding domains that recognise target sequences and bind to them. Comparisons between different transcription factors
suggest that common types of motifs are responsible for DNA binding activity. Motifs are usually quite short and comprise only a small part of the protein structure. Several groups of transcription factors have been described that use particular motifs for DNA binding. The most common types of DNA binding motifs are the zinc fingers, the helix-turn-helix, and the related homeodomain motif, the helix-loop-helix (HLH), and the leucine zippers.

**1.4.2. Homeodomain transcription factors**

The homeodomain is a sequence of around 60 amino acids that is encoded for by the homeobox. It was first identified in *Drosophila*, and its name derives from the homeotic loci whose genes specify the identity of body structures. Many of the genes that control early development in *Drosophila* contain the homeobox sequence, and homologues of many of these genes have been identified in higher eukaryotes. The homeodomain of these transcription factors is responsible for binding to the target DNA sequence, and may also be responsible for the specificity of DNA recognition.

A number of homeobox genes have been shown to control cell identity in specific regions in both vertebrates and invertebrates (McGinnis and Krumlauf, 1992). Among the first to be identified were the Hox family of homeobox genes, which are vertebrate homologues of the *Drosophila* homeotic complex (HOM-C), and have similar organisation and expression (Duboule and Dolle, 1989; Graham *et al.*, 1989). The Hox genes are perhaps the most widely studied and best characterised of the vertebrate homeobox genes isolated so far. The expression of these genes has distinctive anteroposterior boundaries in the hindbrain and spinal cord. Extensive studies have shown that Hox genes play important roles in the development of the spinal cord (Kessel and Gruss 1990), and hindbrain regionalisation (McGinnis and Krumlauf, 1992). In the hindbrain, anterior limits of gene expression coincide with the boundaries
of the rhombomeres (Wilkinson et al., 1989) suggesting that they may play a role in regulating the segmental organisation of the hindbrain.

Whilst Hox genes have been implicated in regulating hindbrain regionalisation, a number of other homeobox genes appear to be important for the development of the forebrain. What is unclear at the moment is whether the forebrain develops in a segmental pattern like the hindbrain. No Hox genes are expressed in the developing telencephalon, although a number of regulatory genes are expressed in the forebrain. An example of this is Pax6, which encodes for a regulatory protein that has two DNA binding domains, a paired domain (Bopp et al., 1986; Treisman et al., 1991) and a paired-like homeodomain (Frigerio et al., 1986), and is expressed early in development (Grindley et al., 1995; Walther & Gruss, 1991). However, in addition to expression in the developing forebrain, Pax6 is also expressed in the midbrain, hindbrain, and the spinal cord. More recently, homeobox genes that have highly localised expression in the forebrain have been described. Amongst the first forebrain-specific homeobox genes to be identified were the Dlx gene family, comprising Dlx1 and Dlx2, mouse homologues of the Drosophila distalless (dll) gene (Cohen et al., 1989). More recently, the Otx and Emx genes, mouse homologues of the orthodenticle (otd) (Finkelstein et al., 1990) and empty spiracles (ems) (Dalton et al., 1989) genes that control head development in Drosophila, were identified in the mouse forebrain and midbrain (Simeone et al., 1992a,b). Members of the mouse Nkx2 family of transcription factors, which is related to the Drosophila NK homeobox gene (Kim and Nirenberg, 1989; Price et al., 1992), are also expressed in the developing forebrain.

1.4.3. Genetic control of cortical development

Early studies of cortical structure produced cytoarchitectural maps that were initially based on differences in structure between various parts of the cortex. Later it was shown that these areas differed not only anatomically, but also in function, and in
the afferent and efferent connections that they maintained. However, the mechanisms involved in generating the diverse, but highly rigid, pattern of cortical areas remains unknown. The discovery of a number of genes that regulate brain development has provided new insights into the processes involved in creating the complex structure of the cerebral cortex.

A number of homeodomain transcription factors have been implicated in regulating the development of the mammalian forebrain. These include the \textit{Dlx}, \textit{Otx}, and \textit{Emx} families of homeobox genes, homologues of which have been shown to have important functions in \textit{Drosophila} head development. The study of the function of these genes in mouse forebrain development has been greatly accelerated by the generation of transgenic “knockout” mutant mice in which the gene has been rendered non-functional. Using these transgenic animals, it has now been possible to show the importance of these homeobox genes in controlling correct forebrain development.

\textbf{Otx homeobox genes in cortical development}

The \textit{Otx} family, comprising \textit{Otx1} and \textit{Otx2} (Simeone \textit{et al.}, 1992a), of homeobox genes have been studied extensively. \textit{Otx1} and \textit{Otx2} are expressed early in the developing mouse embryo (Simeone \textit{et al.}, 1992a). \textit{Otx2} is expressed first, before the onset of gastrulation in the epiblast and visceral endoderm (VE), and expression is progressively restricted to the anterior third of the embryo as gastrulation proceeds. At E9.5 mouse, \textit{Otx2} expression is seen in almost the entire anterior brain, with a posterior boundary at the mesencephalic-metencephalic boundary. As development continues, \textit{Otx2} expression disappears from the dorsal telencephalon, although there is some expression in restricted parts of the adult brain with the exception of the cortex (Simeone \textit{et al.}, 1993; Acampora \textit{et al.}, 1999). \textit{Otx1} begins expression at E8.0 mouse in the neuroepithelium of the developing forebrain and midbrain. The expression of \textit{Otx1} overlaps with that of \textit{Otx2}, although \textit{Otx1} continues to be expressed in the cortical VZ.
from early corticogenesis through to mid-to late gestation stages. At these stages, *Otx1* is expressed strongly in the cortical VZ cells, which at this time are precursors of deep layer neurons. Expression of *Otx1* in the cortical VZ begins to decrease at about the same time that deep layer neurons are being generated, and expression becomes more prominent in the CP, which contains the migratory layer VI and V neurons. *Otx1* is absent from the more superficial layers (layers I through IV) (Simeone *et al.*, 1993; Frantz *et al.*, 1996; Acampora *et al.*, 1999).

Transgenic mutant mice lacking functional copies of the *Otx1* or *Otx2* genes have provided a valuable insight into the importance of these two genes in brain development. In *Otx2*<sup>−/−</sup> animals, the rostral neuroepithelium that is destined to become the forebrain, midbrain, and rostral hindbrain are missing, and there are major abnormalities in the body (Acampora *et al.*, 1995; Matsuo *et al.*, 1995; Ang *et al.*, 1996). These *Otx2*<sup>−/−</sup> null mutants die early in embryogenesis (Acampora *et al.*, 1995; Matsuo *et al.*, 1995; Ang *et al.*, 1996; Acampora *et al.*, 1997; Acampora *et al.*, 1999; Acampora and Simeone, 1999). *Otx1*<sup>−/−</sup> mutants show abnormalities in brain structure and in behaviour, and 30% of animals die before the first postnatal month. The size of the mutant brain is reduced in comparison to the wildtype, and histological analysis revealed large differences in cortical structure. In *Otx1*<sup>−/−</sup> animals, the cortex is reduced in thickness, the sulcus rhinalis is displaced dorsally, and the hippocampus is shrunken with a divaricated dentate gyrus. In the worst affected areas of the cortex, up to 40% reduction in cell numbers was seen in the temporal and perirhinal areas. In these same areas, cortical organisation appeared disrupted with no appreciable cortical lamination. BrdU labelling has revealed a 25% reduction in cell proliferation at E9.75 in the *Otx1*<sup>−/−</sup>, which may contribute to the phenotype seen at later stages (Acampora *et al.*, 1999).

Detailed studies in the cortex has shown that *Otx1* is expressed at high levels in the neurons of layers V and VI, from later stages of gestation through to adulthood.
(Frantz et al., 1994). During corticogenesis, OTX1 protein is localised only within the cytoplasm of cortical progenitor cells. This suggests that *Otx1* does not play a role in the determination of deep layer fates, which are determined in the cortical VZ (McConnell and Kaznowski 1991; Frantz and McConnell, 1996). Recent work by Weimann et al. (1999) shows that cortical lamination and neuronal migration appear to proceed as normal in homozygous mutants of *Otx1*, thus *Otx1* does not appear to be important in establishing laminar fate, or regulating migration of neurons to their correct laminar locations. In the same study, the function of *Otx1* in deep layer neurons was studied using tracer molecules to label axonal projections made by layer V and VI neurons. Injections made in *Otx1* mutant mice showed that callosal and thalamocortical projections appear to be unchanged, but subcortical projections are aberrant. Cortical projections to the thalamus come from layer VI neurons, which also express *Otx1*, although at lower levels than layer V neurons. Subcortical projections come primarily from layer V neurons, as do many of the callosal fibres. However, it has been shown that *Otx1* is only expressed in a subset of layer V projections neurons (Weimann et al., 1999), those that send axons to subcortical targets such as the superior colliculus and pons. Thus, *Otx1* appears to be important in the correct projection of axons from layer V neurons to their appropriate subcortical targets.

**Dlx homeobox genes in cortical development**

*Dlx1* and *Dlx2*, two members of the *Dlx* family of homeobox transcription factors with virtually identical expression patterns in the developing forebrain, have also been studied extensively in the context of telencephalic development. *Dlx2* is the first to be expressed, beginning at approximately E8.75 (mouse) in the mesenchyme of the first and second branchial arches (Bulfone et al., 1993a). The onset of expression of *Dlx1* lags approximately 2 days behind the onset of *Dlx2* expression (Bulfone et al., 1993b;
Eisenstat et al., 1999). \textit{Dlx1} is first detectable at E10 (mouse) in the region of the optic chiasma and the walls of the diencephalons (Price et al., 1991).

By the time \textit{Dlx1} is first expressed, \textit{Dlx2} transcripts can already be detected in the developing forebrain, beginning at E9.5 in the diencephalons in the region of the ventral thalamus (Bulfone et al., 1993b). A short time later, expression of \textit{Dlx2} expands to include the region between the zona limitans intraparencephalica to the postoptic area, and from the preoptic area along the floor of the cerebral vesicles. This rostral zone of expression includes the germinal zone of the medial and lateral ganglionic eminence and the septum. Expression of \textit{Dlx2} appears to be limited to the basal forebrain, and is also limited to the lateral forebrain with little or no expression detected in more medial aspects (Bulfone et al., 1993a,b). The expression of \textit{Dlx1} in the forebrain begins at E12, overlapping the expression domain of \textit{Dlx2}. \textit{Dlx1} transcripts can be detected in the germinative zone located below the cerebral vesicles. This region is the proliferative region of the medial and lateral ganglionic eminence, from which structures such as the globus pallidus, nucleus caudate, and putamen will develop (Price et al., 1991; Bulfone et al., 1993a). A study by Eisenstat et al. (1999) has shown that DLX1 and DLX2 protein are localised to the nucleus, and are expressed by the same cells.

Observations of neuronal migration between the lateral ganglionic eminence and the developing neocortex (De Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997) led Anderson et al. (1997) to investigate the role of \textit{Dlx1} and \textit{Dlx2} in regulating this process. The expression pattern of these two genes, with expression initially in the VZ of the ganglionic eminence followed by expression in the neocortex a few days later, suggests that these genes may play a role in neuronal migration. It has also been demonstrated that DLX1 protein can be detected in GABAergic interneurons located within the developing neocortex (Anderson et al., 1999). Indeed, when
Anderson et al. (1997) investigated the migration of cells from the LGE to the neocortex in mice containing mutations in both Dlx1 and Dlx2 (Dlx1/2<sup>-/-</sup>), they found that no detectable migration occurred in slice preparation containing the lipophilic dye DiI. They also noted that the number of GABA- and calbindin-positive cells in the neocortex of these double mutants was greatly reduced in comparison to the wildtype animals. In addition, the number of cells expressing DLX1, which is also expressed by some GABAergic interneurons in the neocortex, was reduced. More recently, Anderson et al. (1999) have demonstrated that ectopic expression of DLX2 by retroviral gene transfer in Dlx1/2<sup>−/−</sup> mutant mice can increase the population of GABAergic interneurons in vitro. This indicates that Dlx1 and Dlx2 are both required for the migration and/or specification of cortical neurons generated within the germinal zone of the medial and lateral ganglionic eminence.

1.4.4. *Emx* genes in the developing telencephalon

The mouse *Emx* gene family consists of two members, *Emx1* and *Emx2*. These genes are mouse homologues of the *Drosophila* empty spiracles (*ems*) gene, and share an 80% homology at the amino-acid level with the *ems* homeodomain. In addition to the mouse homologues, two *ems* homologous genes have been identified in humans (Simeone et al 1992a,b), xenopus (Pannese et al., 1998), zebrafish (Morita et al., 1995), and newt (Beauchemin et al., 1998). *ems* is expressed in anterior head and interneurons of the trunk and ventral nerve cord (VNC) in *Drosophila*. Mutant analysis of *Drosophila* has shown that *ems* is required for regionalised neurogenesis in the deutocerebral and tritocerebral anlagen in the anterior brain. In addition, *ems* plays an important role in correct axonal pathfinding of specific interneurons in the posterior brain and VNC (Hartmann et al., 2000).

*Emx1* and *Emx2* show a nested pattern of expression, along with two other transcription factors, *Otx1* and *Otx2*, in the developing rostral brain (Simeone et al.,
The expression domains of these genes are overlapping regions of the developing brain contained within each other in the sequence $Emx1 < Emx2 < Otx1 < Otx2$. Interestingly, the temporal expression of these genes also follow a similar sequence, with the two Otx genes being expressed first, $Otx2$ begins as early as E5.5, followed by $Otx1$ and $Emx2$ at E8-8.5, and finally $Emx1$ at E9.5 (Simeone et al., 1992a,b). The spatial and temporal expression patterns of these four homeobox genes suggest that they may play important roles in establishing the identity of various regions of the developing brain.

The expression of $Emx$ genes is restricted to the developing forebrain, including the presumptive cerebral cortex, the hippocampus, olfactory bulbs, and olfactory epithelium. $Emx2$ has a larger expression domain, being expressed in both dorsal and ventral telencephalon and the hypothalamus, whilst $Emx1$ expression is restricted to dorsal telencephalon. The nested pattern of expression of $Emx1$ within the expression domain of $Emx2$ begins at E9.5, continuing through all stages of development until long after birth. In the developing neocortex, $Emx1$ is expressed in both proliferative and postmitotic cells, in all layers of the cerebral wall with the exception of the marginal zone (Layer I) (Gulisano et al., 1996; Briata et al., 1996). Expression of $Emx2$ is restricted to the MZ (layer I) and the proliferative VZ (Gulisano et al., 1996; Mallamaci et al., 1998). The pattern of expression, and the time of onset of expression, of these two genes suggest a role in the development and maturation of the neocortex.

1.5. Aims of the study

The cerebral cortex is an immensely complicated, yet well-defined structure, organised functionally and cytoarchitecturally into distinct areas. These cortical areas are comprised of a multitude of distinct cell types, generated from a pseudostratified neuroepithelium that lines the lateral ventricles. The specification of these distinct areas and cellular identities is thought to be influenced by information intrinsic to the cells,
and by environmental influences in the form of cell-cell interactions, growth factors, neurotrophins, and neurotransmitters (McConnell, 1995a,b, 1988). Recently, much emphasis has been placed on the role of transcription factors in the determination of cellular types and specification of cortical areas. Analysis of transgenic mutant mice lacking particular transcription factors has demonstrated the importance and function of a number of these regulatory genes in the development of the cerebral cortex.

In the present study, we have investigated the expression and function of the Emx1 and Emx2, members of the Emx homeobox gene family, in the development of the cerebral cortex. As a first step towards understanding the role of Emx1, the present study sought to examine the expression of Emx1 during development and postnatal life. Although the expression of Emx1 in the developing cortex has been demonstrated, the expression of this gene in the late postnatal and adult cerebral cortex has not been characterised. Thus, using a variety of techniques such as in-situ hybridisation and immunocytochemistry at the light and electron microscope level, the expression and distribution of Emx1 in the cerebral cortex of postnatal rats was examined. In addition, we sought to characterise the cortical cell types that expressed this gene to determine whether expression was restricted to particular neuronal phenotypes.

Previous studies have demonstrated the importance of Emx2 in regulating radial migration and arealisation in the developing cerebral cortex (Mallamaci et al., 2000a,b; Bishop et al., 2000). In the present study, we sought to investigate whether Emx2 is required for the tangential migration of interneurons from the ganglionic eminence into the developing neocortex. The migration of cortical interneurons was investigated in vitro using Dil tracing in organotypic slice cultures, and by immunocytochemical staining in frozen sections, in Emx2'' mutant mice.

Finally, we investigated the migration of neurons generated in the medial and lateral ganglionic eminence along tangential migratory routes into the neocortex.
Previous studies suggested that these migrating neurons may use the fibres of the corticofugal system as guidance cues into the neocortex. To investigate whether these cortical efferent fibres are important in the migration of the cortical interneurons, we performed immunocytochemical staining in organotypic slice cultures in which migrating neurons were labelled with Dil. In addition, we investigated whether the neural cell adhesion molecule TAG-1 was important in the guidance of neurons into the neocortex using blocking antibodies against TAG-1 in organotypic slice cultures.
CHAPTER TWO

List of Contents

2.1. Introduction .............................................................................................................. 53
2.2. Materials .................................................................................................................... 55
  2.2.1. Tissue fixation and processing: ......................................................................... 55
  2.2.2. In situ hybridisation ...................................................................................... 56
  2.2.3. Immunohistochemistry .................................................................................. 57
  2.2.4. Electron microscopy ...................................................................................... 58
  2.2.5. Primary cell culture ...................................................................................... 58
2.3. Methods ..................................................................................................................... 59
  2.3.1. Tissue preparation ............................................................................................ 59
  2.3.2. In-situ hybridisation ...................................................................................... 59
  2.3.3. EMX1 immunohistochemistry ......................................................................... 64
  2.3.4. Glutamate and GABA immunohistochemistry ................................................ 64
  2.3.5. Electron microscopy ...................................................................................... 65
  2.3.7. Combined immunohistochemistry and in situ hybridisation ......................... 67
  2.3.8. Immunohistochemistry on chimeric mice ...................................................... 67
  2.3.9. Primary cell culture and immunohistochemistry/in situ hybridisation .......... 69
2.4. Results ........................................................................................................................ 71
  2.4.1. Expression of Emx1 mRNA in the developing telencephalon of the rat ...... 71
  2.4.2. Expression of Emx1 in the adult cerebral cortex .............................................. 71
  2.4.3. Emx1 is expressed in pyramidal neurons....................................................... 72
  2.4.4. EMX1 is expressed in radial columns of chimeric mice ................................ 74
  2.4.5. Emx1 is co-localized with glutamate in neurons in cortical cell cultures ... 74
2.5. Discussion .................................................................................................................. 86
EXPRESSION OF *Emx1* IN THE DEVELOPING AND MATURE NEOCORTEX

2.1. Introduction

The mammalian cerebral cortex is a highly complex structure composed of a diverse array of neurons and glia, organised into cytologically and functionally distinct areas. All cortical areas are arranged vertically into cortical layers, or laminae. The pyramidal cells are the dominant neuronal population, and are found in all layers with the exception of layer I (Lorente de Nó, 1949). These are the projection neurons of the cortex and utilise the excitatory amino acid L-glutamate as a neurotransmitter (Parnavelas *et al.*, 1989). The other neuronal type found in the cortex are the nonpyramidal cells which are scattered through all layers. These are the cortical interneurons and utilise the inhibitory neurotransmitter γ-aminobutyric acid, or GABA (Parnavelas *et al.*, 1989). Recent evidence suggests that the two neuronal populations are generated in distinct proliferative zones, with the pyramidal neurons and a small number of interneurons derived from the cortical VZ (Parnavelas, 2000) but the majority of cortical interneurons are generated in the MGE, and migrate tangentially into the neocortex (Anderson *et al.*, 1997; Tamamaki *et al.*, 1997; Lavdas *et al.*, 1999; Parnavelas, 2000).

Little is known about the mechanisms underlying the complex changes that occur during the development of the forebrain and about the genes involved in the control of cell fate. Recently, much emphasis has been placed over the role of transcription factor genes in defining regional and cell identity in developing embryos. Within the last decade, several families of transcription factors, including the homeobox genes, have been cloned and shown to be expressed in overlapping domains within the developing brains of rodents (Rubenstein and Puelles, 1994). A number of these genes,
including members of the *Emx* and *Otx* families, are expressed in the cerebral cortex from early developmental stages (Simeone et al., 1992a,b; Boncinelli et al., 1995). *Emxl*, a member of the *Emx* homeobox gene family, shows widespread expression in both proliferative and postmitotic neurons, in all layers of the cortex with the exception of layer I (Gulisano et al., 1996; Briata et al., 1996). The fact that this gene is expressed from early corticogenesis through to adulthood suggests that it may be involved in maintaining some aspect of cortical neurons. A number of homeobox genes have been shown to control cell identity in invertebrates and vertebrates (McGinnis and Krumlauf, 1992), and we hypothesized that *Emxl* may control the identity of cortical pyramidal neurons. The spatial and temporal pattern of expression of *Emxl* coincides with the pattern of distribution of pyramidal cells from the time of their origin in the VZ to adulthood. Nonpyramidal cells, shown to originate in the ganglionic eminence, express the LIM-homeobox gene *Lhx6* and appear to acquire their morphological and neurochemical identity prior to their arrival in the cortex (Anderson et al., 1997; Lavdas et al., 1999).

Using a variety of immunohistochemical methods, this study demonstrates the expression of *Emxl* in neuronal cell types during the development of the cerebral cortex from early embryonic life through to adulthood.
2.2. Materials

All reagents used for \textit{in situ} hybridisation were of molecular biology (RNase and DNase free) or analytical grade to prevent Rnase contamination of solutions (except for cell culture).

\textit{Animals}: Prenatal tissue was obtained from timed-pregnancy Sprague Dawley rats. The day on which the plug was found was designated E1 (in accordance with Bayer and Altman, 1991). A total of 15 embryos from 3 litters (5 embryos from each litter) of pups at gestational stages E15 and E16 were used for \textit{in situ} hybridisation. For postnatal stages a total of 20 animals were used (P4 \textit{n}=2, P7 \textit{n}=3, P14 \textit{n}=3, P21 \textit{n}=4, P35 \textit{n}=6, P60 \textit{n}=2).

2.2.1. Tissue fixation and processing:

Chemical, reagents, and other materials:

\textit{0.1 M Phosphate buffer (PB), pH 7.4} – a stock solution of 0.2 M was made by dissolving 5.93 g of NaH$_2$PO$_4$.2H$_2$O and 23.0 g of Na$_2$HPO$_4$ in 800 ml of distilled water and pH adjusted. The final volume was made up to 1000 ml.

\textit{4\% paraformaldehyde in 0.1 M PB, pH 7.4} – an 8\% solution was made by dissolving 8 g per 100 ml of distilled water heated to 65°C to which a few drops of 5 M NaOH was added until the paraformaldehyde went into solution. This was then filtered and diluted to 4\% with 0.2 M PB buffer, pH 7.4.

\textit{Phosphate buffered saline (PBS), pH 7.4} – one PBS tablet was dissolved in 100 ml of distilled water.

\textit{Sucrose solutions} – sucrose solutions were made by dissolving 12 g (12\% w/v), 24 g (24\% w/v), or 30 g (30\% w/v) of sucrose in 60 ml of distilled water. One PBS tablet was added and the solution was made up to a final volume of 100 ml.
7.5% Gelatin solution/15% sucrose in PBS — 150 g of sucrose was dissolved in 700 ml of distilled water to which was added 100 ml of a 10× PBS stock solution. 75 g of gelatin was added to the solution and heated until it dissolved. The final volume was made up to 1000 ml with distilled water and the solution was allowed to cool. The gelatin was stored at −20°C until use.

Equipment — Peristatic pump (Marlow Watson), Cryostat (Bright Instruments), Vibrotome (TPI).

2.2.2. In situ hybridisation

Chemicals, reagents, and other materials:

*Diethyl pyrocarbonate (DEPC) treated water* — 1 ml of DEPC was added to 1000 ml of distilled water (0.1%), incubated overnight at RT, and autoclaved before use. All solutions used for *in situ* hybridisation were made with DEPC water.

*DIG-labelling reagents* — RNA DIG-labelling mix (10×) was obtained from Roche, restriction enzymes EcoRI and HindIII, RNA polymerases Sp6 and T7, RNAsin RNase inhibitor, and RQ1 Rnase-free DNase were from Promega.

*3 M Sodium Acetate, pH 5.2* — 40.81 g of sodium acetate was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid, and made up to a final volume of 100 ml. The solution was sterilized by autoclaving.

*50× Tris-Acetate-EDTA (TAE) buffer, pH 8.0* — 242 g of Trizma base (Tris[hydroxymethyl]aminomethane) was dissolved in 600 ml of distilled water. To this was added 100 ml of 0.5 M EDTA (ethylenediaminetetraacetic acid) (pH 8.0) and 57.1 ml of glacial acetic acid, and made up to a final volume of 1000 ml. For working concentration, the 50× stock solution was diluted to 1× with distilled water.

*0.1 M Triethanolamine buffer, pH 8.0* — a 1 M stock solution was made by dissolving 185.7 g of triethanolamine-HCl (2,2’,2”-Nitrilotriethanol) in 800 ml of distilled water,
adjusted to pH 8.0 with NaOH, and made up to a final volume of 1000 ml. The stock solution was diluted to 0.1 M with distilled water.

Hybridisation buffer – a buffer containing 50% formamide, 5× SSC (pH 7.0), 5× Denhardtts solution, 500 µg/ml salmon sperm DNA, and 250 µg/ml yeast RNA was used for prehybridisation and hybridisation.

SSC wash buffers – washing buffers of 5× and 0.2× SSC were prepared by diluting a 20× SSC stock solution with distilled water.

1× Tris-buffered saline (TBS), pH 7.4 – a 10× stock solution was prepared by dissolving 121.1 g of Trizma base (Tris[hydroxymethyl]aminomethane) and 87.66 g of NaCl in 800 ml of distilled water to which was added 70 ml of concentrated HCl. The pH was adjusted to 7.4 and made up to a final volume of 1000 ml. The 10× stock was diluted to 1× with distilled water.

NTM buffer, pH 9.5 – the buffer was prepared freshly prior to use by diluting 1 M Tris buffer, pH 9.5, 5 M NaCl, and 1 M MgCl₂ stock solutions in distilled water to give a final concentration of 0.1 M Tris, 0.15 M NaCl, and 0.05 M MgCl₂.

NBT/BCIP substrate solution – the substrate solution was prepared by diluting 200 µl of NBT/BCIP stock solution (Roche) in 10 ml of NTM buffer.

Equipment – heating block, microcentrifuge, minigel apparatus, hybridisation oven.

2.2.3. Immunohistochemistry

Chemicals, reagents, and other materials:

PBS, pH 7.4 - as above

TBS, pH 7.4 – as above

Diaminobenzidine (DAB) substrate – a 0.025% (w/v) solution was prepared by dissolving 25 mg of DAB in 100 ml of Tris-HCl buffer, pH 7.4. Endogenous peroxidase activity was blocked by the addition of 0.01% H₂O₂.
Immunochemicals and reagents – Primary antibodies against glutamate (Sigma), GABA (Sigma), EMX1 (Briata et al. 1996), and NeuN (Chemicon) were used in this study. Biotinylated anti-Rabbit IgG secondary antibody, biotinylated anti-mouse IgG, Fluorescein-Avidin D, ABC elite kit, and Vectashield™ mounting medium were obtained from Vector. Tyramide signal amplification (TSA) kit was obtained from NEN. Normal Goat Serum (NGS, Harlan Seralab), Streptavidin-Texas Red and streptavidin-FITC (Amersham Pharmacia), anti-rabbit-Cy3 (Zymed), anti-mouse-TRITC, anti-rabbit-Alexa™594 and anti-rabbit-Alexa™488 were from Molecular Probes.

2.2.4. Electron microscopy

Chemicals, reagents, and other materials:

4% Osmium tetroxide – Osmium tetroxide was made by dissolving in distilled water.

0.1M Sodium acetate – 0.82 g of NaAc was dissolved in 100 ml of distilled water.

Aqueous uranyl acetate (w/v) – 2% and 3% aqueous uranyl acetate was made by dissolving in distilled water.

Graded ethanol – Ethanol solutions of decreasing concentration (90%, 75%, 50%, 25% (v/v)) were prepared by diluting absolute alcohol with an appropriate volume of distilled water.

Araldite resin mixture – 53 g of araldite CY212, 47 g of dodecenyl succinic anhydride (DDSA), and 1.5 ml of dibutyl phthalate were mixed for at least 5 min before the addition of 1 ml of benzyl dimethyl amine. The mixture was then mixed well prior to use.

2.2.5. Primary cell culture

See section 2.3.7.
2.3. Methods

2.3.1. Tissue preparation

*Embryonic tissue*: Pregnant Sprague-Dawley rats were killed by overexposure to CO\textsubscript{2} and the uterine horns exposed. Embryos were removed and the brains dissected out carefully and placed into 4% PFA/0.1M PB overnight at 4°C. Following a brief wash in PBS, the fixed brains were cryoprotected in 30% sucrose overnight at 4°C. The brains were then embedded in 15% gelatin and quick frozen in isopentane cooled on dry ice. Frozen tissue was stored at -80°C until use.

*Postnatal tissue*: Postnatal rats were anaesthetised with ether and fixed by transcardial perfusion with a 4% PFA/0.1M PB solution (0.1% glutaraldehyde was added for EM). The brain was removed, post-fixed in the same fixative solution for 3 hr at 4°C, and washed overnight in PBS. For cryosectioning, the tissue was cryoprotected in a 20% sucrose solution for 48 hr at 4°C, then quick frozen in isopentane cooled on dry ice.

*Cryosectioning*: Frozen tissue was removed from storage at -80°C and allowed to equilibrate in the cryostat chamber. Sections of 15-20 µm were cut and collected on Superfrost slides, and stored at -80°C until use.

*Vibrotome sections*: For free-floating sections, postnatal tissue was sectioned at 50µm on a Vibrotome (TPI). Sections were collected in PBS and processed for immunohistochemistry and/or electron microscopy as described below.

2.3.2. *In-situ* hybridisation

A. Preparation of DNA template

i. The *Emx1* cDNA template (1 µg/µl) was linearised at restriction enzyme sites downstream (3’ end of the insert) of the sense and antisense sequences as follows:

2 µl of the appropriate restriction enzyme buffer
2 μl of Bovine Serum Albumin (BSA)

5 μl of pEmxl template plasmid

2 μl of restriction enzyme EcoRI* or HindIII**

9 μl of Ultrapure water

The tubes were placed in a heating block at 37°C for 2 hr.

* For sense probe, EcoRI (20 U/μl) was used as the restriction enzyme with an appropriate buffer

** For antisense probe, HindIII (20 U/μl) was used as the restriction enzyme with an appropriate buffer.

ii. The linearised DNA was checked on a 1% agarose gel to ensure efficient restriction of the plasmid template. 0.5 g of agarose (Gibco) was dissolved in 1× Tris-Acetate EDTA (TAE) buffer by heating the solution until the agarose melted and the solution became clear. The agarose solution was then allowed to cool to approximately 60°C before the addition of 2.5 μl of ethidium bromide (10 mg/ml), swirled, and poured into the gel casting tray. The gel was allowed to cure for 1 hr prior to use. The gel was submerged in TAE buffer in the gel apparatus and loaded with a 1 μl sample of the linearisation reaction diluted 1:3 in gel loading buffer (Sigma). A 1 Kb DNA ladder and a sample of uncut DNA was loaded in adjacent sample lanes to check for appropriate linearisation. The gel was run at 80V for approximately 30-60 min.

iii. The linearisation reaction was stopped by the addition of 2 μl of 10× TE buffer, pH 8.0. Linearised DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the reaction buffer, mixed thoroughly, and spun at 13000 RPM for 5 min. The aqueous layer (20 μl) was removed and placed in a fresh tube, to which 10 μl of 3 M NaOAc and 70 μl of absolute alcohol was added. The
contents were mixed and cooled at -20°C overnight before being centrifuged at 13000 RPM for 15 min at 4°C. The pellet was washed with ice-cold 70% ethanol, air-dried, and resuspended in 10 µl of Ultrapure water (to give approx. 0.5 µg/µl).

B. Preparation of DIG labelled riboprobes

i. *in vitro* transcription:

The cRNA probes were transcribed from the linearised template DNA as follows:

- 4 µl of 5× Transcription buffer
- 2 µl of 0.2 M DTT
- 0.5 µl of RNASin Rnase inhibitor
- 2 µl of linearised DNA (sense or antisense template)
- 2 µl of DIG RNA labelling mixture
- 2 µl of T7* or Sp6** RNA polymerase
- 7.5 µl of ultrapure water

The reaction mixture was incubated at 37°C for 2 hr.

* For sense control probe, T7 RNA polymerase was used

** For antisense probe, Sp6 RNA polymerase was used

ii. The transcription products were checked on a 1% agarose gel (see above for methods).

iii. After transcription, 2 µl of RQ1 Rnase-free DNase I was added to the reaction mixture and incubated for 15 min at 37°C to remove the DNA template. The reaction was stopped by the addition of 2 µl of TE buffer, pH 8.0.

iv. The cRNA probe was precipitated by the addition of 10 µl of lithium chloride and 70 µl of absolute alcohol. The contents were mixed thoroughly and stored at -20°C overnight. The RNA was then centrifuged at 13000 RPM for 15 min at 4°C,
the pellet was washed in ice-cold 70% ethanol, air-dried, and resuspended in 100 μl of ultrapure water.

C. Hybridisation of cRNA probes to RNA sequences in-situ:

Prenatal tissue:

i. Section pretreatments:

*In situ* hybridisation on prenatal tissue was carried out on 20 μm cryostat sections mounted on Superfrost plus slides (BDH). Slides were removed from the storage at -80°C and allowed to warm to room temperature. The sections were fixed in 4% PFA for 10 min at RT and washed 3× 5 min in PBS. They were then acetylated by placing them into 400 ml of TEA buffer, pH 8.0 to which 1 ml of acetic anhydride was added dropwise under constant agitation for 10 min. The slides were then rinsed briefly in PBS, permeabilised in PBS containing 1% Triton X-100 for 30 min at RT, and washed 3× 5 min in PBS.

ii. Prehybridisation/Hybridisation:

The slides were then placed horizontally in a humidified chamber and 500 μl of prehybridisation buffer (hybridisation buffer minus the cRNA probe) was laid on top and incubated for 2 hr at RT. The prehybridisation buffer was then replaced with 200 μl of hybridisation buffer containing 300-500 ng/ml of DIG-labelled cRNA probe and coverslipped with a small piece of parafilm. Prior to use, the hybridisation buffer containing the cRNA probe was heated to 80°C for 5 min and rapidly cooled on ice to denature the probe. Hybridisation was carried out for 16-20 hr at 69°C in a hybridisation oven.

iii. Washes:
On the following day the slides were washed briefly in a pre-heated 5× SSC solution to remove the coverslip, placed into a pre-heated 0.2× SSC solution, and washed at 69°C for 1-2 hr.

iv. Immunological detection:

The slides were washed for in TBS, pH 7.4 for 5 min at RT. The DIG-labelled cRNA probe was visualised using an antibody conjugated to alkaline phosphatase that was directed against the DIG molecule. The sections were blocked for 1 hr in 10% NGS diluted in TBS, pH 7.4 prior to the application of the anti-DIG-AP antibody that was diluted 1:2000 in TBS containing 1% NGS and incubated for 2 hr at RT. The slides were then washed 2× 5 min in TBS, and equilibrated for 5 min in NTM buffer, pH 9.5. A substrate solution for alkaline phosphatase, BCIP/NBT was used to visualise the antibody. The substrate solution was diluted in NTM buffer, pH 9.5 and applied to the slides. The chromogen reaction was allowed to take place overnight at 4°C. When sufficient staining had been achieved, the reaction was stopped by washing in TE buffer, pH 8.0. Slides were then washed briefly in PBS, dehydrated through an ascending ethanol series (70%, 80%, 95%, 100% twice), cleared in Histoclear solution, and mounted in DPX mountant (BDH). Staining was visualised under brightfield illumination on a Leica DMRB microscope.

Postnatal tissue:

For in situ hybridisation on postnatal tissue, the tissue pre-treatment was modified as follows:

In situ hybridisation on postnatal tissue was carried out on 30 μm cryostat sections mounted on superfrost plus slides (BDH). Slides were removed from the storage at −80°C and allowed to warm to room temperature. The sections were fixed in 4% PFA for 10 min at RT and washed 3× 5 min in PBS. To increase the permeability of
the cRNA probes, the sections were incubated in 5 μg/ml proteinase K diluted in PBS for 5-15 min at RT. The slides were washed briefly in PBS and re-fixed in 4% PFA for 10 min at RT. Following fixation, the slides were washed 3× 5 min in PBS before being acetylated by placing them into 400 ml of TEA buffer, pH 8.0 to which 1 ml of acetic anhydride was added dropwise under constant agitation for 10 min. The slides were then rinsed briefly in PBS before proceeding with prehybridisation as described above.

2.3.3. EMX1 immunohistochemistry

Postnatal brains were sectioned on a Vibrotome at 50 μm and processed free-floating in 1.5 ml microtubes. Sections were blocked for non-specific labelling with 10% NGS diluted in TBS, pH 7.4 for 1 hr at RT with constant agitation. The sections were then transferred into fresh tubes containing the EMX1 antibody diluted in TBS, pH 7.4 with 5% NGS and 0.3% Triton X-100 (for electron microscopy, the Triton X-100 was omitted), incubated for 1 hr at RT (with agitation), and then at 4°C for 36 hr. The sections were then processed for DAB visualisation as described above except that washing steps were increased to 3× 10 min.

2.3.4. Glutamate and GABA immunohistochemistry

Immunohistochemistry with antibodies against glutamate or GABA was carried out on 20 or 30 μm cryostat sections mounted on Superfrost plus slides. Sections were removed from storage at −80°C, warmed to RT, and washed in PBS for 5 min. Blocking for non-specific labelling was carried out by incubating the sections in a solution containing 10% NGS and 0.1% Triton X-100 diluted in PBS for 1 hr at RT. Sections were incubated with the anti-glutamate antibody (1:500-1:1000, Sigma) or anti-GABA (1:1000, Sigma) diluted in PBS containing 5% NGS and 0.1% Triton X-100, overnight at 4°C. The sections were then washed 3× 5 min in PBS and incubated in biotinylated
secondary antibody (1:200, Vector) for 2 hr at RT. Following a further wash, the sections were incubated in ABC complex (Vector Labs) for 2 hr at RT. Antibody labelling was revealed by incubating the sections in DAB substrate until a desired level of staining was achieved. Sections were washed, dehydrated through an ascending ethanol series, and mounted in DPX.

2.3.5. Electron microscopy

For electron microscopy, postnatal animals were perfused with 4% paraformaldehyde containing 0.1% glutaraldehyde followed by 3 hr postfixation in 4% paraformaldehyde. The brains were washed thoroughly and processed for EMX1 immunohistochemistry as described in section 2.3.5. The processed sections were then postfixied in 2% osmium tetroxide in 0.1 M phosphate buffer for 30 min followed by staining with aqueous 1% uranyl acetate for 30 min. The tissue sections were then rinsed in cold 0.1 M sodium acetate buffer (2× 5 min), followed by dehydration through an ascending ethanol series; 2 min in 25%, 2 min in 50%, 10 min in 70% (2× 5 min), 30 min in 90% (2× 15 min), and 1 hr in absolute alcohol (4× 15 min). Sections were rinsed in propylene oxide for 5 min and placed overnight in a 1:1 mixture of propylene oxide:araldite, then transferred in fresh araldite resin. Following several changes in fresh resin (3× 1 hr), the tissue sections were flat embedded and allowed to polymerise at 70°C for 48 hr.

Resin embedded sections were re-mounted on araldite stubs using resin mixture and trimmed. Semithin sections of 0.5 µm were cut using glass knives using an ultracut microtome and tissue was selected for ultrathin sectioning. Ribbons of ultrathin sections were cut and collected on carbon coated grids of 200-mesh. To enhance contrast, sections were counterstained with 3% aqueous uranyl acetate and Reynolds lead citrate for 30 min each, and examined on a JEOL 1010 electron microscope.
2.3.6. Double immunofluorescence

Double immunofluorescence was carried out on 20 μm frozen sections through the cortex of P35 rat brains. For EMX1 and glutamate immunohistochemistry, both primary antisera were raised in rabbit. We therefore used the TSA amplification method described in section 2.3.8. to allow simultaneous detection of both molecules on the same tissue. Briefly, sections were blocked in 10% NGS in TBS for 1 hr at RT prior to incubation with rabbit anti-EMX1 diluted 1:5000 in 1% NGS/TBS overnight at 4°C. Following a brief wash in TBS, sections were incubated in biotinylated anti-rabbit secondary antibody for 2 hr at RT, then in ABC-Elite complex for 1 hr at RT. The sections were then washed and incubated in biotinyl tyramide diluted in amplification diluent (NEN Life sciences) for 10 min followed by 1 hr in streptavidin-FITC (Amersham). Control sections in which the primary antibody or TSA amplification was omitted was performed alongside the experimental sections. Following a thorough wash in TBS, sections were incubated overnight with rabbit anti-glutamate diluted 1:500 in 1% NGS/TBS at 4°C. This was followed by incubation in anti-rabbit-Cy3 secondary antibody for 2 hr at RT. Sections were washed and coverslipped with Vectashield mounting medium.

Double immunofluorescence was also carried out with antibodies against glutamate and NeuN, a neuronal marker. Sections were blocked with 10% NGS in TBS for 1 hr followed by overnight incubation with rabbit anti-glutamate (1:500) and mouse anti-NeuN (1:100) diluted in 1% NGS/TBS at 4°C. The sections were washed and incubated with biotinylated anti-rabbit and anti-mouse-TRITC for 2 hr at RT, followed by streptavidin-FITC for 1 hr at RT. Sections were washed and coverslipped with Vectashield™ mounting medium.
All sections were imaged on a Leica TCS-NT laser scanning confocal microscope using the Argon (488 nm) and Krypton (568 nm) laser lines.

2.3.7. Combined immunohistochemistry and in situ hybridisation

Two colour combined immunohistochemistry and in-situ hybridisation was carried out on 30 μm cryostat sections of postnatal tissue mounted on superfrost plus (BDH). Briefly, immunohistochemistry using antibodies against glutamate or GABA was carried out as described above and visualised with DAB. The sections were washed and re-fixed in 4% PFA for 10 min at RT. Slides were then processed for in situ hybridisation as described above. All chemicals used were of molecular biology grade (DNase and RNase free), ultrapure or DEPC treated water was used, and all buffers were autoclaved prior to use.

2.3.8. Immunohistochemistry on chimeric mice

Both primary antisera, anti-EMX1 and anti β-galactosidase were raised in the same species - rabbit. Using a method originally developed by Shindler and Roth (1996), both antigens were detected simultaneously using secondary antibodies with discriminating fluorophores. This method works on the basis that one of the 2 primary antisera is used at such dilute levels that only tyramide signal amplification allows for its detection. The second primary antiserum is used at normal concentration and is detected by conventional immunohistochemistry. The secondary antibody used to detect the second primary antibody is incapable of detecting the first primary antiserum by virtue of the latter’s extreme dilution. Here, tyramide signal amplification was used to detect the anti-EMX1 antibody while the β-galactosidase antigen was detected by conventional means.

Mice were killed by intracardial perfusion with 4% paraformaldehyde. Brains were dissected out and fixed for a further 10 min in the same fixative. They were then
cryoprotected, frozen and sectioned at 20 μm with a cryostat. Sections were collected on AES-coated slides dried for 2 hr and then either stored at −20°C or processed immediately.

Sections were blocked first in 10% normal horse serum (CSL, Melbourne, Australia) in PBS for 1 hr and then in 0.5% Blocking Reagent (Renaissance TSA Indirect, NEN Life Science Products, Boston, MA, USA) in a buffer containing 0.1 M Tris-Cl and 0.15 M NaCl (pH 7.6) for 30 minutes at room temperature. Incubation with rabbit anti-EMX-1 (1:6000 in PBS, 0.3% TX-100) was performed over 36 hours, followed by biotinylated goat anti-rabbit IgG (1:200, Vector laboratories, Burlingame, CA, USA) and Streptavidin-HRP (1:100, Renaissance TSA Indirect, NEN Life Science Products, Boston, MA, USA) for 1 hr each. This was followed by a 10 min amplification of biotinyl tyramide diluted 1:100 in Amplification Diluent (Renaissance TSA Indirect, NEN Life Science Products, Boston, MA, USA) and a 1 hr incubation in Fluorescein-Avidin D (1:200, Vector Laboratories, Burlingame, CA, USA). Following several washes in PBS, the tissue was processed for the detection of the β-galactosidase transgene product using a rabbit polyclonal antibody (1:500 in PBS, 0.3% TX-100, 5 Prime → 3 Prime, Boulder, CO, USA). An overnight incubation in primary antibody was followed by incubation in Alexa Fluor™ 594 conjugated goat anti-rabbit IgG (1:400, Molecular Probes, Eugene, OR, USA). Sections were mounted with Fluoromount-G (Southern Biotechnology, Birmingham, AL, USA).

Controls included the omission of each of the primary antibodies while maintaining the rest of the immunocytochemical protocol. This allowed for the assessment of non-specific background. Additionally, tissue was processed for the detection of EMX-1 as indicated above followed by an incubation in Alexa Fluor™ 594 conjugated goat anti-rabbit IgG (1:400), without the addition of the second primary
antibody. This ensured that the first primary was sufficiently dilute to escape detection by the conventional secondary antibody.

2.3.9. Primary cell culture and immunohistochemistry/in situ hybridisation

Embryonic brain primary cultures were derived from E16 and E18 rat cortices. Cortical cells were dissociated with trypsin in Ca$^{++}$, Mg$^{++}$ free S-MEM medium supplemented with 0.6% BSA, 0.038% MgSO$_4$ and 0.6% glucose. The reaction was terminated with the addition of FCS at a final concentration of 10%. The cells were plated on poly-L-lysine coated 10 mm coverslips at a concentration of 3.5x10$^5$ cells/well in defined medium (DMEM/HAM F12), supplemented with 2 mM glutamine, 5 mM HEPES, 25 mM KCl, 0.6% glucose, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 25 μg/ml insulin, 100 μg/ml transferrin and 5% FCS and cultured for 3 DIV. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PB for 20 min, and kept in PBS in preparation for in situ hybridization and immunocytochemistry.

For in situ hybridization, cells were dehydrated by passing through an ascending series of ethanol. After drying briefly, coverslips were incubated in pre-hybridization buffer at 45°C for 90 min. The buffer contained 50% formamide, 5× SSC, 5× Denhardts, 5 mM EDTA, 25 μg/ml yeast t-RNA and 0.2 mg/ml fish sperm DNA. Hybridization took place at the same temperature for 18 hr, with a probe concentration of 30 ng/ml of hybridization solution (50% formamide, 5× SSC, 5× Denhardts, 5 mM EDTA). Washes were done with 2× SSC (prewarmed) and 0.2× SSC at 45°C. This procedure was followed by glutamate (1:200, Sigma)- or GABA (1:200; Sigma) immunocytochemistry in order to identify the cells that expressed the homeobox gene in culture. Cells were incubated in antibodies in blocking buffer, diluted 1:10 in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) at room temperature for 2 hr. They were then incubated with anti-DIG FITC (1:500) and TRITC conjugated
secondary antibodies at room temperature for 2 hr, mounted in Citifluor, and observed with an epi-fluorescence microscope.
2.4. Results

2.4.1. Expression of *Emx1* mRNA in the developing telencephalon of the rat

The expression pattern of *Emx1* was examined by *in situ* hybridisation on coronal sections through the brains of E15-E18 rat embryos. In agreement with previous studies (Simeone *et al.*, 1992 a,b; Gulisano *et al.*, 1996; Briata *et al.*, 1996), expression was largely restricted to parts of the dorsal telencephalon, including the neocortex, hippocampus, and dentate gyrus. At E15, strong expression of *Emx1* is seen in the neocortex, with transcripts detected through the thickness of the cerebral wall with the exception of the MZ (Fig. 2.1A,B). At later stages, as the neocortex expands, expression can be seen in the VZ, IZ, and CP but not in the MZ.

2.4.2. Expression of *Emx1* in the adult cerebral cortex

Previous studies have shown that *Emx1* is first expressed at the early stages of the embryonic rodent brain (E9.5 mouse), and expression continues into early postnatal life (Briata *et al.*, 1996; Gulisano *et al.*, 1996). Here, we found that the expression of this homeobox gene persists through all stages of postnatal development and in the adult cerebral cortex. Cells expressing *Emx1* were numerous in all layers of the cortex, except layer I. Although at first glance cells appeared to be evenly distributed throughout these layers, careful examination clearly showed that the density, size, and morphology of labelled cells was characteristic for each cortical layer. Layers II & III showed the highest density of *Emx1* expressing cells, most of which had features characteristic of pyramidal neurons. Layer IV showed the lowest density of *Emx1* expressing cells, most showing small round or ovoid cell bodies. Layer V contained mainly a complement of medium and large labelled neurons pyramidal in shape. The medium-sized cells were predominantly in the superficial portion of this layer, whereas the large cells were found chiefly in the deep part of layer V. Labelled cells in layer VI were generally small,
showing a range of shapes and orientations (Fig. 2.2). This pattern of expression appeared consistent throughout different cortical areas along the rostro-caudal axis. Also, the distribution of $Emx1$ transcripts correlated closely to the distribution of EMX1 protein (Fig. 2.3A,B), an observation consistent with previous studies in embryonic cerebral cortex (Briata et al., 1996).

2.4.3. EMX1 is localised to the nucleus of neuronal cell types

In line with previous studies (Gulisano et al., 1996; Briata et al., 1996), we found that EMX1 protein was localised to neurons, demonstrated by double-immunohistochemical staining with antibodies against EMX1 and NeuN, a marker of neuronal cells (Fig. 2.4). Staining with these antibodies revealed that EMX1 was present in the nucleus of the majority of NeuN positive neurons, whilst a small number of neurons did not express EMX1 (Fig. 2.4C). This may indicate that EMX1 is expressed by particular types of neurons, and not by all neurons as suggested in previous studies (Gulisano et al., 1996; Briata et al., 1996).

2.4.3. $Emx1$ is expressed in pyramidal neurons

*In situ* hybridization preparations revealed $Emx1$ transcripts were present in cells whose density, distribution, and soma shapes were identical to those of pyramidal neurons as described for the developing and adult rat neocortex (Parnavelas et al., 1977; Miller, 1988). Their pattern of distribution was also very similar to that of neurons labelled in the cortex with an antibody to neurotransmitter glutamate (Fig. 2.5A), a marker of pyramidal cells (Conti et al., 1987; Dori et al., 1989, 1992). This suggested that $Emx1$ may be expressed by the pyramidal cells of the cerebral cortex.

Analysis of ultrathin sections cut from tissue taken from P35 animals, and processed for electron microscope immunocytochemistry for EMX1, showed that immunoreactive neurons had ultrastructural features characteristic of pyramidal cells (Peters and Kara, 1985). Although some cells only revealed nuclear staining, a
considerable number of neurons showed some degree of cytoplasmic labelling. As is typical of pyramidal neurons, the nuclei were round or slightly oval, and their nuclear envelopes showed smooth contours (Fig. 2.6). Consistent with what is known about the synaptic organization of cortical pyramidal cells (Feldman, 1984), all EMX1-labelled neurons received only symmetrical axosomatic synapses.

To further confirm the identity of the Emx1 expressing cells, we performed double-labelling experiments. Specifically, we processed sections from each postnatal age first for immunocytochemistry with an antibody against glutamate and then for Emx1 in situ hybridisation. We found that, at all ages examined, the vast majority of Emx1 expressing cells were also glutamate immunoreactive (Fig. 2.7A,B). Emx1 transcripts were localised in the perinuclear cytoplasm of the cells, whilst glutamate immunoreactivity was typically present throughout the cell body. However, double labelling experiments that involved Emx1 in situ hybridization and GABA immunocytochemistry showed only a small percentage of double-labelled cells (Fig. 2.7C,D). Cell counts in sections taken from P35 animals showed that 84.6% of the total number of labelled cells were Emx1 positive, 14.5% were GABA positive, and 0.9% expressed both GABA and Emx1.

We also performed double-labelling experiments with antibodies against glutamate and EMX1 in sections through the cortex of P35 rat brains. As both primary antisera were raised in rabbit, we used the tyramide amplification system originally developed by Shindler and Roth (1996), which allows for simultaneous labelling with two antibodies from the same species. Using this method, we found that EMX1 was co-localised in almost all cells that utilised glutamate, a marker of pyramidal neurons (Fig. 2.8 and Fig. 2.9). EMX1 was localised specifically to the nucleus of these cells, whilst glutamate was present throughout the cell body.
2.4.4. EMX1 is expressed in radial columns of chimeric mice

We also used highly unbalanced mouse stem cell chimeras (Tan et al., 1998) to study the expression of EMX1. In these mice, single embryonic stem (ES) cells carrying the lacZ reporter gene are injected into host blastocysts, occasionally resulting in a small number of marked progenitor cells in the embryonic cerebral wall. Some progenitors generate radial columns of pyramidal neurons characterized by their expression of glutamate (Tan et al., 1998), whilst other progenitors give rise to widely scattered GABAergic neurons. Using these chimeric mice, we showed that every radially arranged β-galactosidase-positive cell also expressed EMX1 (Fig. 2.10A-D). In contrast, EMX1 protein was not localized in scattered cells, the GABAergic interneurons (Fig. 2.10E-H).

2.4.5. Emx1 is co-localized with glutamate in neurons in cortical cell cultures

Primary cortical cell cultures were prepared from E15 and E18 rat embryos and maintained for 3 days in the presence of 5% FCS. Consistent with earlier studies (Pappas and Parnavelas, 1997), these cultures contained predominantly neurons and only a small percentage (approximately 1%) of cells of glial origin. Double labelling experiments, using in situ hybridization for Emx1 and immunofluorescence for either glutamate or GABA, indicated that Emx1 mRNA was present in the majority (85% in the E18 cultures) of glutamate containing neurons (Fig. 2.11A,C,E). Only a small percentage (10% in the E18 cultures) of the GABAergic neurons in cultures prepared from cortices of either age were observed to express Emx1 (Fig. 2.11B,D,F).
Figure 2.1. Light micrographs illustrating the distribution of Emx1 mRNA transcripts in (A) coronal and (B) sagittal sections through the developing rat telencephalon at E15. Strong expression of Emx1 is observed in the developing cortex and hippocampal anlage, with no signal detected in the ventral telencephalon. HP – Hippocampal anlage, LGE – Lateral ganglionic eminence, MGE – Medial ganglionic eminence, NCX – Neocortex, OB – Olfactory bulb. Scale bars: A,B, 100μm
Figure 2.2. Light micrographs illustrating the laminar distribution of *Emx1* mRNA transcripts in sections through the motor cortex of rats of various postnatal ages: P7 (A), P21 (B), and P35 (C). *Emx1* is expressed in all layers of the cortex, except layer 1. Scale bar: 100 μm.
Figure 2.3. Light micrographs illustrating the laminar distribution of *Emx1* mRNA transcripts (A) and EMX1 protein (B) in sections through the motor cortex of a rat at P21. The distribution of EMX1 protein correlates closely to that of the transcript, an observation consistent with previous studies of embryonic cerebral cortex (Briata *et al.*, 1996). Scale bar: A,B, 100 μm
Figure 2.4. Confocal microscope images of double-labelled tissue sections with EMX1 antibody (green) and a neuron-specific marker, NeuN (red). Colocalisation of these two antibodies appears yellow. (A) NeuN staining in sections through P35 rat cortex, (B) EMX1 protein is localised to the nucleus, (C) EMX1 is present in the majority of NeuN-positive neurons. Arrows point to NeuN-positive cells that do not express EMX1. There were no EMX1-positive cells that were NeuN-negative, indicating that EMX1 is expressed in only neuronal cell types. Scale bar: 50μm
Figure 2.5. Light micrograph illustrating the distribution of (A) pyramidal (glutamate-positive) and (B) nonpyramidal (GABA-positive) neurons in a coronal section through the cerebral cortex of the rat at P35. Scale bar: 100 μm
Figure 2.6. Electron micrograph of an EMX1-labelled pyramidal neuron (P) in layer 5 of the motor cortex of a rat of the same age. The protein is clearly localized in the nucleus of this cell. Note that the nucleus of an adjacent nonpyramidal neuron (NP) is free of any EMX1 reaction product. Scale bar: 2.5 μm.
Figure 2.7. Light micrographs of sections through the motor cortex of rats at P35 and processed for *Emx1 in situ* hybridization and glutamate immunocytochemistry (A,B), or for *Emx1 in situ* hybridization and GABA immunocytochemistry (C,D). In these double-labelling experiments, *Emx1* transcripts (blue) were localized in the perinuclear cytoplasm of cortical neurons, while glutamate or GABA immunoreactivity (brown) was typically present throughout the cell body. A,B, Nearly all *Emx1* expressing cells were also glutamate labelled and appeared pyramidal in form. Cells shown in B are double-labelled neurons in layer V shown in higher magnification. C,D, Nearly all *Emx1* expressing cells were free of GABA immunoreactivity. In D, three GABA-immunoreactive cells (arrows) did not contain *Emx1* label. Scale bars: A,C, 160 μm; B,D, 20 μm.
Figure 2.8. Confocal microscope images of tissue sections double-labelled with antibodies against EMX1 (green) and the excitatory neurotransmitter, glutamate (red). Colocalisation appears as yellow. (A) EMX1 staining in sections through P35 rat cortex, (B) the same section stained for glutamate, (C) the vast majority of EMX1-positive cells are also immunoreactive for the neurotransmitter glutamate, a marker of pyramidal neurons. Scale bar: 100μm
Figure 2.9. High magnification confocal microscope images of tissue sections double-labelled with antibodies against EMX1 (green) and the excitatory neurotransmitter, glutamate (red). Colocalisation appears as yellow. (A) EMX1 protein is localised to the nucleus of the cell, (B) whilst glutamate immunoreactive is present throughout the cell soma, (C) EMX1 protein is expressed in cortical neurons that utilise the excitatory neurotransmitter glutamate, a marker of pyramidal cells. (D) EMX1 antibody staining was amplified using the TSA system, but no signal is detected when the amplification reagent is omitted. Scale bar: 30μm.
Figure 2.10. EMX1 is expressed by columnar, but not scattered, neurons in the neocortex of ES cell chimeras. A, Antibodies to β-gal (red) reveal a cluster of neurons forming a radial column in the somatosensory cortex. No other β-gal-positive cells besides those depicted in this cluster were observed. At higher magnification of boxed area, individual neurons (arrowheads) staining for β-gal (B) are also immunoreactive for EMX1 (C). D, Double immunofluorescence shows overlapping staining (orange) of EMX1 with every radially migrating cell marked with β-gal. E, In a hemisphere devoid of columns, only scattered cells staining for β-gal (red) are seen. Boxed area is depicted in subsequent panels. F, At higher magnification, individual neurons staining (arrowheads) for β-gal (F) do not react with EMX1 antibodies (G). H, Double immunofluorescence using antibodies to both β-gal and EMX1 clearly reveal that scattered neurons (arrowheads) derived from tangential dispersion do not stain for EMX1. Scale bars: A, E, 250 μm, B, C, D, 70 μm, F, G, H, 200 μm
Figure 2.11. Expression of *Emx1* in dissociated cortical cell cultures prepared from E15 rat embryos and maintained *in vitro* for 3 days. Double-labelling experiments, using *in situ* hybridization for *Emx1* (A,B) and immunofluorescence for either glutamate (C) or GABA (D), revealed the presence of *Emx1* in glutamatergic (E) but not in GABAergic (F) neurons. Scale bar: 20 μm.
2.5. Discussion

*Emx1* is a gene that contains a homeobox analogous to that present in *empty spiracles*, a Drosophila gene expressed in the developing head (Walldorf and Gehring, 1992). Its expression is exclusively confined to the dorsal telencephalic neuroepithelium from the time of its first appearance in mouse embryos at E9.5 (Simeone *et al.*, 1992a,b; Boncinelli *et al.*, 1995; Briata *et al.*, 1996; Gulisano *et al.*, 1996). *Emx1* is one of a handful of forebrain-specific transcription factors that have been identified, and amongst the first to be expressed specifically in the dorsal telencephalon, with no detectable expression in ventral telencephalic structures (Simeone *et al.*, 1992a,b; Gulisano *et al.*, 1996; Briata *et al.*, 1996). Thus *Emx1* is generally considered to be a marker of neocortical and hippocampal structures during development of the forebrain.

It has been reported that *Emx1* transcripts and the protein product are localized in most cells of the cerebral cortex during the process of proliferation, migration, differentiation and maturation (Briata *et al.*, 1996; Gulisano *et al.*, 1996). However, these analyses have focused on the period of corticogenesis during embryonic life and in the early stages of postnatal life in mice. During the early stages of neurogenesis, *Emx1* is expressed in nearly all cells in the proliferative zone and in the other forming layers of the developing cortex. However, there were some unlabelled cells present at the later stages of corticogenesis. The proportion of such cells increased as development proceeds through the stages of differentiation and maturation in postnatal life (Gulisano *et al.*, 1996). Based on this pattern of staining, we suggest that *Emx1* is expressed only by pyramidal neurons from very early in embryonic life. There are several lines of evidence that support this hypothesis.

Experiments by Tan and colleagues (1998), using unbalanced mouse embryonic cell chimeras, have demonstrated that the specification of the pyramidal lineage occurs at the level of the progenitor, before the onset of neurogenesis. Indeed, *Emx1* first
appears in the telencephalic neuroepithelium in mouse embryos at E9.5, one day before
the generation of the first neurons of the neocortex. In addition, our present findings
have shown that this gene is expressed in clones of pyramidal neurons and not in
diffusely scattered nonpyramidal cells. Lineage studies with recombinant retroviruses
have suggested that, at the onset of neurogenesis, the ventricular neuroepithelium of the
dorsal telencephalon is populated almost exclusively by pyramidal cell progenitors and
their progeny (Parnavelas, 2000). There are very few glia progenitors at this stage
(Parnavelas, 1999), and the small number of nonpyramidal neurons generated in this
zone are most likely from progenitors of mixed potential (Mione et al., 1997; Tan et al.,
1998; Parnavelas, 2000). Other studies (Anderson et al., 1997; Lavdas et al., 1999) have
shown that the vast majority of cortical nonpyramidal cells are generated in the medial
ganglionic eminence of the ventral telencephalon, an area free of Emx1 expression.
Tracing experiments (Lavdas et al., 1999) have indicated that these neurons first appear
in the marginal zone, a layer devoid of Emx1-expressing cells, at the onset of
neurogenesis and somewhat later in all other layers of the developing cortex. So, as
cortical development proceeds, there is an increasing number of nonpyramidal neurons
and glial cells in the cortical plate which coincides with the gradual increase of Emx1-
negative cells in the cortex (Briata et al., 1996). Taken together, these studies
demonstrate that the pattern of Emx1 expression in the cortex from early embryonic life
to maturity parallels that shown by pyramidal neurons during their generation in the
dorsal ventricular zone, migration and eventual settling in the cortical plate.

Here, we used a variety of experimental approaches to confirm the identity of
the cells that express Emx1 in the developing and mature cerebral cortex. We found that,
similar to pyramidal neurons, cells expressing Emx1 were distributed in all layers except
layer I. This pattern of distribution was also similar to that of cortical neurons stained
for the neurotransmitter glutamate, a marker of pyramidal cells (Dori et al., 1989, 1992).
Further, \textit{in situ} hybridization and immunocytochemistry at the light and electron microscope levels showed that the density, distribution, soma shape, and ultrastructural features of these cells were identical to those of pyramidal neurons (Parnavelas \textit{et al.}, 1977; Peters and Kara, 1985). Immunohistochemical staining with antibodies against EMX1 and NeuN, a marker of neuronal cells, revealed that the majority of, but not all, neuronal cells expressed EMX1. Double-labelling experiments confirmed that the vast majority of \textit{Emx1}-expressing cells were also glutamate immunoreactive. We also observed that \textit{Emx1} is expressed by most of glutamate-containing neurons in dissociated cortical cell cultures and the vast majority of cells in radially arranged clones of pyramidal neurons in the cortices of chimeric mice. In summary, we have used a multitude of experimental approaches, all of which have shown that the homeobox gene \textit{Emx1} is expressed almost exclusively by pyramidal neurons from the early stages of embryonic life to adulthood and, therefore, can be reliably used as a marker of the pyramidal cell lineage.

Although we have demonstrated that the vast majority of \textit{Emx1}-expressing cells were pyramidal, a small percentage was observed to contain GABA, conventionally a marker of nonpyramidal neurons. This observation suggests that some of the cells that express this homeobox gene may be cortical interneurons. Extensive electron microscopic analysis of \textit{Emx1}-stained sections taken through the cortex of 35 day-old animals showed no labelled cells with features characteristic of nonpyramidal neurons (Fairen \textit{et al.}, 1984). However, double-labelling experiments in animals of the same age showed that slightly less than 1\% of the \textit{Emx1}-expressing cells were GABAergic. This does not necessarily imply that these cells are nonpyramidal neurons. Evidence now suggests that a small proportion of neurons in the developing and adult cerebral cortex express both neurotransmitter glutamate and GABA, markers of pyramidal and nonpyramidal neurons, respectively (Lavdas \textit{et al.}, 1996; Hill \textit{et al.}, 2000). The
percentage is highest at the early stages of development (3.7% at P7, declining to 0.6% in the adult) (Lavdas et al., 1996). It is not known why some cells in the cortex and in other areas of the brain (see Lavdas et al., 1996 for references) express both neurotransmitters. However, it is interesting to note that some GABAergic cells in the cortical plate of the rhesus monkey were found to exhibit pyramidal morphology (Schwartz and Meinecke, 1992), prompting speculation that some projection neurons and interneurons of the cortex share phenotypic traits in early development.

The possibility cannot be excluded that the GABA-containing neurons expressing Emxl represent the relatively few interneurons generated, together with all pyramidal cells, in the cortical ventricular zone (Mione et al., 1997; Parnavelas, 2000). These cells, most likely produced by progenitors of mixed potential, have the tendency to be diffusely scattered in the cortex and, similar to their pyramidal relatives, may express Emxl. Why does the proportion of these cells declines as development proceeds in postnatal life? The decline in their frequency parallels the decrease in the percentage of mixed pyramidal/nonpyramidal clones observed in the rat cerebral cortex during postnatal development, and may be due to selective cell death (see Lavdas et al., 1996 for discussion). On the basis of the present and previous findings, it may be concluded that the homeobox gene Emxl is expressed by pyramidal neurons and, possibly, by the relatively few GABA-containing cells generated in the cortical ventricular zone.

The expression of Emxl exclusively in the telencephalic cortex has prompted the suggestion that this gene plays a role within the dorsal part of the forebrain. The appearance of Emxl in the dorsal forebrain even before the generation of the first cortical neurons may suggest that this transcription factor is essential of the pyramidal phenotype. However, mutant mice that lack functional Emxl protein show only subtle differences in cortical cytoarchitecture and no change in the expression of other transcription factors, such as Otx1, Tbr1 and Id2 (Qiu et al., 1996). Further, expression
of the partially deleted *Emx1* gene in mutant mice is normal, suggesting that EMX1 is not important in regulating its own expression at the transcriptional level (Briata *et al.* 1996). One noticeable difference in the mutant mice is the reduction or absence of the corpus callosum formed by the axons of cortical pyramidal neurons located predominantly in layers II & III, V and VI (Jacobson and Trojanowski, 1974). Yoshida et al (1997) noted the absence of *Emx1* expression in a group of cells that form the glial sling, a structure that is essential for the guidance of callosal fibres (Hankin and Silver 1988). This suggests that the absence of *Emx1* in the glial sling results in the inability of the callosal fibres to cross the midline resulting in aberrant bundles of fibres. However, it is also possible that *Emx1* is required by pyramidal cells in order to help establish connections to cortical and subcortical targets, as in the case of *Otx1* which is required for the establishment of subcortical projections from layer V pyramidal neurons.
CHAPTER THREE

List of Contents

3.1. Introduction ................................................................................................................92
3.2. Materials .....................................................................................................................95
  3.2.1. Tissue fixation and processing: .........................................................................95
  3.2.2. In-situ hybridisation ...........................................................................................96
  3.2.3. Immunohistochemistry .......................................................................................97
  3.2.4. Organotypic slice cultures ...............................................................................98
3.3. Methods ......................................................................................................................99
  3.3.1. Tissue fixation and processing ..........................................................................99
  3.3.2. In-situ hybridisation ...........................................................................................99
  3.3.3. Immunohistochemistry .....................................................................................103
  3.3.4. Double immunofluorescence ............................................................................103
  3.3.5. Organotypic slice cultures ..............................................................................104
  3.3.6. Placement of Dil ...............................................................................................105
  3.3.7. Fixation of slice preparations and imaging of Dil .........................................105
  3.3.8. Immunohistochemical staining of Dil labelled slices ...................................105
3.4. Results ......................................................................................................................107
  3.4.1. Migration of neurons from the ganglionic eminence to the neocortex .....107
  3.4.2. Expression of Lhx6 mRNA in the developing cortex ...................................108
  3.4.3. Immunohistochemical localisation of GABA in the developing cortex ....108
  3.4.4. Expression of TAG-1 mRNA in the developing cortex ...............................109
  3.4.5. Immunohistochemical localisation of TAG-1 ................................................109
  3.4.6. Corticofugal fibres in tangential migration ....................................................110
  3.4.7. Role of TAG-1 in neuronal migration ..........................................................111
3.5. Discussion ................................................................................................................125
MIGRATION OF INTERNEURONS FROM THE GANGLIONIC EMINENCE INTO THE DEVELOPING NEOCORTEX

3.1. Introduction

The cerebral cortex is comprised of two principal neuronal cell types, the excitatory pyramidal cells that utilise the neurotransmitter glutamate, and the inhibitory nonpyramidal cells that utilise GABA. Previously it was generally thought that the neurons that comprise the cortex arose from the germinal ventricular zone (VZ) of the developing cortex, but recent evidence demonstrates a subcortical origin for a large number of cortical interneurons (De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999; Wichterle et al., 1999). These neurons are generated within the proliferative epithelium of the MGE and LGE, the primordium of the basal ganglia, and subsequently migrate along a tangential pathway into the developing neocortex. DiI tracing studies indicate that these cells appear to follow specific routes into, firstly the MZ, and then the IZ of the developing cortex (Lavdas et al., 1999). The cells that migrate from the MGE to the neocortex contain GABA, the neurochemical signature of cortical interneurons, and also express the LIM-homeodomain gene Lhx6 (Lavdas et al., 1999). Early migrating cells that end up in the MZ also express reelin. The homeobox genes, Dlx1 and Dlx2 have also been shown to be required for the migration of these neurons from their site of origin in the ventral telencephalon to the developing neocortex (Anderson et al., 1997).

In the developing cortex, newly generated neurons use the fibres of radial glial cells to migrate out of the cortical VZ towards the CP. These radial glial fibres span the thickness of the cerebral wall and provide a substratum for radially migrating neurons. Studies of the developing monkey cortex at the electron microscope level reveal intimate contact between migrating neurons and the radial glial fibre to which they are
attached (Rakic, 1972). Recent studies have revealed a host of proteins, such as reelin, astrotactin, and GGF, that are involved in the guidance and regulation of radial migration in the developing cortex (discussed in detail in chapter 1.3.4.). In contrast, very little is known about the molecular mechanisms that regulate the tangential migration of cortical interneurons from the MGE into the neocortex. The migration of cortical interneurons along distinct migratory pathways (Lavdas et al., 1999; Parnavelas, 2000) is suggestive of a specific guidance mechanism for this migration. Metin and Godement (1996) noted the close apposition of tangentially orientated cells in the neocortex to bundles of axons belonging to the corticofugal system. This has led to speculation that the corticofugal fibre system may be involved in guiding the migration of these cortical interneurons.

The corticofugal system is made up of cortical efferent fibres that project back to the thalamus, and it has been shown that the neural adhesion molecule of the immunoglobulin superfamily, TAG-1 (Dodd et al., 1988; Furley et al., 1990), is a marker of these cortical efferent fibres (Fukuda et al., 1997; Kawano et al., 1999). These studies revealed that TAG-1 is expressed extensively in the neocortex, with TAG-1 immunopositive fibres present in the MZ, CP, and IZ. The regions of TAG-1 immunoreactivity in the developing neocortex correlate closely to the areas in which streams of tangentially migrating cells have been observed (Anderson et al., 1997; Lavdas et al., 1999).

In this study, we sought to examine the relationship between migrating neurons originating from the MGE and the fibres of the corticofugal system using DiI tracing and immunohistochemical staining methods in organotypic slice culture preparations. We also investigated the possibility that the neural adhesion molecule TAG-1, expressed by the corticofugal fibres, may be important in the guidance of these
migrating neurons using antibodies to block the action of TAG-1 in culture preparations.
3.2. Materials

All reagents used for in-situ hybridisation were of molecular biology (RNase and DNase free) or analytical grade to prevent RNase contamination of solutions (except for cell culture).

Animals: Prenatal tissue was obtained from timed-pregnant Sprague-Dawley rats. The day on which the plug was found was designated E1 in accordance with Bayer and Altman (1991). A total of 31 litters of gestational stages E15-E18 (E15 n=10, E16 n=15, E17 n=3, E18 n=3) were used for organotypic slice cultures. A further 15 litters of gestational stages E15-E20 (E15 n=3, E16 n=3, E17 n=3, E18 n=2, E19 n=2, E20 n=2) were used for histological preparations.

3.2.1. Tissue fixation and processing:

Chemicals, reagents, and materials:

0.1 M Phosphate buffer (PB), pH 7.4 – a stock solution of 0.2 M was made by dissolving 5.93 g of NaH$_2$PO$_4$·2H$_2$O and 23.0 g of Na$_2$HPO$_4$ in 800 ml of distilled water and pH adjusted. The final volume was made up to 1000 ml.

4% paraformaldehyde in 0.1 M PB, pH 7.4 – an 8% solution was made by dissolving 8g per 100 ml of distilled water heated to 65° to which a few drops of 5 M NaOH was added until the paraformaldehyde went into solution. This was then filtered and diluted to 4% with 0.2 M PB buffer, pH 7.4.

Phosphate buffered saline (PBS), pH 7.4 – one PBS tablet was dissolved in 100 ml of distilled water.

30% Sucrose in PBS, pH 7.4 – 30 g of sucrose along with one PBS tablet was dissolved in 70 ml of DEPC-treated water. The final volume was made up to 100 ml.

7.5% Gelatin solution/15% sucrose in PBS – 150 g of sucrose was dissolved in 700 ml of distilled water to which was added 100 ml of 10× PBS stock solution. 75 g of gelatin
was added to the solution and heated until it dissolved. The final volume was made up to 1000 ml with distilled water and the solution was allowed to cool. The gelatin was stored at -20°C until use.

3% agar in PBS – 1.5 g of agar (BDH) was dissolved in 50 ml of PBS by heating until the solution boiled.

3.2.2. In-situ hybridisation

Chemicals, reagents, and other materials:

*Diethyl pyrocarbonate (DEPC) treated water* – 1 ml of DEPC was added to 1000 ml of distilled water (0.1%), incubated overnight at RT, and autoclaved before use. All solutions used for *in-situ* hybridisation were made with DEPC water.

*DIG-labelling reagents* – RNA DIG-labelling mix (10×) was obtained from Roche, restriction enzymes *EcoRI* and *HindIII*, RNA polymerases Sp6 and T7, RNAsin RNase inhibitor, and RQ1 Rnase-free DNase were from Promega.

3 M Sodium Acetate, pH 5.2 – 40.81 g of sodium acetate was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid, and made up to a final volume of 100 ml. The solution was sterilized by autoclaving.

50× Tris-Acetate-EDTA (TAE) buffer, pH 8.0 – 242 g of Trizma base (Tris[hydroxymethyl]aminomethane) was dissolved in 600 ml of distilled water. To this was added 100 ml of 0.5 M EDTA (ethylenediaminetetraacetic acid), pH 8.0, and 57.1 ml of glacial acetic acid, and made up to a final volume of 1000 ml. For working concentration, the 50× stock solution was diluted to 1× with distilled water.

0.1 M Triethanolamine buffer, pH 8.0 – a 1 M stock solution was made by dissolving 185.7 g of triethanolamine-HCl (2,2',2''-Nitrilotriethanol) in 800 ml of distilled water, adjusted to pH 8.0 with NaOH, and made up to a final volume of 1000 ml. The stock solution was diluted to 0.1 M with distilled water.
**Hybridisation buffer** – a buffer containing 50% formamide, 5× SSC (pH 7.0), 5× Denhardt's solution, 500 μg/ml salmon sperm DNA, and 250 μg/ml yeast RNA was used for prehybridisation and hybridisation.

**SSC wash buffers** – washing buffers of 5× and 0.2× SSC were prepared by diluting a 20× SSC stock solution with distilled water.

1× Tris-buffered saline (TBS), pH 7.4 – a 10× stock solution was prepared by dissolving 121.1 g of Trizma base (Tris[hydroxymethyl]aminomethane) and 87.66 g of NaCl in 800 ml of distilled water to which was added 70 ml of concentrated HCl. The pH was adjusted to 7.4 and made up to a final volume of 1000 ml. The 10× stock was diluted to 1× with distilled water.

**NTM buffer, pH 9.5** – the buffer was prepared freshly prior to use by diluting 1 M Tris buffer, pH 9.5, 5 M NaCl, and 1 M MgCl₂ stock solutions in distilled water to give a final concentration of 0.1 M Tris, 0.15 M NaCl, and 0.05 M MgCl₂.

**NBT/BCIP substrate solution** – the substrate solution was prepared by diluting 200 μl of NBT/BCIP stock solution (Roche) in 10 ml of NTM buffer.

**Equipment** – heating block, microcentrifuge, minigel apparatus, hybridisation oven.

### 3.2.3. Immunohistochemistry

Chemicals, reagents, and other materials:

**PBS, pH 7.4** - as above

**TBS, pH 7.4** – as above

**Diaminobenzidine (DAB) substrate** – a 0.025% (w/v) solution was prepared by dissolving 25 mg of DAB in 100 ml of Tris-HCl buffer, pH 7.4. Endogenous peroxidase activity was blocked by the addition of 0.01% H₂O₂.

**Immunochemicals and reagents** – Primary antibodies against GABA (Sigma), mouse monoclonal TAG-1 (1C12, 4D7) or rabbit polyclonal TAG-1 (Tg-1, Tg-2, Tg-3) were
used in this study. Biotinylated anti-Rabbit IgG secondary antibody, biotinylated anti-mouse IgG and IgM, and ABC elite kit were obtained from Vector. Normal Goat Serum (NGS, Harlan SeraLab), Streptavidin-Texas Red (Amersham Pharmacia), anti-mouse-FITC and anti-rabbit-FITC were from Molecular Probes, Vectashield mounting medium (Vector).

3.2.4. Organotypic slice cultures

Chemicals, reagents, and other materials:

Artificial Cerebro-Spinal Fluid (ACSF) – ACSF was made by dissolving 1.864 g KCl (25 mM), 0.272 g KH₂PO₄ (2 mM), 5.957 g HEPES (25 mM), 6.667 g D(+)-glucose (37 mM), 1.204 g MgSO₄ (10 mM), and 59.902 g sucrose (175mM) in 800 ml of distilled water. The pH was adjusted using 5 M NaOH, and 0.074 g CaCl₂·2H₂O (0.5 mM) and 1 ml of penicillin/streptomycin cocktail was added, and made up to a final volume of 1000 ml. The solution was sterilised by filtration through a 0.22 μm filter and stored at 4°C.

3% Agarose in PBS – 1.5 g of low-melting point agarose (Sigma) was dissolved in 50 ml of PBS by heating until the solution became clear. The 3% agarose solution was then kept warm (60°C) in a water bath until use.

DMEM/F12 medium – culture medium was prepared by adding 120 mg D-glucose, 25 μl of 200 mM glutamine, 125 μl of N2 supplement (Gibco), 50 μl of penicillin/streptomycin solution, and 5 ml of Heat-inactivated Foetal Calf Serum (FCS) to 45 ml of DMEM/F12 medium (sigma).

1,1-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine (dil) – Fine tungsten powder was coated with a solution of DiI dissolved in a small volume of dichloroethane. The dichloroethane was allowed to evaporate, leaving a coating of DiI on the tungsten particles.
3.3. Methods

3.3.1. Tissue fixation and processing

Embryonic tissue: Pregnant Sprague-Dawley rats were killed by overexposure to CO$_2$ and the uterine horns exposed. Embryos were removed and the brains dissected out carefully and placed into 4% PFA/0.1 M PB overnight at 4°C.

Frozen sections: For frozen sections, the fixed brains were cryoprotected in 30% sucrose overnight at 4°C. The brains were then embedded in 15% gelatin and quick frozen in isopentane cooled on dry ice. Frozen tissue was stored at −80°C until use.

Free-floating sections: Free-floating sections were prepared by embedding the fixed brains in 3% agar. The agar blocks were trimmed, mounted, and sectioned at 100 μm on a Vibrotome.

3.3.2. In-situ hybridisation

A. Preparation of DNA template

i. The Emx1 cDNA template (1 μg/μl) was linearised at restriction enzyme sites downstream (3’ end of the insert) of the sense and antisense sequences as follows:

2μl of the appropriate restriction enzyme buffer

2μl of Bovine Serum Albumin (BSA)

5μl of pEmx1 template plasmid

2μl of restriction enzyme EcoRI* or HindIII**

9μl of Ultrapure water

The tubes were placed in a heating block at 37°C for 2 hr.

* For sense probe, EcoRI (20 U/μl) was used as the restriction enzyme with an appropriate buffer
** For antisense probe, *HindIII* (20 U/μl) was used as the restriction enzyme with an appropriate buffer.

ii. The linearisation reaction was checked by running a sample of the reaction buffer on a 1% agarose gel. 0.5 g of agarose (Gibco) was dissolved in 1× Tris-Acetate EDTA (TAE) buffer by heating the solution until the agarose melted and the solution became clear. The agarose solution was then allowed to cool to approximately 60°C before the addition of 2.5 μl of ethidium bromide (10 mg/ml), swirled, and poured into the gel casting tray. The gel was allowed to cure for 1 hr prior to use. The gel was submerged in TAE buffer in the gel apparatus and loaded with a 1 μl sample of the linearisation reaction diluted 1:3 in gel loading buffer (Sigma). A 1 Kb DNA ladder and a sample of uncut DNA was loaded in adjacent sample lanes to check for appropriate linearisation. The gel was run at 80V for approximately 30-60 min.

iii. The linearisation reaction was stopped by the addition of 2 μl of 10× TE buffer, pH 8.0. Linearised DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the reaction buffer, mixed thoroughly, and spun at 13000 RPM for 5 min. The aqueous layer (20 μl) was removed and placed in a fresh tube, to which 10 μl of 3 M NaOAc and 70 μl of absolute alcohol was added. The contents were mixed and cooled at −20°C overnight before being centrifuged at 13000 RPM for 15 min at 4°C. The pellet was washed with ice-cold 70% ethanol, air-dried, and resuspended in 10 μl of Ultrapure water (to give approx. 0.5 μg/μl).

B. Preparation of DIG labelled riboprobes

i. *in vitro* transcription:

The cRNA probes were transcribed from the linearised template DNA as follows:
4 μl of 5× Transcription buffer

2μl of 0.2M DTT

0.5μl of RNAsin Rnase inhibitor

2μl of linearised DNA (sense or antisense template)

2μl of DIG RNA labelling mixture

2μl of T7* or Sp6** RNA polymerase

7.5μl of ultrapure water

The reaction mixture was incubated at 37°C for 2 hr.

* For sense control probe, T7 RNA polymerase was used

** For antisense probe, Sp6 RNA polymerase was used

ii. The reaction was checked by running a small sample (1 μl) on a 1% agarose gel (see above for methods).

iii. After transcription, 2 μl of RQ1 Rnase-free DNase I was added to the reaction mixture and incubated for 15 min at 37°C to remove the DNA template. The reaction was stopped by the addition of 2 μl of TE buffer, pH 8.0.

iv. The cRNA probe was precipitated by the addition of 10 μl of lithium chloride and 70 μl of absolute alcohol. The contents were mixed thoroughly and stored at –20°C overnight. The RNA was then centrifuged at 13000 RPM for 15 min at 4°C, the pellet was washed in ice-cold 70% ethanol, air-dried, and resuspended in 100 μl of ultrapure water.

C. Hybridisation of cRNA probes to RNA sequences in-situ:

i. Section pretreatments:

*In-situ* hybridisation on prenatal tissue was carried out on 20 μm cryostat sections mounted on Superfrost plus slides (BDH). Slides were removed from the storage at –80°C and allowed to warm to room temperature. The sections were fixed in 4%
PFA for 10 min at RT and washed 3× 5 min in PBS. They were then acetylated by placing them into 400 ml of TEA buffer, pH 8.0 to which 1 ml of acetic anhydride was added drop wise under constant agitation for 10 min. The slides were then rinsed briefly in PBS, permeabilised in PBS containing 1% Triton X-100 for 30 min at RT, and washed 3× 5 min in PBS.

ii. Prehybridisation/Hybridisation:

The slides were then placed horizontally in a humidified chamber and 500 µl of prehybridisation buffer (hybridisation buffer minus the cRNA probe) was laid on top and incubated for 2 hr at RT. The prehybridisation buffer was then replaced with 200 µl of hybridisation buffer containing 300-500 ng/ml of DIG-labelled cRNA probe and coverslipped with a small piece of parafilm. Prior to use, the hybridisation buffer containing the cRNA probe was heated to 80°C for 5 min and rapidly cooled on ice to denature the probe. Hybridisation was carried out for 16-20 hr at 69°C in a hybridisation oven.

iii. Washes:

On the following day the slides were washed briefly in a pre-heated 5× SSC solution to remove the coverslip, placed into a pre-heated 0.2× SSC solution, and washed at 69°C for 1-2 hr.

iv. Immunological detection:

The slides were washed for in TBS, pH 7.4 for 5 min at RT. The DIG-labelled cRNA probe was visualised using an antibody conjugated to alkaline phosphatase that was directed against the DIG molecule. The sections were blocked for 1 hr in 10% NGS diluted in TBS, pH 7.4, prior to the application of the anti-DIG-AP antibody that was diluted 1:2000 in TBS containing 1% NGS and incubated for 2 hr at RT. The slides were then washed 2× 5 min in TBS, and equilibrated for 5 min in NTM buffer, pH 9.5. A substrate solution for alkaline phosphatase, BCIP/NBT was
used to visualise the antibody. The substrate solution was diluted in NTM buffer, pH 9.5 and applied to the slides. The chromogen reaction was allowed to take place overnight at 4°C. When sufficient staining had been achieved, the reaction was stopped by washing in TE buffer, pH 8.0. Slides were then washed briefly in PBS, dehydrated through an ascending ethanol series (70%, 80%, 95%, 100% twice), cleared in Histoclear solution, and mounted in DPX mountant (BDH). Staining was visualised under brightfield illumination on a Leica DMRB microscope.

3.3.3. Immunohistochemistry

Immunohistochemical staining of sections with 4D7/TAG-1 antibody was carried out on 20 μm cryostat sections of embryonic mouse brains. Sections stored at −80°C were allowed to warm to RT and blocked for 1 hr with 10% NGS in TBS, pH 7.4 at RT. The blocking solution was replaced with primary antibody solution containing 4D7/TAG-1 antibody (1:50, mouse IgM, DSHB) diluted in TBS containing 1% NGS and 0.1% Triton X-100 and incubated overnight at 4°C. The sections were then washed 3× 5 min in TBS, and incubated in biotinylated secondary antibodies (1:200 in TBS) for 2 hr at RT. Following a further wash with TBS, the sections were incubated in ABC elite complex for 2 hr at RT. Antibody staining was revealed by incubation in DAB substrate until a desired level of colour development was obtained. Sections were then dehydrated through an ascending alcohol series, and mounted with DPX.

3.3.4. Double immunofluorescence

Double immunofluorescence was performed with antibodies against TAG-1 and GABA in free-floating sections of E16 embryonic brains. Sections were blocked in 10% NGS in TBS for 1 hr at RT with constant agitation prior to incubation with polyclonal anti-GABA (1:1000) and mouse monoclonal anti-TAG-1 (1C12, 1:10,000) diluted in 1% NGS/TBS overnight at 4°C. Following a brief wash in TBS, the sections were
incubated with anti-mouse-FITC and biotinylated anti-rabbit secondary antibodies for 2 hr at RT. Following a further wash in TBS, the sections were incubated with streptavidin-Texas Red™ for 2 hr at RT, washed, and mounted in Vectashield™ mounting medium. Sections were imaged on a Leica TCS-SP confocal microscope.

3.3.5. Organotypic slice cultures

Organotypic slice cultures were prepared from brains of embryonic rat at developmental stages E15-E18. Embryonic mice were staged with the day of plug equivalent to E1. Timed pregnant Sprague Dawley rats were killed by exposure to CO₂ and the uterine horns were removed and placed into ice-cold ACSF. Embryos were removed carefully from the uterus and placed into ACSF bubbled with oxygen. The brain was removed under a dissecting microscope and kept in cold oxygenated ACSF. To embed the brains, a 3% solution of low-melting point agarose was placed into small cylindrical moulds on ice, and allowed to cool to approximately 35-40°C. The brains were carefully transferred into the cooled agarose, orientated, and the agarose was allowed to set. The agarose block was mounted on a stage, placed into the bath containing ice cold ACSF, and sectioned at 300 μm on a Vibroslicer (Campden Instruments). The cut sections were then collected, mounted on 13 mm nitrocellulose filters (Millipore), and placed into a multiwell plate. The sections were kept moist by overlaying a small volume of ACSF. Following placement of Dil (see section 3.3.6) the slices were maintained in DMEM/F12 medium at 37°C for 24-36 hr.

For antibody blocking experiments, the slices were maintained in DMEM/F12 medium containing antibodies against TAG-1 (4D7, 1C12, 1C12 Fab fragments, Tg1, Tg2, and Tg3) and incubated at 37°C. To ensure that TAG-1 was blocked in these cultures, the medium was supplemented with fresh antibodies after 24 hr in culture.
3.3.6. Placement of Dil

Fine tungsten powder was coated with Dil prior to placement into the slice preparations. Dil crystals were dissolved in dichloroethane, mixed with a small amount of tungsten powder, and allowed to evaporate. The tip of a small glass micropipette was used to apply the Dil-coated tungsten powder to the slice, and the Dil particles were pushed slightly below the surface. Placements of Dil were made into the MGE or LGE, taking care not to place the Dil too close to the corticostriatal boundary.

3.3.7. Fixation of slice preparations and imaging of Dil

After 24-36 hr, the slice preparations were rinsed in PBS, and fixed in 4% PFA for 1-2 hr at RT. The slices were then washed thoroughly in PBS, gently removed from the filter membranes, and placed onto glass slides. They were then mounted in either hydromount (BDH) or PBS. Dil labelled cells were imaged on either a Leica TCS-SP laser scanning confocal microscope, or on a Leica DMRB microscope equipped with a cooled CCD camera (Quantix 1401E, Roper Scientific) and the Metamorph acquisition software (Universal Imaging Corp.).

3.3.8. Immunohistochemical staining of Dil labelled slices

A number of slices were also processed for immunohistochemical staining for TAG-1. In these cases, the Dil labelled slices were rinsed in PBS and then fixed in 4% PFA overnight at 4°C. The slices were then washed in PBS, removed from the filter membranes, and embedded in 3% agar. The agar blocks were then sectioned at 50 μm on a Vibrotome and collected in PBS.

The sections were blocked in 10% NGS diluted in PBS, pH 7.4 for 1 hr at RT. Blocking solution was removed and replaced with primary antibody solution containing either 4D7/TAG-1 antibody (1:50, Ms IgM) or 1C12 antibody (1:10000, Ms IgG) diluted in PBS containing 1% NGS (Triton X-100 was omitted to prevent leaching of
the Dil label) and incubated for 1 hr at RT. Sections were then transferred to 4°C and incubated for a further 16-36 hr. Following incubation sections were washed 3 x 10 min in TBS, and then transferred into biotinylated secondary antibodies (1:200 in TBS) for 2 hr at RT. Following a further wash in TBS, they were incubated in streptavidin conjugated to FITC for 2 hr at RT. They were then washed and mounted in hydromount mountant on glass slides, and imaged on a Leica TCS NT laser scanning confocal microscope.
3.4. Results

3.4.1. Migration of neurons from the ganglionic eminence to the neocortex

We traced the migration of MGE derived neurons into the neocortex using the carbocyanine marker 1,1-dioctodecyl-3,3',3',3'-tetramethylindocarbocyanine (Dil). This fluorescent marker is lipophilic and incorporates into the cell membrane without impairing normal cell functions. Tungsten particles coated with Dil were inserted into either the MGE or LGE in order to label migrating neurons derived from this region. Organotypic slice cultures were prepared from E15 to E18 rat brains and maintained in cell culture for 24-36 hr.

Injections of Dil in slices prepared from E15 rat brains labelled cells that migrated predominantly to a position just below the pial surface, contributing to the MZ population of cells (Lavdas et al., 1999). Migrating cells were easily recognised because of their characteristic long leading process, which was often branched, and their elongated ovoid cell bodies (Fig. 3.1A,B). A stream of tangentially orientated cells could often be seen emanating from the region of the ganglionic eminence, crossing the corticostriatal boundary, and directed towards the MZ of the neocortex (Fig. 3.2A). Some cells also migrated to take up positions within the newly developed IZ although these cells were usually located close to the corticostriatal boundary presumably because they had less time to migrate.

At E16, many more Dil labelled cells could be seen invading the neocortex from the ganglionic eminence. At this stage labelled cells were found to populate all layers of the developing neocortex, the VZ, the IZ, the CP, and the MZ (Fig. 3.2B). The numbers of labelled cells in each layer varied, with the majority of cells located in the IZ where they adopted a predominantly tangential orientation. The CP contained slightly fewer cells, with those located in this layer having a tangential or radial orientation. The MZ
contained few cells at this stage all of which were located just below the pia, and orientated tangentially. Interestingly, Dil labelled cells were also found in the cortical VZ, the site of proliferation of cortical neurons. Many of the cells had a tangential orientation within the VZ, but a few had a downward morphology with the leading process extending towards the ventricular surface.

3.4.2. Expression of Lhx6 mRNA in the developing cortex

The distribution of Lhx6 transcripts was also analysed by in-situ hybridisation. As previously reported, the LIM-domain gene Lhx6 is expressed in the MGE and in the developing neocortex (Grigoriou et al, 1999; Lavdas et al, 1999). At E15, expression of Lhx6 is largely restricted to cells located in the MGE and parts of the septum. At this stage, some light expression can also be detected in the MZ of the neocortex (Fig. 3.3A). At E16, two streams of Lhx6 positive cells can be seen to extend out laterally from the MGE towards the neocortex. Lhx6 positive cells in the neocortex were found to be located predominantly in the lower part of the IZ, extending from the corticostriatal boundary up as far as the dorsal cortex (fig. 3.3B).

3.4.3. Immunohistochemical localisation of GABA in the developing cortex

Previous studies have shown that many neurons that migrate tangentially into the neocortex utilise the neurotransmitter GABA (Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999; Parnavelas, 2000). An antibody against GABA was used to show the distribution of GABAergic neurons in the developing cortex at various stages of corticogenesis, from E15-E17. At E15, at a time when the cerebral wall is comprised mainly of the VZ and the SP/MZ, GABA immunoreactivity is restricted to a small number of cells located just below the pia, in a region corresponding to the presumptive MZ. Two small clusters of GABAergic neurons were observed located within the region of the corticostriatal boundary (Fig. 3.4A,B), one immediately below
the pial surface and another located in the IZ. One day later, at E16, the IZ is formed below the SP, and at this stage, a row of GABAergic neurons can be seen in the lower part of the IZ in addition to those present in the MZ (Fig. 3.5A,B), giving the impression of two streams of cells migrating into the neocortex. As corticogenesis proceeds, GABAergic neurons are found predominantly in the lower IZ, with scattered cells located in the VZ, CP and MZ (Fig. 3.6A,B). The majority of GABAergic neurons found in the IZ had a similar morphology, with a long leading process that was sometimes branched, and an ovoid, elongated cell soma. The leading process was nearly always directed towards the dorsal cortex and appeared to indicate the direction of migration. The morphology of these cells also resembled that of Dil labelled migrating neurons observed in slice culture preparations (Fig. 3.1).

3.4.4. Expression of TAG-1 mRNA in the developing cortex

The expression of TAG-1 was examined at various developmental stages (E15-E20) in the rat forebrain. In-situ hybridisation using a riboprobe against the TAG-1 mRNA transcripts was carried out in coronal sections through the embryonic brain. At E15, expression can be detected in a thin stripe of cells located in the marginal zone, just beneath the pial surface (Fig. 3.7A). One day later, at E16, a second band of TAG-1 expressing cells can be seen in the IZ, extending from the dorsal cortex to the region of the corticostriatal boundary (Fig. 3.7B). The same pattern was seen at subsequent stages of embryonic development (E17-E20, data not shown).

3.4.5. Immunohistochemical localisation of TAG-1

TAG-1 immunohistochemistry, using the mouse monoclonal 4D7 antibody (Yamamoto et al., 1986), was performed in order to visualise the location of the cortical efferent fibres that expressed this protein. The distribution of TAG-1 expressing corticofugal fibres correlated closely with the expression domain of the TAG-1 gene.
Immunochemical staining with the 4D7 antibody was performed in coronal sections through the cortex of E15-E19 rat cortices. At E15, a single row of TAG-1 immunoreactive cells and axons could be seen in the MZ, just below the pia (Fig. 3.8A). One day later, at E16, another band of cells and axons were labelled, this time in the IZ. These fibres were seen to project towards the ganglionic eminence, but stopped in a sharp boundary in the region of the internal capsule (Fig. 3.8B). At E17, distinct fascicles of radially orientated fibres could be seen spanning the thickness of the newly formed CP (Fig. 3.9A). At this stage, immunoreactivity could be seen in the MZ and IZ, where TAG-1 fibres were arranged tangentially, and in the CP where the fibres were arranged radially, as if connecting the IZ and MZ. The same staining pattern was observed at later stages (Fig. 3.9B).

### 3.4.6. Corticofugal fibres in tangential migration

As previously reported by Metin and Godement (1996), tangentially orientated cells located in the IZ of the neocortex are often seen to be closely apposed to the fibres of the corticofugal system. To determine whether migrating cells originating from the MGE were associated with the corticofugal fibres, we used a combination of organotypic slice culture and immunohistochemical staining for TAG-1.

Coronal slices were taken through the brains of E15 - E17 rat embryos and the lipophilic dye, DiI, was placed into the ganglionic eminence. The slices were then maintained in culture for 36 hr in order to allow cells to migrate into the neocortex from the MGE. Immunohistochemical staining with an antibody against TAG-1 was performed to reveal the location of the corticofugal fibres in these slice preparations. TAG-1 immunoreactivity appeared to be normal in these preparations and were comparable to the distribution seen in fixed sections of a equivalent age. At E15, DiI labelled cells were typically found in the MZ with leading processes pointing in the
direction of migration. Immunohistochemical staining of TAG-1 showed that these Dil labelled cells and their leading process were usually found amongst bundles of TAG-1 positive axons that ran tangentially just beneath the pia. Experiments were then performed on slices taken through E16 embryos, a stage when TAG-1 immunoreactivity can also be seen in the IZ. Dil labelled cells could be seen in the MZ but, in addition, a number of migrating cells were observed in the lower part of the IZ, in close association with tangentially orientated fibres positive for TAG-1 (Fig. 3.10A). In slices prepared from E17 embryos, Dil labelled cells were also seen in the cortical plate, with the leading process orientated in a radial direction, perpendicular to the pial surface (Fig. 3.10B). The leading processes of these Dil labelled cells were found to be in close apposition to the radially arrange bundles of TAG-1 positive fibres that are present in the CP at this age.

Previous reports have shown that cells migrating into the neocortex from the LGE/MGE express the neurotransmitter molecule GABA (Anderson et al., 1997; Lavdas et al., 1999). We, therefore, performed double immunohistochemistry with antibodies against GABA and TAG-1 in order to investigate the relationship between these GABAergic cells and the corticofugal fibres. At E16, we found that the majority of GABAergic cells, with features characteristic of migrating neurons, were located within the IZ, amongst a band of tangentially orientated TAG-1 IR fibres (Fig. 3.11A). At E17 and later stages, GABAergic neurons were found mainly within the IZ but also scattered throughout the CP and MZ. These cells were located within areas where TAG-1 IR fibres were also located and the majority of GABAergic cells appeared to be in close apposition to the fibres (Fig. 3.11B).

3.4.7. Role of TAG-1 in neuronal migration

Recent reports have shown that the fibres of the corticofugal system express the neural cell adhesion molecule TAG-1 (Fukada et al., 1997; Kawano et al., 1999). We,
therefore, sought to investigate whether this molecule was involved in the migration of
cortical interneurons from the MGE to the neocortex. Organotypic slice cultures were
prepared from E16 rat embryos, labelled with Dil, and cultured in the presence of
various TAG-1 antibodies. Antibodies of the same isotype as the TAG-1 antibodies
were used in control cultures to eliminate the possibility that any effects seen may be
due to the presence of the antibody molecules themselves. After 36 hr in culture, the
slices were examined to determine if there had been any effect on the migration of
cortical interneurons. Migration of MGE cells in slices cultured in the presence of TAG-
1 antibodies appeared to be disrupted, with a marked decrease in the number of Dil
labelled cells going into the neocortex (Fig. 3.12A,B). This effect was seen with both
monoclonal (4D7 and 1C12) and polyclonal (Tg1 and Tg3) antibodies although one
polyclonal antibody, Tg2, did not appear to have any effect. Control experiments using
monoclonal and polyclonal antibodies of the same isotype as the TAG-1 antibodies did
not block migration.

Immunohistochemical staining for GABA was also performed on slice culture
preparations that had been incubated in TAG-1 antibodies. Control cultures showed
large numbers of GABAergic cells throughout the thickness of the cortical wall, the
majority of which had features characteristic of migrating neurons. In contrast, TAG-1
treated cultures had very few GABAergic neurons present in the cortex, but instead
large numbers of GABAergic cells were clustered in the region of the internal capsule.
The effect was most profound when slices were cultured in the presence of the 4D7
monoclonal antibody, with almost no GABAergic neurons present in the cortex (Fig.
3.12C,D).
Figure 3.1. Confocal microscope images of single DiI labelled cells in organotypic slice culture preparations. DiI was placed into the MGE or LGE and labelled cells were allowed to migrate out of the injection site. Migrating cells are easily recognised by their characteristic long leading process and elongated ovoid cell bodies. (B) Some migrating neurons had two leading processes, with a slightly more rounded cell body. Arrows point to branch points in the leading process. Scale bar: 30 μm.
Figure 3.2. Confocal microscope images of organotypic slice cultures labelled with DiI. DiI labelled cells migrate predominantly into the MZ of the cortex in slices prepared from E15 rat embryos. A steady stream of cells can be seen emanating from the site of DiI placement in the ganglionic eminence heading towards the neocortex. The broken white line demarcates the position of the ventricle. (B) In slices prepared from E16 rat embryos, the majority of DiI labelled cells migrate into the IZ of the neocortex. A small number of cells are also visible in the CP and VZ. Scale bar: 100 µm.
Figure 3.3. Expression of the LIM-homeodomain gene Lhx6 in coronal sections through the developing rat forebrain. (A) At E15, Lhx6 is expressed in high levels in the MGE and the region of the septum. (B) At E16, expression is still strong in the MGE and septum, but expression is also detected in the LGE and neocortex. Two streams of Lhx6-positive cells appear to extend out laterally from the MGE, passing through the LGE, and into the MZ and IZ of the neocortex. Scale bar: 100 μm.
Figure 3.4. Confocal microscope images of GABA immunohistochemistry on coronal sections through E15 rat brains. (A) Two streams of GABAergic neurons can be observed in the region of the corticostriatal boundary. (B) Higher power of the same region shows two groups of labelled cells are visible in this region, one located just below the pial surface, and the other in the IZ of the neocortex in the region of the corticostriatal notch. Scale bars: A, 100 μm, B, 50 μm.
Figure 3.5. Confocal microscope images of GABA immunohistochemistry on coronal sections through E16 rat brains. (A) Two streams of GABAergic neurons can be observed in the neocortex. One group of cells is located in the MZ, just below the pial surface, whilst another group of cells can be seen populating the lower IZ. (B) At higher magnification, GABA expression was predominantly seen in the MZ, and IZ, with scattered cells in the CP. The GABAergic cells located in the IZ have distinct morphologies, with leading processes pointing towards the dorsal cortex, whereas cells located in the CP display a variety of orientations. Scale bars: A, 100 μm, B, 50 μm.
Figure 3.6. (A) Confocal microscope images of GABA immunohistochemistry on coronal sections through E17 rat brains. Three different GABAergic populations are visible in the neocortex, located in the MZ, IZ, and CP. (B) This distribution seen at higher magnification. Note that at this age, the number of GABAergic neurons in the CP has increased, with cells orientated in various directions. Scale bars: A, 100 μm, B, 50 μm.
Figure 3.7. Expression of TAG-1 mRNA in the coronal sections through the developing rat forebrain. (A) At E15, expression is strong in a thin stripe of cells located in the MZ, just below the pial surface. (B) At E16, a second band of expression can be seen in the IZ of the developing neocortex. LGE – Lateral ganglionic eminence, MGE – Medial ganglionic eminence, NCX – Neocortex. Scale bar: 100 µm.
Figure 3.8. Light micrograph illustrating the expression of TAG-1 in the developing rat forebrain. Immunohistochemistry for TAG-1 in coronal sections of E15 (A) and E16 (B) rat cortex. TAG-1 is expressed by the developing corticofugal fibres. (A) Labelled cells in the PP project to the ventral telencephalon in the region of the IC. (B) Labelled axons, oriented tangentially in the MZ and IZ, and radially in the CP. IC – Internal capsule, IZ – Intermediate zone, NCX – Neocortex. Scale bar: 100 μm
Figure 3.9. Light micrograph illustrating the expression of TAG-1 in the developing rat forebrain. Immunohistochemistry for TAG-1 in coronal sections of E17 (A) and E19 (B) rat cortex. At these later stages of development, TAG-1 immunoreactivity is absent in the MZ, but is still present in fibres located in the IZ, orientated tangentially, and the CP, arranged radially. CP – cortical plate, IZ – Intermediate zone. Scale bar: 100 μm.
Figure 3.10. Confocal microscope images of tissue sections double-labelled with DiI (red) and TAG-1, a marker of corticofugal fibres (green). (A) Tangentially migrating neurons in close association with TAG-1 fibres in the IZ. (B) DiI labelled migrating neuron in the CP, with the leading process oriented perpendicular to the pial surface and in close apposition to the radially arranged bundles of the TAG-1 immunopositive axons. Scale bars: 100 μm.
Figure 3.11. Double immunohistochemistry with antibodies against GABA (red) and TAG-1 (green), in coronal sections through E15 (A) or E17 (B) rat forebrain. (A) Tangentially oriented GABAergic neurons dispersed amongst TAG-1 labelled axons, in the IZ. (B) GABAergic neurons and TAG-1 labelled fibers in the IZ, CP and MZ. In IZ and CP, cells show tangential orientation, whereas, in the CP, they tend to be radially organized apposed to TAG-1 positive axons. The arrows point to TAG-1 immunolabelled, radially oriented fibres. Scale bars: 100 μm.
Figure 3.12. TAG-1 mediates the tangential migration of MGE neurons along the fibres of the corticofugal system. The migration of the MGE cells was examined in cortical slice cultures by placing a crystal of Dil in the LGE (A,B), as well as by using immunohistochemistry against GABA (C,D). Cortical slices were cultured for two days in the presence of antibodies against TAG-1 (A,C) or control antibodies (B,D). Very few Dil labelled migrating neurons (A) and GABAergic neurons (C) are detected in the cortex when slices are cultured in the presence of antibodies against TAG-1 in E15 rat (A) or E13.5 mouse (C), when compared to the controls (B and D respectively). Scale bar: 100 µm
through the IZ and accumulate just below the SP. At this age, the differences in cortical size were less apparent than at E13.5, but we found that the length of the cortical arm was reduced on average by 10%. Cell counts obtained from rostral, medial, and caudal regions were adjusted to reflect this decrease in cortical size, and analysed. Statistical analysis, using the student's t-test, revealed no significant differences between the mutant and WT in rostral and medial regions, but there was a significant reduction in the number of the number of calbindin IR cells in mutant caudal cortex (student's t-test, two-tail: \( p < 0.05 \)) (Table 3 and Fig. 4.21). Despite the apparent lack of difference in calbindin IR cells between WT and mutant cortex in the rostral and medial regions, we observed a difference in the distribution of cells in the different layers of the cortex when the proportion of cells located in each layer was calculated (Fig. 4.22). This revealed differences in the spatial location of the calbindin IR cells in the mutant cortex in comparison to the WT. The most apparent difference was in the upper IZ, where on average 25% of calbindin IR cells were located in this region in the mutant, but this population made up only 12% of cells in the WT (Fig. 4.22). In addition, the SP/CP also contained a smaller proportion of calbindin IR cells in the mutant (30%) in comparison to the WT (40%). This is reflected in the thickness of the CP, which appears to be reduced in the mutant cortex.
medial, and caudal regions demonstrating a consistent reduction along the whole rostrocaudal length (Table 2, Fig. 4.16). The proportion of cells located in each layer of the cortex was also analysed (Fig. 4.17). No significant differences in the proportion of cells in the VZ, lower IZ, or upper IZ, were observed although a proportion of cells in the WT were located in the SP/CP, a layer that was not present in the mutant cortex.

In order to determine whether the rate of migration had changed, we made a plot of the number of cells located in each counting bin vs. the bin number, and extrapolated the data by linear regression (Fig. 4.18). At this early stage, the calbindin IR cells located in the IZ have not yet fully penetrated the full length of the cortex, and the leading neurons can clearly be seen. If it is assumed that these migrating neurons move at a steady rate, the number of neurons in each 200 μm bin should be related to the rate at which these cells migrate. Therefore, extrapolation of the data and plotting the linear regression curve will provide an indication of the rate of migration, although this value is arbitrary and does not impart a real indication of the actual dynamics of cell migration. We found that there was a drop in the rate of cell migration in all cortical regions studied, as indicated by the distance these cells had moved into the cortex (Fig. 4.15). Calbindin IR cells had penetrated much further into the cortex in comparison to the mutant, indicating that they were travelling at a faster rate. The fall in rates of migration was not uniform, with the rostral cortex showing the least amount of change and the caudal cortex showing the most (Fig. 4.18).

At E15.5, calbindin IR cells were found through the full thickness of the cerebral wall, predominately located in the lower IZ and the SP/CP, whilst the VZ and upper IZ had much fewer cells (Fig. 4.19). Calbindin immunoreactivity was also observed in bundles of fibres located in the upper IZ, just below the SP, in the WT cortex, but did not appear to be present in the mutant cortex (Fig. 4.19 and Fig 4.20). These calbindin IR fibres may be part of the thalamocortical fibre system, which invade the cortex
In order to visualise these migrating neurons, we performed immunohistochemical staining for calbindin on sections through the cortices of E13.5 and E15.5 WT/heterozygous and homozygous knockout embryos. Calbindin IR cells had morphology typical of migrating neurons (Fig. 4.14A-D). The majority of cells located in the intermediate zone had a long leading process, often branched, and an elongate, ovoid cell body (Fig. 4.14C,D), other cells appeared to have two long leading processes (Fig. 4.14B). A small number of cells in the ventricular had a downward morphology, with the tip of the leading process in contact with the ventricle (Fig. 4.14A). The number of calbindin immunoreactive cells in each layer of the developing cortex, the VZ, the IZ, split into upper and lower regions, and the SP/CP were counted in a series of 200 μm bins extending from the corticostriatal notch up to the most dorsal aspect of the cortex. The data tested using the Shapiro-Wilks W-test, which showed that the number of cells in the cortex was distributed normally, thus allowing the use of parametric statistical tests.

At E13.5, Calbindin IR cells with morphology typical of migrating neurons were observed in both WT and mutant cortices (Fig. 4.15A,B). They were predominately found in a stream of cells located in the lower IZ, with some cells also being present in the upper IZ, and in the VZ. Calbindin IR cells were also found in the early SP/CP, but were absent in the mutant, which did not appear to have a developed SP/CP. In addition, the length of the cortical arm was reduced by 30-50%, with the caudal cortex showing the most dramatic reductions. Thus, the cell counts obtained from rostral, medial, and caudal points through the cortex were adjusted accordingly to reflect this decrease. The normalised data was analysed statistically, using the student’s t-test, which showed that there was a significant reduction in the number of calbindin IR cells found in the cortices of mutant embryos in comparison to the WT (student’s t-test, two-tail: Rostral p < 0.01; Medial p < 0.01; Caudal p < 0.01). This reduction was observed in the rostral,
samples taken at the time of dissection. DiI was placed into the VZ of the MGE and slices were cultured for 36 hrs. The slice preparations were examined on a laser scanning confocal microscope, and images were captured of each cerebral hemisphere.

Confocal images of each hemisphere of each slice were analysed, and the number of DiI labelled cells in each layer of the developing cortex, the VZ, the IZ, the CP, and the MZ were counted (Fig. 4.9A,B). In these experiments, the number of DiI labelled cells were compared only between littermates in order to eliminate variations that may occur due to slight differences in developmental age and in culture conditions. At the developmental stages studied, there is an approximately 25% reduction in the external dimensions of the mutant cerebral cortex in comparison to the WT (Fig. 4.10 and Fig. 4.11). We, therefore, normalised the data by adjusting the cell counts from mutant slices by the appropriate factor to reflect this decrease in cortical volume. The number of DiI labelled cells in mutant and WT slices were analysed and compared statistically using the non-parametric Mann-Whitney U-test for two independent groups which revealed that there was a significant reduction between the number of migrating neurons in mutant and WT embryos (Table 1, Fig. 4.12). We also looked at the number of cells migrating into the various layers of the developing cortex. The majority of cells migrated into IZ, with fewer cells found in the CP, MZ, and VZ in accordance with previous reports (Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999; Parnavelas, 2000). No differences in the number of cells migrating into the MZ or VZ were observed. However, there was a significant reduction in the number of cells found in the CP and the IZ, with the latter showing the largest decreases (Fig. 4.13).

4.4.6. Localisation of Calbindin expressing cells in the neocortex of Emx2 mutants

Previous studies have shown that cortical interneurons migrating from the ganglionic eminence into the cortex express Dlx, Lhx6, GAD67, GABA, calbindin, and calretinin (DeDiego et al., 1994; Anderson et al., 1997; Lavdas et al., 1999; Sussel et
corticofugal fibres may project to abnormal positions in the basal telencephalon. We would therefore expect to see alterations in the immunohistochemical localisation of TAG-1 in the brains of mutant animals. However, immunohistochemical staining in sections through E13.5 (Fig. 4.5A,B) and E15.5 (Fig. 4.6A,B) brains revealed a very similar pattern of expression, with no apparent changes in the trajectory of the corticofugal fibres.

4.4.5. Expression of L1 protein in Emx2<sup>−/−</sup> mutants

The path taken by thalamocortical fibres, those that originate from the thalamic nuclei, was examined by immunohistochemical staining with an antibody against L1, a cell adhesion molecule that is expressed by cortical afferents (Fukuda et al., 1997). Staining of sections through E15.5 cortices of WT and homozygous knockout animals revealed alterations in the trajectory taken by the thalamocortical fibres. In WT sections taken through caudal cortex, weak L1 IR was observed in the IZ of the cortex, and in the dorsal thalamus, in addition to a strongly labelled bundle of fibres in the region of the internal capsule, connecting the dorsal thalamus and cortex (Fig. 4.7A and Fig. 4.8A). In contrast, corresponding sections through Emx2<sup>−/−</sup> cortex revealed an aberrant bundle of L1 IR fibres connecting the dorsal thalamus to the basal telencephalon, with little or no L1 IR detectable in the cortex (Fig. 4.7B and Fig. 4.8B). Staining of sections through the whole length of the telencephalon revealed a failure of the L1 IR thalamocortical fibres to round the corticostriatal notch and enter into the cortex.

4.4.6. Migration of cortical interneurons in Emx2<sup>−/−</sup> mutants

We studied the migration of MGE derived cells into the neocortex in organotypic slice culture preparations labelled with Dil. Coronal slices were taken through the brains of E13.5 and E14.5 mouse embryos from Emx2 mutant litters. Genotyping was carried out visually and later confirmed by PCR genotyping of tissue
anterograde labelling of cortical efferent fibres. Injections of DiI into the rostral cortex revealed no apparent differences between wildtype (WT) (Fig. 4.3A,C) and mutant brains (Fig. 4.3B,D), with retrograde labelling of cell bodies located in the ventrobasal complex (VBC) (Fig. 4.3A-D). In contrast, injections into the caudal cortex of WT and mutant brains showed alterations in thalamocortical connections. DiI placed into WT cortex resulted in retrograde labelling of cell bodies located in the lateral geniculate nucleus (LGN) (Fig. 4.3E) but the same injections into mutant cortex resulted in labelling of cells in the presumptive VBC (Fig. 4.3F).

Interestingly, although no differences in thalamocortical connections could be seen in the rostral cortex, the corticofugal fibres appeared to be altered in the mutant brains. In addition to retrograde labelling the thalamocortical fibres, injections into the rostral cortex also labelled a small aberrant bundle of fibres projecting out of the cortex that is not seen in the WT brain (Fig. 4.4B). In the WT brain, DiI labels the fibres of the anterior commissure, which normally round the base of the ventricle (Fig. 4.4A). However, in the mutant brain, these anterior commissural fibres are absent or misrouted, with a bundle of DiI-labelled fibres directed towards the base of the telencephalon (Fig. 4.4B).

4.4.4. Immunohistochemical localisation of TAG-1 protein in Emx2−/− mutants

Previous studies have shown that the lack of Emx2 results in abnormalities in some forebrain structures. The most striking features are the severe reduction in size of the cortical hemispheres and the disruption in cortical lamination (Yoshida et al., 1997; Mallamaci et al., 2000a). We, therefore, sought to investigate whether the abnormal development of the neocortex would also result in alterations in the connectivity between the cortex and subcortical structures, primarily the thalamus. We examined the trajectory of corticofugal fibres in mutant brains by immunohistochemical staining with an antibody against TAG-1. DiI tracing in these mutants indicate that a number of these
4.4. Results

4.4.1. Expression of Emx2 in the developing telencephalon

The distribution of Emx2 transcripts was previously analysed in detail in the developing cortex by Gulisano *et al.* (1996). Here, we describe the expression of this gene in the cortex as well other areas of the developing telencephalon of the rat. At E15, strong expression of Emx2 was observed in the developing neocortex and hippocampus (Fig. 4.2A). In the cortex, expression spanned almost the entire thickness of the cerebral wall, which at this stage consists mainly of the germinal neuroepithelium or VZ, and the early-generated MZ (Fig. 4.2A). As development continues, the cerebral wall expands with the formation of the IZ and CP. At E16, the expression of Emx2 is confined to the proliferating neuroblasts that populate the VZ and in cell bodies located within the MZ (Fig 4.2B), with no transcripts detectable in cells of the IZ and CP. Emx2 also shows graded expression in a high caudal-low rostral and high medial-low lateral gradients.

Emx2 is also expressed in parts of the ventral telencephalon during forebrain development. At E16 expression of Emx2 is detected in the germinal zones of the LGE and MGE and the septum, although at levels below that observed in the cortical VZ (Fig. 4.2B). Expression of Emx2 in these regions continues through later stages of development. In addition to this, a second expression domain can be seen at E16 in the ventrolateral part of the telencephalon, covering a small area just below the pia. This paleocortical region corresponds to the medial part of the amygdala, and expression in this region persists through to birth.

4.4.5. Corticothalamic and thalamocortical connections in Emx2<sup>−/−</sup> mutants

The connections between the thalamus and the cortex were studied by making placements of Dil crystals into rostral and caudal parts of the cortex. Placements of Dil into the cortex results in retrograde labelling of neuronal cell bodies in the thalamus and
(see section 4.3.2) the slices were maintained in DMEM/F12 medium at 37°C for 24-36 hrs.

4.3.7. Placement of Dil

Fine tungsten powder was coated with Dil prior to placement into the slice preparations. Dil crystals were dissolved in dichloroethane, mixed with a small amount of tungsten powder, and allowed to evaporate. The tip of a small glass micropipette was used to apply the Dil-coated tungsten powder to the slice, and the Dil particles were pushed slightly below the surface. Placements of Dil were made into the MGE, close to the lateral ventricle.

4.3.8. Fixation of slice preparations and imaging of Dil

After 24-36 hr, the slice preparations were rinsed in PBS, and fixed in 4% PFA for 1-2 hr at RT. The slices were then washed thoroughly in PBS, gently removed from the filter membranes, and placed onto glass slides. They were then mounted in either hydromount (BDH) or PBS. The Dil was imaged on either a Leica TCS-NT confocal microscope using the Krypton (568 nm) laser line, or on a Leica DMRB equipped with a cooled CCD camera (Quantix 1401E, Roper Scientific) and the Metamorph acquisition and analysis software (Universal Imaging Corp.).
ascending alcohol series, and mounted with DPX. Images were captured on a Leica DMRB microscope equipped with a cooled CCD camera using the Metamorph image acquisition software.

For cell counting, sections through the cortex were divided into a series of 200 μm bins, starting from the position of the corticostriatal notch. Cells located in the VZ, lower IZ, upper IZ, and the SP/CP were counted and data analysed statistically using the GB-stat software.

4.3.6. Organotypic slice cultures

Organotypic slice cultures were prepared from brains of embryonic mice at developmental stages E13.5-E15.5. To generate homozygous mutant mice, a heterozygous male was mated with a heterozygous female, and pups were born in a normal Mendelian ratio. Embryonic mice were staged with the day of plug equivalent to E0.5. Timed pregnant Emx2 mice were killed by cervical dislocation and the uterine horns were removed and placed into ice-cold ACSF. Embryos were carefully removed with their yolk sacs intact and placed into individual dishes with ACSF bubbled with oxygen. The yolk sac was removed carefully and kept for genotyping. The brain was removed under a dissecting microscope and kept in cold oxygenated ACSF (brains were assigned numbers to maintain identity with yolk sac samples). To embed the brains, a 3% solution of low-melting point agarose was placed into small cylindrical moulds on ice, and allowed to cool to approximately 35-40°C. The brains were carefully transferred into the cooled agarose, orientated, and the agarose was allowed to set. The agarose block was mounted on a stage, placed into the bath containing ice cold ACSF, and sectioned at 300 μm on a Vibroslicer. The cut sections were then collected, mounted on 13 mm nitrocellulose filters, and placed into a multiwell plate. The sections were kept moist by overlaying a small volume of ACSF. Following placement of Dil
340 bp corresponds to heterozygous samples, and those containing one band at 340 bp corresponds to homozygous mutant samples.

4.3.4. Dil tracing in fixed brains

Small crystals of Dil were placed into the rostral (presumptive parietal) or caudal (presumptive occipital) cortex of E19 WT and \textit{Emx2}\textsuperscript{−/−} mutant embryos. The brains were dissected out and fixed in 4\% paraformaldehyde for 24-48 hr before rinsing in PBS. A small hole was made in the injection site using an insect pin, and a small crystal of Dil was inserted into the hole. Brains were transferred into 2\% paraformaldehyde and incubated at RT for 3-4 weeks in the dark. Following several washes in PBS, the brains were embedded in 3\% agarose and sectioned at 100 \textmu m on a Vibrotome. Sections were mounted onto slides, coverslipped with Vectashield\textsuperscript{TM} mounting medium containing DAPI, and imaged on a Leica TCS-SP confocal microscope.

4.3.5. Immunohistochemistry

Immunohistochemical staining of sections with TAG-1 and calbindin antibodies was carried out on 20 \textmu m cryostat sections of embryonic mouse brains. Sections stored at \textminus 80°C were allowed to warm to RT and blocked for 1 hr with 10\% NGS in TBS, pH 7.4 at RT. The blocking solution was replaced with primary antibody solution containing either 4D7/TAG-1 antibody (1:50, mouse IgM, DSHB) or calbindin antibody (1:1000, Swant) diluted in TBS containing 1\% NGS and 0.1\% Triton X-100 and incubated overnight at 4°C. The sections were then washed 3× 5 min in TBS, and incubated in biotinylated secondary antibodies (1:200 in TBS) for 2 hr at RT. Following a further wash with TBS, the sections were incubated in ABC elite complex for 2 hr at RT. Antibody staining was revealed by incubation in DAB substrate until a desired level of colour development was obtained. Sections were then dehydrated through an
**Genotyping of embryonic mice**

Screening of embryonic mice was carried out by coamplification of DNA fragments corresponding to the WT and mutant *Emx2* gene sequences. To achieve this, one forward primer was used for both WT and mutant amplification, but two different primer sequences, corresponding to the WT and the mutant sequences, were used as reverse primers. Genomic DNA was isolated from tissue samples collected from embryonic mice and amplified as follows:

<table>
<thead>
<tr>
<th>Stock</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× TaqPol buffer (MgCl₂ free)</td>
<td>5 µl</td>
<td>1×</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>3 µl</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>2 mM dNTPs</td>
<td>5 µl</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>8 µM E2F oligo primer</td>
<td>5 µl</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>8 µM E2R/WT oligo primer</td>
<td>5 µl</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>8 µM mg-AS oligo primer</td>
<td>5 µl</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>50 ng/µl genomic DNA</td>
<td>2 µl</td>
<td>100 ng</td>
</tr>
<tr>
<td>Ultrapure H₂O</td>
<td>15 µl</td>
<td></td>
</tr>
<tr>
<td>5 U/µl TaqPol</td>
<td>0.5 µl</td>
<td>2.5 U</td>
</tr>
</tbody>
</table>

The PCR mixture was overlaid with 50 µl of mineral oil to prevent evaporation during the amplification cycle. The PCR reaction was carried out in a Hybaid PCR machine using the following program: 98°C for 5 min, 1×; 98°C for 1 min, 72°C for 2 min, 5×; 94°C for 1 min, 72°C for 2 min, 30×; 72°C for 10 min, 1×.

The PCR products were analysed on a 2% agarose gel (Fig. 4.1B). The amplified WT sequence is approximately 180 bp and the mutant gene sequence is about 340 bp. Gel lanes with one band at 180 bp corresponds to WT samples, two bands at 180 bp and
isolated from mouse tail preparations were used as templates for amplification as follows:

<table>
<thead>
<tr>
<th>Stock</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× TaqPol buffer (MgCl₂ free)</td>
<td>5 μl</td>
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<tr>
<td>25 mM MgCl₂</td>
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<td>1.5 mM</td>
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<tr>
<td>2 mM dNTPs</td>
<td>5 μl</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>6 μM neo-S oligo primer</td>
<td>5 μl</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>6 μM neo-AS oligo primer</td>
<td>5 μl</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>4 μM mg-S oligo primer</td>
<td>5 μl</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>4 μM mg-AS oligo primer</td>
<td>5 μl</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>50 ng/μl genomic DNA</td>
<td>2 μl</td>
<td>100 ng</td>
</tr>
<tr>
<td>Ultrapure H₂O</td>
<td>15 μl</td>
<td></td>
</tr>
<tr>
<td>5 U/μl TaqPol</td>
<td>0.5 μl</td>
<td>2.5 U</td>
</tr>
</tbody>
</table>

The PCR mixture was overlaid with 50 μl of mineral oil to prevent evaporation during the amplification cycle. The PCR reaction was carried out in a Hybaid PCR machine using the following program: 95°C for 5 min, 1×; 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, 35×; 72°C for 10 min, 1×.

The PCR products were run on a 2% agarose gel to analyse the results (Fig. 4.1A). The myogenin gene fragment (internal positive control) results in lighter fragment in the 150-250 bp size range, whilst the neomycin gene product produces a heavier fragment in the same size range. Thus, gel lanes with only one band close to 150 bp correspond to wildtype (WT) samples, and those containing two bands, one heavy and one light, correspond to the heterozygous samples.
used to visualise the antibody. The substrate solution was diluted in NTM buffer, pH 9.5 and applied to the slides. The chromogen reaction was allowed to take place overnight at 4°C. When sufficient staining had been achieved, the reaction was stopped by washing in TE buffer, pH 8.0. Slides were then washed briefly in PBS, dehydrated through an ascending ethanol series (70%, 80%, 95%, 100% twice), cleared in Histoclear solution, and mounted in DPX mountant (BDH). Staining was visualised under brightfield illumination on a Leica DMRB microscope.

4.3.3. Genotyping of \textit{Emx2} mice

\textbf{A. Extraction and purification of genomic DNA}

Genomic DNA was isolated from tail samples (adult mice) or from the yolk sac (embryos) for use in PCR genotyping. The tissue samples were placed into lysis buffer containing 0.5 mg/ml proteinase K, and incubated overnight at 55°C with agitation. The DNA was purified by phenol/chloroform extraction. An equal volume of Phenol:chloroform:isoamyl alcohol (25:24:1) was added to the lysis buffer and mixed gently by inversion for 5 min. The tube was centrifuged at 13000 RPM for 5 min and the top aqueous layer was removed and placed into a fresh tube. To precipitate the DNA, 1 ml of ice-cold absolute alcohol was added which resulted in the appearance of a flocculus. The tubes were gently inverted until the flocculus disappeared, and centrifuged at 13000 RPM for 15 min. The pellet was washed with ice-cold 70% ethanol, air-dried, and resuspended in 100 µl of 10 mM Tris buffer, pH 7.4.

\textbf{B. Polymerase chain reaction}

\textbf{Genotyping of adult mice}

Screening of heterozygous adult mice was performed by co-amplification of the neo sequence and the myogenin sequence by polymerase chain reaction. Genomic DNA
PFA for 10 min at RT and washed 3× 5 min in PBS. They were then acetylated by placing them into 400 ml of TEA buffer, pH 8.0 to which 1 ml of acetic anhydride was added drop wise under constant agitation for 10 min. The slides were then rinsed briefly in PBS, permeabilised in PBS containing 1% Triton X-100 for 30 min at RT, and washed 3× 5 min in PBS.

**ii. Prehybridisation/Hybridisation:**

The slides were then placed horizontally in a humidified chamber and 500 µl of prehybridisation buffer (hybridisation buffer minus the cRNA probe) was laid on top and incubated for 2 hr at RT. The prehybridisation buffer was then replaced with 200 µl of hybridisation buffer containing 300-500 ng/ml of DIG-labelled cRNA probe and coverslipped with a small piece of parafilm. Prior to use, the hybridisation buffer containing the cRNA probe was heated to 80°C for 5 min and rapidly cooled on ice to denature the probe. Hybridisation was carried out for 16-20 hr at 69°C in a hybridisation oven.

**iii. Washes:**

On the following day the slides were washed briefly in a pre-heated 5× SSC solution to remove the coverslip, placed into a pre-heated 0.2× SSC solution, and washed at 69°C for 1-2 hr.

**iv. Immunological detection:**

The slides were washed for in TBS, pH 7.4 for 5 min at RT. The DIG-labelled cRNA probe was visualised using an antibody conjugated to alkaline phosphatase that was directed against the DIG molecule. The sections were blocked for 1 hr in 10% NGS diluted in TBS, pH 7.4 prior to the application of the anti-DIG-AP antibody that was diluted 1:2000 in TBS containing 1% NGS and incubated for 2 hr at RT. The slides were then washed 2× 5 min in TBS, and equilibrated for 5 min in NTM buffer, pH 9.5. A substrate solution for alkaline phosphatase, BCIP/NBT was
0.5 µl of RNasin Rnase inhibitor

2 µl of linearised DNA (sense or antisense template)

2 µl of DIG RNA labelling mixture

2 µl of T7* or Sp6** RNA polymerase

7.5 µl of ultrapure water

The reaction mixture was incubated at 37°C for 2 hr.

* For sense control probe, Sp6 RNA polymerase was used

** For antisense probe, T7 RNA polymerase was used

ii. The transcription products were checked on a 1% agarose gel (see above for methods).

iii. After transcription, 2 µl of RQ1 Rnase-free DNase I was added to the reaction mixture and incubated for 15 min at 37°C to remove the DNA template. The reaction was stopped by the addition of 2 µl of TE buffer, pH 8.0.

iv. The cRNA probe was precipitated by the addition of 10 µl of lithium chloride and 70 µl of absolute alcohol. The contents were mixed thoroughly and stored at −20°C overnight. The RNA was then centrifuged at 13000 RPM for 15 min at 4°C, the pellet was washed in ice-cold 70% ethanol, air-dried, and resuspended in 100 µl of ultrapure water.

C. Hybridisation of cRNA probes to RNA sequences in-situ:

Prenatal Tissue:

i. Section pretreatments:

        In-situ hybridisation on prenatal tissue was carried out on 20 µm cryostat sections mounted on Superfrost plus slides (BDH). Slides were removed from the storage at −80°C and allowed to warm to room temperature. The sections were fixed in 4%
ii. The linearised DNA was checked on a 1% agarose gel to ensure efficient restriction of the plasmid template. 0.5 g of agarose (Gibco) was dissolved in 1x Tris-Acetate EDTA (TAE) buffer by heating the solution until the agarose melted and the solution became clear. The agarose solution was then allowed to cool to approximately 60°C before the addition of 2.5 μl of ethidium bromide (10 mg/ml), swirled, and poured into the gel casting tray. The gel was allowed to cure for 1 hr prior to use. The gel was submerged in TAE buffer in the gel apparatus and loaded with a 1 μl sample of the linearisation reaction diluted 1:3 in gel loading buffer (Sigma). A 1 Kb DNA ladder and a sample of uncut DNA was loaded in adjacent sample lanes to check for appropriate linearisation. The gel was run at 80V for approximately 30-60 min.

iii. The linearisation reaction was stopped by the addition of 2 μl of 10× TE buffer, pH 8.0. Linearised DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the reaction buffer, mixed thoroughly, and spun at 13000 RPM for 5 min. The aqueous layer (20 μl) was removed and placed in a fresh tube, to which 10 μl of 3 M NaOAc and 70 μl of absolute alcohol was added. The contents were mixed and cooled at −20°C overnight before being centrifuged at 13000 RPM for 15 min at 4°C. The pellet was washed with ice-cold 70% ethanol, air-dried, and resuspended in 10 μl of Ultrapure water (to give approx. 0.5 μg/μl).

B. Preparation of DIG labelled riboprobes

i. *in vitro* transcription:

The cRNA probes were transcribed from the linearised template DNA as follows:

4 μl of 5× Transcription buffer

2 μl of 0.2 M DTT
4.3. Methods

4.3.1. Tissue fixation and processing

*Embryonic tissue*: Pregnant mice were killed by cervical dislocation and the uterine horns exposed. Embryos were removed and the brains dissected out carefully and placed into 4% PFA/0.1 M PB overnight at 4°C.

*Frozen sections*: For frozen sections, the fixed brains were cryoprotected in 30% sucrose overnight at 4°C. The brains were then embedded in 15% gelatin and quick frozen in isopentane cooled on dry ice. Frozen tissue was stored at −80°C until use.

4.3.2. *In-situ* hybridisation

A. Preparation of DNA template

i. The *Emx2* cDNA template (1μg/μl) was linearised at restriction enzyme sites downstream (3' end of the insert) of the sense and antisense sequences as follows:

2 μl of the appropriate restriction enzyme buffer

2 μl of Bovine Serum Albumin (BSA)

5 μl of pEmx2 template plasmid

2 μl of restriction enzyme *EcoRI* or *HindIII***

9 μl of Ultrapure water

The tubes were placed in a heating block at 37°C for 2 hr.

* For sense probe, *HindIII* (20 U/μl) was used as the restriction enzyme with an appropriate buffer

** For antisense probe, *EcoRI* (20 U/μl) was used as the restriction enzyme with an appropriate buffer.
Diaminobenzidine (DAB) substrate – a 0.025% (w/v) solution was prepared by dissolving 25 mg of DAB in 100 ml of Tris-HCl buffer, pH 7.4. Endogenous peroxidase activity was blocked by the addition of 0.01% H₂O₂.

Immunochemicals and reagents – Primary antibodies against Calbindin (Swant), mouse monoclonal TAG-1 (4D7), and rat monoclonal L1 were used in this study. Biotinylated anti-mouse IgM, biotinylated anti-rat IgG, and ABC elite kit were obtained from Vector, and Normal Goat Serum (NGS, Harlan Seralab).

4.2.5. Cell culture

Chemicals, reagents, and other materials:

Artificial Cerebro-Spinal Fluid (ACSF) – ACSF was prepared by dissolving 1.864 g of KCl (25 mM), 0.272 g of KH₂PO₄ (2 mM), 5.957 g of HEPES (25 mM), 6.667 g of D-Glucose (37 mM), 1.204 g of MgSO₄ (10 mM), and 59.902 g of sucrose (175 mM) in 800 ml of distilled H₂O. The pH was adjusted to 7.4 before the addition of 0.07351 g of CaCl₂.2H₂O (0.5 mM), and 1 ml of 100× Penicillin/streptomycin (5000 U and 5 mg/ml). The volume was made up to 1000 ml and filtered through a 0.22 μm sterile filter.

3% Agarose in PBS – 1.5 g of low-melting point agarose (Sigma) was dissolved in 50 ml of PBS by heating until the solution became clear. The 3% agarose solution was then kept warm (60°C) in a water bath until use.

DMEM/F12 medium – culture medium was prepared by adding 120 mg D-glucose, 25 μl of 200 mM glutamine, 125 μl of N2 supplement (Gibco), 50 μl of 100× penicillin/streptomycin solution, and 5 ml of heat-inactivated Foetal Calf Serum (FCS) (Gibco) to 45 ml of DMEM/F12 medium (Sigma).

1,1-dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine (dil) – Fine tungsten powder was coated with a solution of Dil dissolved in a small volume of dichloroethane. The dichloroethane was allowed to evaporate, leaving a coating of Dil on the tungsten particles.
Genotyping of *Emx2* embryos

Oligonucleotide primer sequences – for the genotyping of embryonic mice, one forward primer and 2 reverse primers were used, with one primer corresponding to a sequence contained within the *Emx2* allele, and the other primer corresponding to a sequence contained within the neo cassette. The sequences used for the primers were as follows:

*Emx2F* 5' CAC AAG TCC CGA GAG TTT CCT TTT GCA CAA CG 3'

*Emx2R/WT* 5' ACC TGA GTT TCC GTA AGA CTG AGA CTG TGA GC 3'

*Emx2R/KO* 5' ACT TCC TGA CTA GGG GAG GAG TAG AAG GTG G 3'

Lysis buffer, pH 8.0 – lysis buffer was made by diluting stock solutions to a final concentration of 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% SDS. To this was added DNase-free proteinase K (Sigma) to a concentration of 0.5 mg/ml.

DNA purification – Phenol:Chloroform:Isoamyl alcohol (25:24:1) (Sigma), Absolute alcohol, 70% ethanol.

Polymerase chain reaction (PCR) - 10× thermophilic reaction buffer, 2 mM dNTPs, 25 mM MgCl₂, 5 U/μl Taq Polymerase were from Promega.

2% agarose gel – a 2% agarose gel was prepared by dissolving 1 g of agarose in 50 ml of TAE buffer by heating until the solution was clear. The gel solution was allowed to cool to 60°C before addition of 2.5 μl of 10 mM ethidium bromide and mixed by swirling. The gel was then poured into the minigel apparatus and allowed to cure for 1 hr prior to use.

Equipment – heating block, microcentrifuge, PCR machine (Hybaid), Minigel apparatus.

4.2.4. Immunohistochemistry

Chemicals, reagents, and other materials:

*PBS, pH 7.4* - as above

*TBS, pH 7.4* – as above
SSC wash buffers – washing buffers of 5× and 0.2× SSC were prepared by diluting a 20× SSC stock solution with distilled water.

1× Tris-buffered saline (TBS), pH 7.4 – a 10× stock solution was prepared by dissolving 121.1 g of Trizma base (Tris[hydroxymethyl]aminomethane) and 87.66 g of NaCl in 800 ml of distilled water to which was added 70 ml of concentrated HCl. The pH was adjusted to 7.4 and made up to a final volume of 1000 ml. The 10× stock was diluted to 1× with distilled water.

NTM buffer, pH 9.5 – the buffer was prepared freshly prior to use by diluting 1 M Tris buffer, pH 9.5, 5 M NaCl, and 1 M MgCl₂ stock solutions in distilled water to give a final concentration of 0.1 M Tris, 0.15 M NaCl, and 0.05 M MgCl₂.

NBT/BCIP substrate solution – the substrate solution was prepared by diluting 200 μl of NBT/BCIP stock solution (Roche) in 10 ml of NTM buffer.

Equipment – heating block, microcentrifuge, minigel apparatus, hybridisation oven.

4.2.3. Genotyping

Chemicals, reagents, and materials:

Genotyping of Emx2 colony

Oligonucleotide primer sequences – for the genotyping of adult mice, 2 primer sets were used to amplify a sequence contained within the neomycin cassette (neo), and an internal myogenin (Mg) control sequence The sequences used for the primers were as follows:

Neo-S 5’ CTT TTT GTC AAG ACC GAC CTG TCC 3’
Neo-AS 5’ CCG CAT TGC ATC AGC CAT GAT GG 3’
Mg-S 5’ CCC CCA AGT TGG TGT CAA AAG CC 3’
Mg-AS 5’ ATG CTC TCT GCT TTA AGG AGT CAG 3’
3% agar in PBS – 1.5 g of agar (BDH) was dissolved in 50 ml of PBS by heating until the solution boiled.

4.2.2. In-situ hybridisation

Chemicals, reagents, and other materials:

Diethyl pyrocarbonate (DEPC) treated water – 1 ml of DEPC was added to 1000 ml of distilled water (0.1%), incubated overnight at RT, and autoclaved before use. All solutions used for in-situ hybridisation were made with DEPC water.

DIG-labelling reagents – RNA DIG-labelling mix (10×) was obtained from Roche, restriction enzymes EcoRI and HindIII, RNA polymerases Sp6 and T7, RNAsin RNase inhibitor, and RQ1 Rnase-free DNase were from Promega.

3 M Sodium Acetate, pH 5.2 – 40.81 g of sodium acetate was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid, and made up to a final volume of 100 ml. The solution was sterilized by autoclaving.

50× Tris-Acetate-EDTA (TAE) buffer, pH 8.0 – 242 g of Trizma base (Tris[hydroxymethyl]aminomethane) was dissolved in 600 ml of distilled water. To this was added 100 ml of 0.5 M EDTA (ethylenediaminetetraacetic acid) (pH 8.0) and 57.1 ml of glacial acetic acid, and made up to a final volume of 1000 ml. For working concentration, the 50× stock solution was diluted to 1× with distilled water.

0.1 M Triethanolamine buffer, pH 8.0 – a 1 M stock solution was made by dissolving 185.7 g of triethanolamine-HCl (2,2',2''-Nitrilotriethanol) in 800 ml of distilled water, adjusted to pH 8.0 with NaOH, and made up to a final volume of 1000 ml. The stock solution was diluted to 0.1 M with distilled water.

Hybridisation buffer – a buffer containing 50% formamide, 5× SSC (pH 7.0), 5× Denhardt's solution, 500 μg/ml salmon sperm DNA, and 250 μg/ml yeast RNA was used for prehybridisation and hybridisation.
4.2. Materials

All reagents used for *in-situ* hybridisation were of molecular biology (RNase and DNase free) or analytical grade to prevent RNase contamination of solutions (except for cell culture).

*Animals:* A total of 24 timed pregnant female C57/B16 mice were used in this study (E12.5 n=2, E13.5 n=7, E14.5 n=10, E15.5 n=3, E19 n=2). The day on which the plug was found was designated E0.5.

4.2.1. Tissue fixation and processing:

Chemicals, reagents, and materials:

0.1 M Phosphate buffer (PB), *pH* 7.4 — a stock solution of 0.2 M was made by dissolving 5.93 g of NaH$_2$PO$_4$.2H$_2$O and 23.0 g of Na$_2$HPO$_4$ in 800 ml of distilled water and *pH* adjusted. The final volume was made up to 1000 ml.

4% paraformaldehyde in 0.1 M PB, *pH* 7.4 — an 8% solution was made by dissolving 8 g per 100 ml of distilled water heated to 65°C to which a few drops of 5 M NaOH was added until the paraformaldehyde went into solution. This was then filtered and diluted to 4% with 0.2 M PB buffer, *pH* 7.4.

*Phosphate buffered saline (PBS), *pH* 7.4* — one PBS tablet was dissolved in 100 ml of distilled water.

*Sucrose solutions* — sucrose solutions were made by dissolving 18 g (18% w/v) or 30 g (30% w/v) of sucrose in 60 ml of distilled water. One PBS tablet was added and the solution was made up to a final volume of 100 ml.

7.5% Gelatin solution/15% sucrose in PBS — 150 g of sucrose was dissolved in 700 ml of distilled water to which was added 100 ml of a 10x PBS stock solution. 75 g of gelatin was added to the solution and heated until it dissolved. The final volume was made up to 1000 ml with distilled water and the solution was allowed to cool. The gelatin was stored at −20°C until use.
MZ and IZ of the cortex. Thus, disruptions in these corticofugal fibres may result in abnormal migration of the MGE-derived neurons. A recent study by Mallamaci et al. (2000b) demonstrated errors in the direction taken by thalamocortical fibres during early stages of development, with fibres being directed towards the basal telencephalon.

In this study we sought to investigate whether Emx2 may play a role in the migration of MGE-derived cortical neurons. We used DiI to retrogradely label thalamocortical fibres in fixed brains of Emx2 mutant to examine abnormalities in this fibre system. To test whether the lack of Emx2 may result in disruptions in tangential migration, we used DiI to label and trace MGE cells in organotypic slice culture preparations.
mutant, this defect appears to occur during later stages of corticogenesis (Mallamaci et al., 2000a). Thus, without reelin, newly generated cortical neurons that migrate radially out from the cortical VZ are unable to overcome earlier generated cells in the CP and settle below them, similar to that observed in the reeler mouse (Caviness and Sidman, 1973; Ogawa et al., 1995).

Recent work by Anderson et al (1997) has demonstrated the dependence of this tangential migration on the Dlx1 and Dlx2 homeobox genes, although it is not known how these genes function to promote the migration of cortical interneurons. It has also been suggested that the LIM-homeobox gene, Lhx6, may also be involved in the regulation of migration of cells from the MGE to the neocortex (Lavdas et al., 1999). The Lhx6 homeobox gene is expressed heavily in the MGE, with expression spreading into the neocortex a few days later. The appearance of Lhx6 in the neocortex correlates spatially and temporally with the migration of cells into the neocortex as determined by DiI tracing experiments (Lavdas et al., 1999). In the ganglionic eminence of the ventral telencephalon, there is an overlap of expression between Emx2 and Dlx1 and Dlx2 in the proliferative VZ of the ganglionic eminence (Gulisano et al., 1996; Bulfone et al., 1993a,b). Thus, it is possible that Emx2 may also have a role in regulating the tangential migration of cortical interneurons from their site of generation in the ganglionic eminence into the developing neocortex.

Recently, there has been speculation that the fibres of the corticofugal system may play a key role in the migration of the MGE-derived neurons into the neocortex (Metin and Godement, 1996; Anderson et al., 1999; Parnavelas, 2000). In chapter 3, I described our work investigating the role of these corticofugal fibres in providing a pathway for these migrating cells, and the importance of the neural cell adhesion molecule TAG-1 in these process. MGE-derived cortical interneurons appear to enter this fibre system in the region of the internal capsule, and follow their trajectory into the
Emx2 IN THE DEVELOPMENT OF THE NEOCORTEX

4.1. Introduction

*Emx2*, a member of the *Emx* homeobox gene family, is expressed early in telencephalic development, with its expression domain including the cortical VZ where it is expressed heavily. In addition, there is expression in the VZ of the MGE and LGE, although at lower levels than that seen in the cortical VZ. The widespread expression of *Emx2* in the proliferative VZ of the cortex suggested a role in cell proliferative (Simeone *et al.*, 1992a,b), with later studies demonstrating a gradient of expression along both the rostro-caudal and dorso-lateral axes, with strong expression in the posterior dorsal cortex and gradually decreasing in anterior and ventrolateral regions (Gulisano *et al.*, 1996; Mallamaci *et al.*, 1998). The expression gradient of *Emx2* is in the opposite gradient to that followed by neurogenesis, which has a rostro-lateral maximum and a caudo-medial minimum (Bayer and Altman, 1991). Thus, it was suggested that *Emx2* may have a role in suppressing cell proliferation or promoting cell differentiation, and may contribute to regulating cortical arealisation (Rakic, 1988; O'leary, 1989). Recent studies have demonstrated that cortical arealisation in *Emx2*\textsuperscript{+/-} mutants is disrupted along the rostro-caudal axes, with the expansion of rostral cortical areas and contraction of caudal ones (Mallamaci *et al.*, 2000b; Bishop *et al.*, 2000).

Additional functions of *Emx2* have also been described in the developing neocortex. Defects in cortical lamination have been described in *Emx2*\textsuperscript{+/-} mutant embryos (Pellegrini *et al.*, 1996; Yoshida *et al.*, 1997) as well as defects in the hippocampus. A recent study by Mallamaci *et al.* (2000a) demonstrated a reeler-like phenotype in the *Emx2*\textsuperscript{+/-} mutant cortex, with inversion of cortical layers although unlike the reeler mutation, the PP is split into the MZ and SP. The defects in cortical lamination result from the lack of reelin signalling in the MZ, although in the *Emx2*\textsuperscript{+/-}
CHAPTER FOUR

List of Contents

4.1. Introduction ................................................................. 133
4.2. Materials........................................................................ 136
  4.2.1. Tissue fixation and processing:................................. 136
  4.2.2. In-situ hybridisation ................................................. 137
  4.2.3. Genotyping ............................................................. 138
  4.2.4. Immunohistochemistry .............................................. 139
  4.2.5. Cell culture ............................................................ 140
4.3. Methods.......................................................................... 141
  4.3.1. Tissue fixation and processing .................................. 141
  4.3.2. In-situ hybridisation ................................................. 141
  4.3.3. Genotyping of Emx2 mice ....................................... 145
  4.3.4. Dil tracing in fixed brains ........................................ 148
  4.3.5. Immunohistochemistry .............................................. 148
  4.3.6. Organotypic slice cultures ....................................... 149
  4.3.7. Placement of Dil ..................................................... 150
  4.3.8. Fixation of slice preparations and imaging of Dil ....... 150
4.4. Results........................................................................... 151
  4.4.1. Expression of Emx2 in the developing telencephalon .... 151
  4.4.5. Corticothalamic and Thalamocortical connections in Emx2+/− mutants .... 151
  4.4.4. Immunohistochemical localisation of TAG-1 protein in Emx2+/− mutants . 152
  4.4.5. Expression of L1 protein in Emx2+/− mutants .................. 153
  4.4.6. Migration of cortical interneurons in Emx2+/− mutants ........ 153
  4.4.6. Localisation of Calbindin expressing cells in the neocortex of Emx2+/− mutants ....................................................... 154
4.5. Discussion ................................................................. 183
demonstrated that the cell adhesion molecule TAG-1, expressed by the corticofugal fibres, provides an attractive substrate for neurons migrating from the ganglionic eminence to the neocortex.
Expression in the mouse has also been associated with the developing cranial nerves and some central fibre tracts, to the developing corpus callosum, and to precerebellar neuronal migratory streams (Yamamoto et al., 1986; Wolfer et al., 1994).

Presumably, contact between the migrating neurons and corticofugal fibres is mediated by receptors or adhesion molecules that are able to bind to TAG-1. The effect of blocking antibodies may therefore come about due to the binding of the antibody to the TAG-1 molecule located on the surface of corticofugal fibres, and thus inhibiting the interaction between migrating neurons and the TAG-1 fibres. We do not know the molecular mechanisms behind the recognition of TAG-1 by its receptors, although both homophilic and heterophilic binding of TAG-1 has been shown to take place in vivo and in vitro (Felsenfeld et al., 1994; Suter et al., 1995; Tsiotra et al., 1996; Buchstaller et al., 1996; Dhar-majotra et al., 1998; Lustig et al., 1999). Immunohistochemical analysis does not show the presence of TAG-1 IR on the migrating neurons, suggesting that homophilic binding is not likely to be the mechanism by which these cells respond to TAG-1. Interestingly, a cluster of GABAergic cells with features characteristic of migrating neurons was observed in the region of the internal capsule. This area corresponds to the region where the fibres of the corticofugal system appear to terminate as revealed by TAG-1 IR. This suggests that in the absence of TAG-1, the migrating neurons may be unable to recognise or utilise the corticofugal fibres, resulting in the suspension of migration, and become “stranded” in the LGE.

The present study provides evidence from tracing and immunohistochemical analyses, and from functional experiments to show that cortical interneurons generated in the ganglionic eminence utilise the corticofugal system as a scaffold for directed migration into the different layers of the developing cortex. In addition, we have
(Fishell and Hatten, 1991) so it is conceivable that TAG-1 may be important in tangential migration. To test the role of TAG-1 in tangential migration, we used blocking antibodies to TAG-1 in slice culture preparations labelled with Dil. We found that the addition of TAG-1 antibodies to the culture medium resulted in a dramatic decrease in the number of Dil labelled cells migrating into the neocortex. Reductions in the number of migrating neurons were observed in cultures incubated in both monoclonal and polyclonal antibodies although the effect varied between different antibodies. In contrast, we found that in control cultures incubated in the presence of antibodies of the same isotype migration appeared normal. Thus, the migration of neurons is not hindered by the presence of antibody molecules in the culture medium, but is impaired in the presence of antibodies specific to TAG-1. Immunohistochemical staining of slice cultures incubated in TAG-1 antibody for GABA revealed a large decrease in the numbers of GABAergic neurons present in the neocortex.

The impairment of migration by TAG-1 antibodies suggests that this cell adhesion molecule plays an important role in tangential migration. TAG-1 protein has been localised in many fibre systems as well as in migrating neurons in the developing CNS of a variety of species (Yamamoto et al., 1986; Dodd et al., 1988; Wolfer et al., 1994; Bailly et al., 1995). It has been suggested that TAG-1 may be involved in adhesive processes required for migration based on its expression on migrating neurons, especially at points of contact between the migrating neuron and the axons that they migrate upon (Yamamoto et al., 1990; Wolfer et al., 1994; Bailly et al., 1995). TAG-1, and its chicken homologue axonin-1, is expressed by extending axons (Ruegg et al., 1989a,b; Stoeckli et al., 1985), and has been shown to be associated with the development of ascending and commissural spinal fibre systems, spinal nerves, and the retinotectal system in rat (Dodd et al., 1988; Furley et al., 1990; Chen and Chiu, 1992; Vaughn et al., 1992; Yoshihara et al., 1995), mouse (Wolfer et al., 1994), and chick
cells and fibres located in the IZ, with the fibres projecting towards the ganglionic eminence. These fibres ended in a large fan-shaped pattern in the region of the internal capsule. Also at this stage, with the splitting of the preplate by the newly arrived CP neurons, distinct fascicles of TAG-1 immunopositive fibres were seen to span the thickness of the CP. At later stages, the expression of TAG-1 was mainly restricted to the IZ and CP. The distribution of TAG-1 seen in the cortex is in accordance with previous reports of TAG-1 expression (Wolfer et al., 1994; Fukuda et al., 1997; Kawano et al., 1999), and correlates to results of tracing studies of corticofugal fibre development (Molnar and Cordery, 1999; Auladell et al., 2000).

The temporal and spatial appearance of TAG-1 immunopositive fibres in the developing telencephalon correlates closely to the appearance of Lhx6 positive cells and GABAergic neurons in the neocortex. We investigated whether the migrating neurons may utilise the fibres of the corticofugal system using DiI labelling and immunohistochemical staining in slice culture preparations. Migrating neurons were labelled with DiI and allowed to migrate away from the ganglionic eminence and into the neocortex. The location of the corticofugal fibres was revealed by immunohistochemical staining with an antibody against TAG-1 and immunofluorescence. We found that DiI labelled cells in the neocortex were closely apposed to fibres that were TAG-1 immunopositive, with their leading processes orientated along the direction of the TAG-1 fibres. These results suggested that migrating neurons derived from the MGE may utilise the fibres of the corticofugal system as a substrate upon which they migrate into the neocortex.

We also investigated whether TAG-1, a neural cell adhesion molecule belonging to the immunoglobulin superfamily expressed on corticofugal fibres, may be involved in the process of tangential migration into the neocortex. Cell adhesion molecules, such as astrotactin, have been shown to play an important role in radial migration in the cortex.
that appear to be in the process of migration. At later stages (E17 onwards), some scattered cells are also detectable in the CP, with most cells orientated radially as opposed to the tangential orientation of MZ and IZ cells.

The mechanisms that guide the migration of cortical neurons from the ventral to the dorsal telencephalon are not well understood. Presumably there must be some sort of guidance mechanism that these cells follow in order to reach their correct position within the cortex. In the neocortex, fibres of radial glial cells that span the thickness of the cerebral wall provide a substratum for newly generated neurons to migrate radially towards their final position within the cortical plate. However, there is uncertainty as to whether radial glial fibres also play a role in nonradial migration in the neocortex as described by O'Rourke et al. (1992, 1995, 1997), and it has been suggested that axons may provide a substratum for nonradial migration (Rakic, 1985; Gray, 1990). Godement and Metin (1996) noted the close apposition of GABAergic neurons to fibres of the corticofugal system, and suggested that these fibres may play an important role in guiding these migrating neurons. The corticofugal fibre system would provide an ideal guidance cue for cells migrating into the cortex as fibres of the corticofugal system extend the whole length of the cortex.

In this study, we investigated the possibility that neurons migrating from the ganglionic eminence may utilise the fibres of the corticofugal system to navigate their way into the neocortex. We studied the development of the corticofugal fibres by immunohistochemical staining with an antibody directed against TAG-1 (Dodd et al., 1988; Furley et al., 1990). TAG-1, a neural cell adhesion molecule belonging to the immunoglobulin superfamily, is expressed on the axons of cortical efferent fibres (Fukuda et al., 1997; Kawano et al., 1999). During early stages of development (E14-15 rat), TAG-1 expression is restricted to a single row of cells and their axons in the MZ, just below the pia. A few days later, at E16, TAG-1 immunoreactivity was also seen in
form the MGE into the neocortex, although whether or not all migrating neurons express this gene is not known. We studied the expression of this gene by in-situ hybridisation in coronal sections through the brains of E15 and E16 rat embryos. *Lhx6* is expressed abundantly in the ventral telencephalon, in a large region corresponding to the MGE. At E15, *Lhx6* transcripts were not readily detectable in the germinal VZ of the MGE, nor was it present in the LGE. At E16, *Lhx6* was still abundantly expressed in the MGE, but transcripts were also detected in the LGE and in the IZ of the neocortex, but at lower levels. Interestingly, the expression of this gene revealed two streams of *Lhx6* positive cells leaving the MGE, one from the dorsolateral and the other from the ventrolateral aspect of the MGE. The two streams of *Lhx6* cells appeared to converge in the region of the internal capsule, combining into a narrower stream of cells entering the MZ and IZ. The presence of these two streams may suggest that cells destined for the MZ and IZ may originate from slightly different parts of the MGE, or that different parts of the MGE may provide different subpopulations of GABAergic neurons to the neocortex. At later stages, *Lhx6* was still abundant in the MGE, and continued to be expressed at lower levels in the neocortex.

In accordance with these previous reports, immunohistochemical staining with an antibody against GABA in fixed sections through the neocortex revealed cells with morphology characteristic of Dil labelled migrating neurons, with a long leading process and elongated cell body. At E15, these GABAergic neurons were restricted mainly to the MZ of the neocortex, with long leading processes that were directed towards the dorsal cortex. Embryonic day 16 (E16) marked the appearance of a row of GABA-positive neurons in the lower part of the IZ. These GABAergic cells had morphology typical of migrating neurons with leading processes directed towards the dorsal cortex. Thus, immunohistochemical localisation of GABA at this age gives an impression of two streams of GABAergic cells, one in the MZ and the other in the IZ,
3.5. Discussion

The results of this investigation clearly show that a significant number of cortical interneurons arise from the ganglionic eminence and subsequently migrate to take up positions within the developing neocortex. During early stages of neurogenesis, subcortically generated neurons migrate to take up positions within the PP or MZ of the neocortex (Fig. 2A, Lavdas et al., 1999; Parnavelas, 2000). As development proceeds, later born cortical interneurons migrate to populate all layers of the neocortex although the majority are found within the IZ (Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999; Parnavelas, 2000). However, these cells appear to assume different orientations dependent upon the layer in which they are found. Migrating cells in the MZ and IZ are orientated tangentially, usually with long leading processes directed towards the dorsal cortex. In the CP, Dil labelled cells were often found to be orientated either radially or obliquely, with their leading process directed towards the pia, and were rarely orientated tangentially. The cortical VZ also contained a small number of Dil labelled cells, most of which were tangentially orientated. Interestingly, of cells found within the VZ, a small number also adopted a “downward” morphology with a long leading process that was directed towards the ventricular surface.

Previous reports have shown that the subcortically generated neurons that migrate to the neocortex utilise the neurotransmitter GABA (Anderson et al., 1997; Lavdas et al., 1999; Parnavelas, 2000), and express the calcium binding protein calbindin (Anderson et al., 1997). In addition, these neurons also express the LIM-homeobox gene Lhx6 (Lavdas et al., 1999), and those that end up in the MZ also express Reelin (Lavdas et al., 1999).

The LIM homeobox gene Lhx6 has been proposed as a molecular marker of tangentially migrating neurons originating from the MGE. Lavdas et al. (1999) demonstrated the presence of this transcription factor in Dil labelled cells that migrated
Figure 4.1. Gel analysis of PCR products. (A) Genotyping of adult mice to distinguish between WT and $Emx2^{+/-}$ heterozygous animals. Arrows indicate bands in the 150-250 bp, the lighter band corresponds to the myogenin internal control, and the heavier band corresponds to the inserted neomycin gene (knockout allele). Samples in lanes 2 and 6 are WT whilst lanes 1, 3, 4, and 5 are heterozygous. (B) Genotyping of embryonic mice to distinguish between WT, $Emx2^{+/-}$ heterozygous and $Emx2^{-/-}$ mutant embryos. Arrows indicate bands at 180 bp (WT allele) and 340 bp (mutant allele). Samples in lane 5 are WT, lanes 2, 3, and 7 are heterozygous, and lanes 1, 4, 6, and 8 are homozygous mutants.
Figure 4.2. Light micrographs illustrating the distribution of $Emx2$ mRNA transcripts in sections through the developing rat telencephalon at (A) E15 and (B) E16. Strong expression of $Emx2$ is observed in the developing cortex and hippocampus, with weaker expression in the ventral telencephalon. (A) Sagittal section through E15 cortex showing a clear gradient of expression along the rostrocaudal axes, with higher levels of expression in the hippocampus and caudal cortex. (B) Coronal section through E16 cortex shows a gradient of expression along the mediolateral axes, with highest levels of expression in the hippocampus and dorsal cortex. HP – Hippocampal anlage, LGE – lateral ganglionic eminence, MGE – Medial ganglionic eminence, NCX – Neocortex, PA – Pallidum. Scale bar: A, B, 100 μm
Figure 4.3. Dil tracing of thalamocortical connections in E19 WT and $Emx2^{-/-}$ mutant embryos. Small crystals of Dil placed into the presumptive parietal cortex (A,B, asterisks), retrogradely labelled cell bodies located in the presumptive ventrobasal complex (VBC) in the dorsal thalamus (C,D). Placements of crystals into the presumptive occipital cortex retrogradely labelled cells in the lateral geniculate nucleus (LGN) in WT brains (E), but labelled cells in the ventrobasal complex in $Emx2^{-/-}$ mutants (F). Scale bar, 500µm.
Figure 4.4. Dil tracing of thalamocortical and corticothalamic connections in E19 WT and $Emx2^{+/-}$ mutant embryos. Placements of Dil into the presumptive parietal cortex labelled fibres connecting the thalamus and cortex, and those of the anterior commissure. (A) In WT brains bundles of fibres are directed towards the thalamus and the anterior commissure (asterisk), but (B) fibres appear to be directed towards the basal telencephalon in $Emx2^{+/-}$ mutants (asterisk). Scale bar, 200 μm
Figure 4.5. Immunohistochemical localisation of TAG-1 in coronal sections through E13.5 WT and Emx2<sup>−/−</sup> cortex. TAG-1 is expressed by the developing corticofugal fibres. (A) TAG-1 positive fibres present in the IZ of the WT cortex. These fibres are present along the whole length of the cortical arm and terminate in the region of the internal capsule. (B) In the Emx2<sup>−/−</sup> mutant cortex, TAG-1 fibres are present in the IZ. IC – Internal capsule, IZ – Intermediate zone, LGE – lateral ganglionic eminence, MGE – Medial ganglionic eminence, NCX – Neocortex. Scale bar, 100 μm.
Figure 4.6. Immunohistochemical localisation of TAG-1 in coronal sections through E15.5 WT and $Emx2^{+/−}$ cortex. Labelled axons, orientated tangentially in the IZ and MZ, and radially in the CP, are present in both the (A) WT and (B) $Emx2^{+/−}$ mutants. CP, Cortical plate, IC – Internal capsule, IZ – Intermediate zone, NCX – Neocortex, VZ – Ventricular zone. Scale bar, 100 μm.
Figure 4.7. L1 immunohistochemical staining in coronal sections through E15.5 WT and Emx2⁻/⁻ mutant forebrain. L1 is expressed by developing thalamocortical fibres. (A) L1 stains a large fan-shaped axonal bundle in the region of the internal capsule, connecting the dorsal thalamus and cortex. (B) In Emx2⁻/⁻ mutants, the L1-positive axonal bundle appears to be directed towards the basal telencephalon. Arrows point to L1-positive thalamocortical fibres in the IZ of WT cortex (A) but are absent in Emx2⁻/⁻ cortex (B). DT – Dorsal thalamus, NCX – Neocortex. Scale bar, 100 μm.
Figure 4.8. L1 immunohistochemical staining in coronal sections through E15.5 WT and $Emx2^+\text{-}^{-}$ mutant cortex. L1 is expressed by developing thalamocortical fibres. (A) L1-positive axons are present in the upper IZ, just below the subplate, of WT cortex. (B) No L1 immunoreactivity is observed in the IZ of $Emx2^+\text{-}^{-}$ mutant cortex. Scale bar, 200 μm.
Figure 4.9. Confocal microscope images of organotypic slice cultures of E14.5 WT and
Emx2\textsuperscript{−/−} mutant forebrain. DiI was placed into the MGE to label cells migrating into the
developing neocortex. DiI labelled cells migrate predominantly into the IZ of the cortex
in both (A) WT and (B) Emx2\textsuperscript{−/−} mutant slices. Scale bar, 100 \textmu m.
Figure 4.10. Whole-brain views of E12.5 WT and $Emx2^{-/-}$ mutant embryos. (A,B) Dorsal (A,B) and side-view (C,D) show a reduction in the size of the cerebral hemispheres in the $Emx2^{-/-}$ mutant (B) in comparison to the WT (A) (black arrowheads), and an expansion in the width of the diencephalons (white arrowheads).
Figure 4.11. Whole-brain views of E13.5 WT and Emx2\(^{+/-}\) mutant embryos. (A,B) Dorsal (A,B) and side-view (C,D) show a reduction in the size of the cerebral hemispheres in the Emx2\(^{+/-}\) mutant (B) in comparison to the WT (A) (black arrowheads), and an expansion in the width of the diencephalons (white arrowheads). Development of the olfactory bulbs also appears to be affected.
<table>
<thead>
<tr>
<th>Zone</th>
<th>Number of Dil labelled cells (± S.E.M.)</th>
<th>Wildtype (n=14 slices)</th>
<th>Emx2&lt;sup&gt;-/-&lt;/sup&gt; (n=23 slices)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marginal Zone</td>
<td>3.50 ± 0.59</td>
<td>2.57 ± 0.44</td>
<td></td>
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<tr>
<td>Cortical Plate</td>
<td>6.93 ± 1.16</td>
<td>4.43 ± 0.71</td>
<td></td>
</tr>
<tr>
<td>Intermediate Zone</td>
<td>22.14 ± 2.75</td>
<td>14.21 ± 1.51</td>
<td></td>
</tr>
<tr>
<td>Ventricular Zone</td>
<td>3.00 ± 0.68</td>
<td>3.22 ± 0.58</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.12. The total number of Dil labelled cells in the cortex of WT and Emx2<sup>+</sup> mutant slices were counted. The cell counts were corrected to take into account the reduction in cortical size observed in the Emx2<sup>+</sup> mutant. Error bars represent S.E.M.
Number of Dil labelled cells migrating from the Ganglionic Eminence into the Neocortex

![Bar chart showing the number of Dil labelled cells migrating from the Ganglionic Eminence into the Neocortex. The chart compares WT and KO genotypes. The WT group has a higher number of Dil labelled cells compared to the KO group.](chart.png)
Figure 4.13. The number of Dil labelled cells in the MZ, CP, IZ, and VZ of the cortex of WT and $Emx2^{-/-}$ mutant slices were counted. The cell counts were corrected to take into account the reduction in cortical size observed in the $Emx2^{-/-}$ mutant. Error bars represent S.E.M.
Number of Dil labelled cells migrating from the Ganglionic Eminence into different layers of the developing Neocortex
Figure 4.14. Calbindin immunohistochemical staining in sections through the developing cortex. Calbindin labels cells with morphology resembling that of migrating neurons. (A) A number of cells found in the VZ of the cortex had a downward morphology, with the tip of the leading process in contact with the ventricle. (B) A small number of cells appeared to have two long leading processes, one directed towards the pial surface (top) and the other orientated tangentially. (C,D) The majority of cells were orientated tangentially within the IZ, with a long leading process directed towards the dorsal cortex. Scale bar, 40 µm.
Figure 4.15. Immunohistochemical localisation of calbindin-positive cells in coronal sections through E13.5 WT (A) and $Emx2^{+/}$ mutant (B) cortex. Calbindin IR cells were present in the MZ and IZ of the developing cortex (A,B). Cells were present in the IZ of ventrolateral cortex but not in the dorsomedial cortex. Calbindin-positive cells had penetrated 50% of the length of the cortical arm in both WT and $Emx2^{+/}$ mutant. Scale bar, 100 µm.
Table 2. Calbindin immunoreactive cells in the cortex of E13.5 WT and *Emx2*<sup>−/−</sup> mutant mice

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<tr>
<th></th>
<th>Number of calbindin-positive cells (± S.E.M.)</th>
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<tbody>
<tr>
<td></td>
<td>Wildtype (n=3 embryos)</td>
</tr>
<tr>
<td></td>
<td>Rostral 142.00 ± 4.73</td>
</tr>
<tr>
<td></td>
<td>Medial 211.50 ± 6.26</td>
</tr>
<tr>
<td></td>
<td>Caudal 227.00 ± 3.61</td>
</tr>
</tbody>
</table>
Figure 4.16. The total number of calbindin labelled cells in the cortex of E13.5 WT and Emx2−/− mutants were counted. Cell counts were made in coronal sections through rostral, medial, and caudal parts of the cortex. The cell counts were corrected to take into account the reduction in cortical size observed in the Emx2−/− mutant. Significant reductions (p < 0.01) in the number of calbindin-positive cells were observed in all regions of the cortex along the rostrocaudal axes. Error bars represent S.E.M.
Figure 4.17. Graphical representation of the proportion of calbindin-positive cells in different layers of the developing neocortex of E13.5 WT and Emx2<sup>−/−</sup> mutants. At this age, the majority of cells were located in the lower IZ, with a small proportion of cells located in the upper IZ, and VZ. No differences were observed in the proportion of cells located in each layer of the WT and Emx2<sup>−/−</sup> mutant cortex.
Figure 4.18. Graphical illustration to show the relative rate of migration in E13.5 WT and $Emx2^{+/}$ mutant cortex. The number of calbindin-positive cells were counted in a series of 200 μm bins along the cortical arm and plotted. Extrapolation of the data points provides an indication of the relative rates of migration in the WT and $Emx2^{+/}$ mutants. The shallower slope indicates a greater rate of migration, as seen in the WT cortex, with steeper slopes indicating a decreased rate of migration, as demonstrated in the $Emx2^{+/}$ mutants.
Figure 4.19. Immunohistochemical localisation of calbindin-positive cells in coronal sections through E15.5 WT (A) and Emx2/+ mutant (B) cortex. Calbindin IR cells are present in the MZ, SP/CP, IZ, and VZ of the developing cortex (A, B). (A) The majority of cells in the WT cortex were located in the lower IZ, and SP/CP, with fewer cells observed in the VZ or upper IZ. Calbindin also labelled thalamocortical fibres that were located in the upper IZ, just below the SP. (B) Calbindin-positive cells were observed in all layers of the developing cortex, but were more scattered in the IZ, with a greater number of cells located in the upper IZ in comparison to the WT. Scale bar, 100 μm.
Figure 4.20. Higher magnification of a coronal section through E15.5 WT and $Emx2^{+/}$ mutant cortex stained with an antibody against calbindin. Cells were clearly visible in all layers of the developing cortex (A,B), the majority of which had morphology typical of migrating neurons. (A) In WT cortex, a large stream of cells can be seen in the lower IZ whilst the upper IZ is relatively cell sparse. Arrows point to calbindin-positive thalamocortical fibres. (B) In the $Emx2^{+/}$ mutant cortex, streams of cells were still present in the lower IZ, but a large number of cells are also located in the upper IZ. Note the absence of calbindin-labelled thalamocortical fibres in the $Emx2^{+/}$ mutant. Scale bar, 100 μm.
Table 3. Calbindin immunoreactive cells in the cortex of E15.5 WT and *Emx2<sup>−/−</sup>* mutant mice.

<table>
<thead>
<tr>
<th></th>
<th>Number of calbindin-positive cells (± S.E.M.)</th>
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<tbody>
<tr>
<td></td>
<td>Wildtype</td>
</tr>
<tr>
<td></td>
<td>(n=3 embryos)</td>
</tr>
<tr>
<td>Rostral</td>
<td>314.67 ± 17.29</td>
</tr>
<tr>
<td>Medial</td>
<td>372.33 ± 16.19</td>
</tr>
<tr>
<td>Caudal</td>
<td>430.67 ± 3.18</td>
</tr>
</tbody>
</table>
Figure 4.21. The total number of calbindin labelled cells in the cortex of E15.5 WT and Emx2\textsuperscript{−/−} mutants were counted. Cell counts were made in coronal sections through rostral, medial, and caudal parts of the cortex. The cell counts were corrected to take into account the reduction in cortical size observed in the Emx2\textsuperscript{−/−} mutant. No significant differences were observed in rostral and medial cortex. As significant reduction (p < 0.05) in the number of calbindin-positive cells was observed in the caudal cortex of the Emx2\textsuperscript{−/−} mutant. Error bars represent S.E.M.
Calbindin Immunoreactive Cells in E15.5 Wildtype and Emx2-/- Cortex

Average Number of Cells

Rostral  Medial  Caudal

WT  KO

Rostral

Medial

Caudal
Figure 4.22. Graphical representation of the proportion of calbindin-positive cells in different layers of the developing neocortex of E15.5 WT and Emx2^−/− mutants. In both WT and Emx2^−/− mutant cortex, the majority of the cells were located in the lower IZ, although the proportion of cells located in this region in the Emx2^−/− mutant was slightly reduced. A small reduction was also observed in the SP/CP of the Emx2^−/− mutant. However, the proportion of cells located in the upper IZ of the Emx2^−/− mutant was increased in comparison to the WT.
4.5. Discussion

In the present study, we have used a variety of techniques to investigate the role of the homeobox gene *Emx2* in corticogenesis. More specifically, we studied the phenomenon of tangential migration of cortical interneurons from the ganglionic eminence to the cortex by DiI tracing in organotypic slice culture preparations. Previous studies have shown that the homeobox genes of the *Dlx* family, *Dlx1* and *Dlx2*, are important in regulating the migration of cortical interneurons from their place of birth in the ganglionic eminence to their final destination in the cortex. In *Dlx1/2*−/− mice, little or no migration of cells from the ganglionic eminence to the cortex is observed in culture, with the number of GABAergic cells in the cortex reduced by almost two-thirds. It is not known how these two genes function in regulating cell migration, and migration in mice with mutations in only one gene appears to be normal. Both genes are expressed abundantly in the ganglionic eminence before the onset of migration suggesting that these genes may have an early influence on cell fate.

Evidence that the *Dlx1* and *Dlx2* transcription factors play a role in cell migration led us to investigate whether the *Emx2* transcription factor may also be involved in this mechanism. *Emx2* is a member of the *Emx* homeobox gene family, which are mouse homologues of Drosophila empty spiracles gene. *Emx2* is expressed abundantly in the dorsal telencephalon, in the hippocampus, in the VZ of the neocortex, and in some cells of the MZ (Gulisano *et al.*, 1996; Mallamaci *et al.*, 1998; Mallamaci *et al.*, 2000a). In addition, some expression is also detectable in the ventral telencephalon, most notably in the VZ of the ganglionic eminence (Fig. 4.2B), and in the region of the amygdala. Expression in the germinal zone of the ganglionic eminence, where cortical interneurons are generated, suggests that *Emx2* may be a candidate for genes involved in the generation of cortical interneurons, or in the regulation of their migration into the cortex.
It has been suggested that tangentially migrating neurons utilise the fibres of the corticofugal system to navigate their way into the cortex (Metin and Godement 1996; Anderson et al., 1997). In the previous chapter we describe work in which we demonstrate the role of the corticofugal fibres, and the importance of the cell adhesion molecule TAG-1 in tangential migration. Previous reports have shown that the lack of Emx2 results in gross abnormalities in the structure of the cortex and hippocampus (Yoshida et al. 1997; Pellegrini et al., 1996; Mallamaci et al., 2000a,b), and alterations in the connections between the thalamus and the cortex (Mallamaci et al., 2000b; Bishop et al., 2000). We used Dil tracing in organotypic slice cultures and immunohistochemical staining in Emx2+ mice to determine whether alterations in corticofugal and thalamocortical fibres would result in disruptions in tangential migration.

The connections between the thalamus and cortex were examined by Dil tracing and immunohistochemical staining with antibodies specific for corticofugal (TAG-1) and thalamocortical (L1) fibres. Tracing studies revealed alterations in the route taken by these fibre systems in the mutant animals. No differences were seen when injections were made into rostral cortical areas, but injections into caudal regions revealed misrouted thalamocortical connections (Fig. 4.3E,F) in the brains of mutant animals. L1 immunohistochemical staining revealed a large aberrant bundle of fibres connecting dorsal cortex to the basal telencephalon in mutant animals (Fig. 4.4B). The presence of such a bundle suggests errors in axonal pathfinding into the cortex in mutant mice. In contrast, the corticofugal fibre system did not appear to be affected in the Emx2+ mice as demonstrated by TAG-1 immunohistochemistry. Tangentially arranged TAG-1 IR fibres were present in the IZ and MZ, and were seen to project into the region of the internal capsule. This suggests that the lack of Emx2 may result in misrouting of the cortical afferents, whilst the cortical efferents are not affected.
In the previous chapter, we discussed the importance of corticofugal fibres in the guidance of tangentially migrating neurons into the neocortex. Our experiments indicate that the fibres of the corticofugal system are unaffected by the *Emx2* mutation and, thus, tangential migration into the cortex should be able to proceed as normal. However, our present data reveals that tangential migration of cortical interneurons from the MGE is reduced in *Emx2<sup>−/−</sup>* mice. Using DiI to label migrating neurons in slices prepared from E14.5 WT and *Emx2<sup>−/−</sup>* mice, we found that there was a significant reduction in the number of DiI labelled cells in the cortices of slices from *Emx2<sup>−/−</sup>* embryos in comparison to the WT (Table 1, Fig. 4.9, Fig. 4.12). This decrease in overall number of DiI labelled cells was reflected in the number of cells migrating into specific layers of the developing cortex, with significantly fewer cells migrating into the IZ and CP of the *Emx2<sup>−/−</sup>* mutant. These results suggest that the presence of the corticofugal fibres is essential, but not sufficient, to orchestrate the proper migration of cortical interneurons into the neocortex. The reduction in the number of migrating cells in spite of an apparently normal corticofugal system would seem to suggest that some other factor is affecting the migratory process.

We also used an antibody against calbindin, a calcium binding protein, as a marker of migrating neurons. Previous reports have suggested that cells migrating from the ganglionic eminence to the neocortex express this calcium-binding protein (Anderson *et al.*, 1997; Chapouton *et al.*, 2000). Furthermore, the number of calbindin positive cells in the neocortex is reduced in slice preparations that have been transected at the corticostriatal boundary resulting in disruptions in tangential migration, and in *Dlx1/2<sup>−/−</sup>* double knockout mice which have a reduced number of tangentially migrating neurons (Anderson *et al.*, 1997). It should be noted that calbindin is not an ideal marker of tangentially migrating neurons as some cells expressing this protein in the neocortex have morphology resembling that of pyramidal cells (DeFilipe, 1997; Hof *et al.*, 1999).
However, Anderson et al. (1997) have shown that the reduction of calbindin positive cells in the cortex of new-born (P0) Dlx1/2\(^{-/-}\) double knockouts affects the interneuron population, with numbers of calbindin IR cells with pyramidal morphology remaining unchanged. This suggests that changes in tangential migration does not affect the calbindin expressing pyramidal population of cells in the cortex, and that the majority of the calbindin IR cells demonstrating tangential migratory morphology are likely to be interneurons.

We studied the localisation of calbindin expressing cells in the neocortex of WT and Emx2\(^{+/-}\) mice at E13.5, near the onset of tangential migration into the cortex, and E15.5, a stage when migration should be at a peak. We found significant reductions in the number of calbindin IR cells in the cortex of E13.5 mutants, with the largest changes occurring in the caudal regions of the cortex. These reductions in cell numbers may in part be explained by the observed decline in the rate of migration in the mutant brains, with the effect being most apparent in the caudal regions. It is possible that the reduction in the number of calbindin IR cells may also be related to the reduction in the size of the mutant cortex. At E13.5, we found that the cortical arm was reduced in length dramatically, by between 30-50\%, and the number of calbindin IR cells was significantly decreased accordingly. At later stages, E15.5, the cortical arm was reduced by only 10\%, and no significant differences in cell numbers was observed in rostral and medial cortex, with a small decrease in the caudal cortex.

At both ages examined, we found that the largest differences occurred in the caudal-most cortex. This appears to be in keeping with what is known about the functions of Emx2 in the development of the cortex. Previous studies have shown that both the Emx2 mRNA transcript and the EMX2 protein are expressed in low rostral to high caudal gradient, as well as a high medial to low lateral gradient in the developing cortex (Gulisano et al., 1996; Mallamaci et al., 1998; Mallamaci et al., 2000a,b; Bishop
et al., 2000). Studies in loss-of-function Emx2<sup>−/−</sup> mutant mice, has demonstrated changes in cortical arealisation, with expansion of rostral cortex and contraction of caudal cortex (Bishop et al., 2000; Mallamaci et al., 2000b). Gene expression studies and DiI tracing of fibre thalamocortical and corticothalamic fibres, in these mice have shown that there is a caudal shift in the limit of the somatosensory cortex with a reduction in the size of the visual cortex (Bishop et al., 2000; Mallamaci et al., 2000b). In this study, we found that during early stages of development, all cortical regions appear to be reduced in size, with an accompanying reduction in the number of cells migrating from the ganglionic eminence into the neocortex. At later stages, the changes are restricted to the caudal-most cortex, an area that is known to be most affected by the lack of Emx2, with no apparent differences observed in rostral and medial cortical areas.

Whilst little change was observed in the number of calbindin IR cells in the cortex of E15.5 mutant mice, we did notice that there was a difference in the proportion of cells located in each layer. It is possible that these changes in the proportion of cells in the upper IZ could be a result of laminar disorganisation previously reported in Emx2<sup>−/−</sup> mice (Mallamaci et al., 2000a). In these mice, the ‘inside-out’ formation of the cortical layers is not observed resulting in an inverted cortex similar to that seen in the reeler mutant (Caviness and Sidman, 1973). However, one important difference between the reeler and Emx2<sup>−/−</sup> cortical mutation is that, unlike the reeler cortex in which the preplate fails to split, neurons destined for the CP are able to penetrate the SP and form cortical layers below the MZ. However, it seems unlikely that defects in cortical lamination would affect the migratory routes used by the incoming interneurons to travel into the cortex. Such defects are more likely to affect the final positioning of the interneurons within the cortical layers in the mature cortex.

Alternatively, changes in migratory pathways may result from alterations in guidance cues such as chemoattractants, chemorepellents, or migratory substrates. One
intriguing possibility is that incoming cortical afferents may create a non-permissive region in the upper IZ where cortical interneurons are unable to migrate, or at least during their initial migration into the cortex. This hypothesis is supported by the observation that thalamocortical fibres, revealed by immunohistochemical staining with an antibody against L1, are severely disrupted in E15.5 mutant embryos. In such mutants, the L1-IR fibres appear to be directed towards the basal telencephalon as opposed to the cortex, and close examination of the cortex reveals the lack of most, if not all, of these thalamocortical fibres. In the WT mouse, these thalamocortical fibres normally invade the cortex in the upper region of the IZ, immediately below the subplate (Auladell et al., 2000), and few migrating neurons are located in this region. However, in the mutant cortex, lacking these thalamocortical fibres, an increase in the number of migrating neurons is observed in the upper IZ. The lack of thalamocortical fibres in the upper IZ and the increased number of migrating neurons in this region suggest that perhaps the presence of the fibres may be prohibitive for migration. This may arise through a repellant action, whereby the fibres may express a chemorepellent surface molecule that makes them an unattractive substrate for migration, or the migrating neurons may simply lack receptors required for the recognition of these fibres. Another possibility is that the migrating neurons and incoming thalamocortical fibres may compete to bind to the corticofugal fibres that are present throughout the whole thickness of the intermediate zone. As described in the previous chapter, neurons migrating tangentially into the neocortex utilise the corticofugal fibres for guidance, mediated by the cell adhesion molecule TAG-1. The "handshake" hypothesis, as proposed by Molnar and Blakemore (1995), postulates that interactions between the early corticofugal and thalamocortical fibres may be important in the guidance of the thalamocortical fibres beyond the internal capsule and into the cortex. This suggests that the thalamocortical fibres would need to be apposed close to the corticofugal fibres, and
may physically obstruct tangentially migrating neurons from attaching to the corticofugal fibres. Such an interaction would inhibit the tangential migration of neurons within the upper IZ whilst still allowing the cells to migrate radially into the CP, assuming that these cells utilise other systems, such as the radial glial fibres, for their radial migration.

The current data suggests that the lack of \textit{Emx2} results in disruptions in the tangential migration of cortical interneurons from the ganglionic eminence into the neocortex. This appears to be more severe during the earlier stages of corticogenesis, with all cortical areas demonstrating a decrease in migrating neurons at E13.5 but only caudal regions being affected at E15.5. This is mirrored by the changes in cortical size, with large reductions observed at E13.5 but much smaller reductions at E15.5. These observations suggest that the migration of neurons derived from the ganglionic eminence may be related to the size of the cortex, perhaps controlled through a feedback mechanism. However, it is difficult to determine the exact cause for the disruption in tangential migration due to the vast changes that occur in the cortex as a result of the absence of \textit{Emx2}. As this gene is also expressed within the proliferative VZ of the ganglionic eminence, it is possible that it is expressed in the progenitors of the cortical interneurons. Thus, the defects in tangential migration could arise either through intrinsic effects of the progenitors cells, perhaps causing a failure to generate sufficient daughter cells, or maybe due to misspecification of the cortical neurons prior to leaving the ganglionic eminence. The changes in the structure and development of the neocortex in the \textit{Emx2}^{+/} mutants may cause direct or indirect effects on the rate and number of tangentially migrating neurons. It is possible that, as mentioned earlier, the migration of neurons into the cortex may be controlled by a feedback mechanism, possibly linked to the development and size of the cortex. However, it is also possible that the migration of cortical interneurons is independent of the events taking place in the cortex, and that the
disruptions in cell migration may be an indirect effect of the alterations in cortical
development in $Emx2^{+/}$ mutants. Our current study provides evidence for disruptions in
tangential migration of cortical interneurons into the cortex of $Emx2^{+/}$ mutants. However, we cannot determine at this stage whether these changes are caused by a cell autonomous defect, or whether migration is determined by events that occur in the
developing neocortex.
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