AN *IN VIVO* AND *IN VITRO* INVESTIGATION OF DEVELOPMENT OF THE CEREBRAL NEOCORTEX IN THE MOUSE BRAIN

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DISCLAIMER

All of the experiments presented in this thesis (except some of those in Chapter 3 which were done in collaboration with Teresa Levers) were performed by me (Julia Edgar) under the direct supervision of Dr David J. Price. Katy Gillies, Grace Grant and Vivien Allison provided assistance with histology for the work presented in Chapter 3.

Julia M. Edgar
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ABSTRACT

Abstract of thesis submitted for the degree of Doctor of Philosophy entitled: an *in vivo* and *in vitro* investigation of development of the cerebral neocortex in the mouse brain

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The mature cerebral neocortex comprises six clearly defined neuronal layers or laminae. It receives its main afferent input from the dorsal thalamus. The neurones and glia that populate the cerebral neocortex and underlying cerebral wall originate in the proliferative region that lines the ventricles of the developing cerebral neocortex. This proliferative region consists of two distinct proliferative populations: the pseudostratified ventricular epithelium (PVE) and the secondary proliferative population (SSP). Both the neurons and radial glia of the cerebral neocortex are thought to derive from the PVE. The SPP gives rise to many of the late born glial cells.

During development, the neuronal precursors that will form the cortical laminae become terminally postmitotic in the proliferative zone. From there they migrate along radial glia cells to the marginal zone which contains Cajal-Retzius cells that are important for normal lamination. They detach from the radial glia and get displaced downwards by later born cells that migrate through them. Thus, the cortical laminae develop inside-out. The birthdate of the neuronal precursors correlates strongly with their ultimate laminar fate. This consistent and predetermined pattern of laminar development can be exploited to elucidate the factors that regulate cell migration and lamination.

I used 5-bromo-2-deoxyuridine (BrdU) to label proliferating neuronal and glial precursors *in vivo* and used immunohistochemical techniques to investigate their distribution within the developing mouse neocortex at embryonic and early postnatal stages. I developed a double labelling protocol to study the antigenic characteristics of these cells. I showed how neuronal and glial precursors begin to invade the developing neocortex, characterised some of the cells in terms of their antigenic properties and investigated their proliferative behaviour.

To investigate the factors that regulate the formation of the cortical layers, I developed a tissue culture technique using embryonic cortical slices and used BrdU labelling to study a population of neuronal precursors that give rise to cortical layers III and IV. These cortical layers receive direct thalamic innervation *in vivo*. I characterised the culture system and showed that it provided a satisfactory model for the study of the cell migration. I then showed that the thalamus was important for the normal migration of layer III/IV cells but that new cortical layeres did not form in culture. I investigated the numbers and distribution of Cajal-Retzius cells in cultured

cortical slices and showed that they were highly disrupted. I concluded that this might contribute to the failure of cells to form new laminae *in vitro*.

To ascertain if there was an *in vivo* correlate for my *in vitro* results, I used an immunohistochemical technique to characterise the thalamus, thalamocortical innervation and Cajal-Retzius cells in the mutant mouse Small eye (*Sey*), in which migration of late born cortical cells is aberrant. I showed that in the *Sey* mouse the Cajal-Retzius cells appeared normal in terms of location and morphology. However, both the dorsal thalamus and thalamocortical innervation were abnormal. Thus, the results of both the *in vitro* and *in vivo* investigations suggest a role for the thalamus in the normal formation of cortical layers III and IV.

TABLE OF CONTENTS

List of contents **Page number**

ABBREVIATIONS

BrdU 5-bromo-2-deoxyuridine

C caudal

Cmt centromedial thalamic nucleus

cp caudate putamen

CP cortical plate

CTX cortex

DAB 3,3'-diaminobenzidine

E embryonic day

GE ganglionic eminence

IZ intermediate zone

L lateral

ldt lateral dorsal thalamic nucleus

MZ marginal zone

O olfactory bulb

P postnatal day

PBS phosphate buffered saline

PZ proliferative zone

R rostral

Re reuniens nucleus

Rh rhomboids nucleus

SP subplate

SVZ subventricular zone

Th thalamus

TBS tris buffered saline

vpl ventral posterior lateral thalamic nucleus

vpm ventral posterior medial thalamic nucleus

V ventricular edge

VZ ventricular zone

WM white matter

CHAPTER 1: DEVELOPMENT OF THE MAMMALIAN NEOCORTEX

1.1 Development of the nervous system from a single layer of cells

The complexity of the central nervous system is immense. And yet it derives, like the rest of the body's organs and tissues, from a simple sheet of cells. How an apparently homogenous population of cells gives rise to a structure of such amazing complexity is the subject of extensive research, speculation and debate.

The embryo begins as a flat disc of three layers of cells, termed the endoderm, mesoderm and ectoderm. The endoderm gives rise to the viscera (the lining of many internal organs); the mesoderm gives rise to the muscles and bones of the body; the ectoderm gives rise to the nervous system and skin.

Very early on in development, a specialised piece of the ectoderm, called the neural plate, forms a groove along the rostrocaudal extent of the ectodermal sheet. This is called the neural groove. The edges of the groove, termed the neural folds, move together and fuse dorsally to form the neural tube. The entire central nervous system develops from the neural tube. The peripheral nervous system derives from the neural crest, which is a small part of the neural folds that are not incorporated into the neural tube. The neural crest lies dorsal and lateral to the neural tube.

Three swellings form at the rostral end of the neural tube. These are called the primary vesicles. The most rostral vesicle is called the prosencephalon or forebrain, the middle is the mesencephalon or midbrain and the most caudal is the rhombencephalon or hindbrain. The rhombencephalon connects with the caudal part of the neural tube which gives rise to the spinal cord.

As development progresses, the prosencephalon divides into the telencephalon, two lateral swellings at its most rostral part, and the diencephalon. From the diencephalon, two small vesicles project laterally. These are the optic vesicles. The telencephalon gives rise to the two cerebral hemispheres. The

diencephalon gives rise to the thalamus, the hypothalamus, the epithalamus and the subthalamus.

1.2.1 The cerebral cortex

As stated above, the cerebral cortex derives from the telencephalic vesicles. The neocortex, with which this thesis is mainly concerned, is that part of the cerebral cortex that is visible on the external surface of the mammalian brain. It processes sensory information, governs motor output and is responsible for complex cognitive functions such as language and spatial awareness. The complexity of its functions belies the elegant simplicity of its basic structure. The mature cortex comprises six clearly defined neuronal layers or laminae: each layer contains neurons that share several characteristics, including size, shape, physiological properties and packing density (reviewed in Gilbert, 1983). All cortical laminae contain cells of two main types: pyramidal cells or projection neurons and nonpyramindal cells or interneurons.

The cortex is also divided radially into regional and functional units. Large sulci or folds in the cortical wall define the regions or lobes. The functional units are found within these lobes. For example, the parts of the cortex responsible for processing visual information are located in the occipital lobe and those for processing and producing speech are found in the parietal and frontal lobes respectively.

1.2.2 Development of the cerebral cortex

The developing telencephalic wall (subsequently referred to as the cortical wall) is divided into a number of zones (Boulder Committee, 1970). In its earliest stages it consists of a layer of neuroepithelial cells that divide prolifically. Remarkably, all the neurons and glia that form the cerebral cortex arise from this one population of apparently identical precursors (His, 1889; Sauer, 1935; Sauer and Walker, 1950; Fujita, 1963; Stensaas, 1967; Hinds and Ruffett, 1971, all cited in Takahashi *et al.*, 1992; Sidman and Rakic, 1982; Bayer and Altman, 1991; Takahashi *et al.*, 1992, 1994). This layer of neuroepithelial cells is termed the ventricular zone (VZ) because

it lies right at the edge of the ventricle (the hollow, fluid filled cavity formed by the neural tube). Ventricular zone cells undergo interkinetic movement as they divide (His, 1904, cited in Takahashi *et al.*, 1993; Sauer, 1935; Sauer and Walker, 1959; Angevine and Sidman, 1961; Berry *et al.*, 1964; Boulder Committee, 1970; Misson *et al.* 1988a; Takahashi *et al.*, 1992). They migrate to the outer part of the ventricular zone during gap phase one or G1 of the cell cycle. They synthesise DNA during the synthetic or S-phase of the cell cycle. Then they migrate to the ventricular edge during the second gap phase, or G2. They undergo mitosis (M phase) at the ventricular edge. The daughter cells then either re-enter the cell cycle and migrate back out to the outer region of the zone during G1 and/or they become terminally postmitotic and migrate away to form the neurons of the cortical wall. This interkinetic movement is illustrated in Figure 1.

The first neurons to leave the ventricular zone form a layer of tissue called the primordial plexiform zone or preplate (PPL). The intermediate zone (IZ) develops between the ventricular zone and the preplate. This is a cell sparse region that contains cortical afferents and efferents and cells migrating away from the ventricular zone. The subventricular zone (SVZ) forms above the ventricular zone, from ventricular zone cells (Smart, 1972; Altman and Bayer, 1990; Halliday and Cepko, 1992 and Takahashi *et al.*, 1993). Like the ventricular zone, it is a region rich in proliferating cells. However, unlike the ventricular zone, its cells do not undergo interkinetic movement as they progress through the stages of the cell cycle.

As the intermediate zone and subventricular zone form, the neurons of the preplate mature and receive synaptic contacts from cortical afferents. The preplate is considered to represent a primitive cortical organisation shared by amphibians, reptiles and mammals (Marin-Padilla, 1992, 1998). Cells of the cortical plate (CP; future neocortex) split the prelate into two separate layers, by accumulating within it (Luskin and Shatz, 1985a). The outer layer lies directly beneath the pia and is known as the marginal zone (MZ; future layer I). The lower layer is a transient structure that lies immediately above the intermediate zone and is known as the subplate (SP).

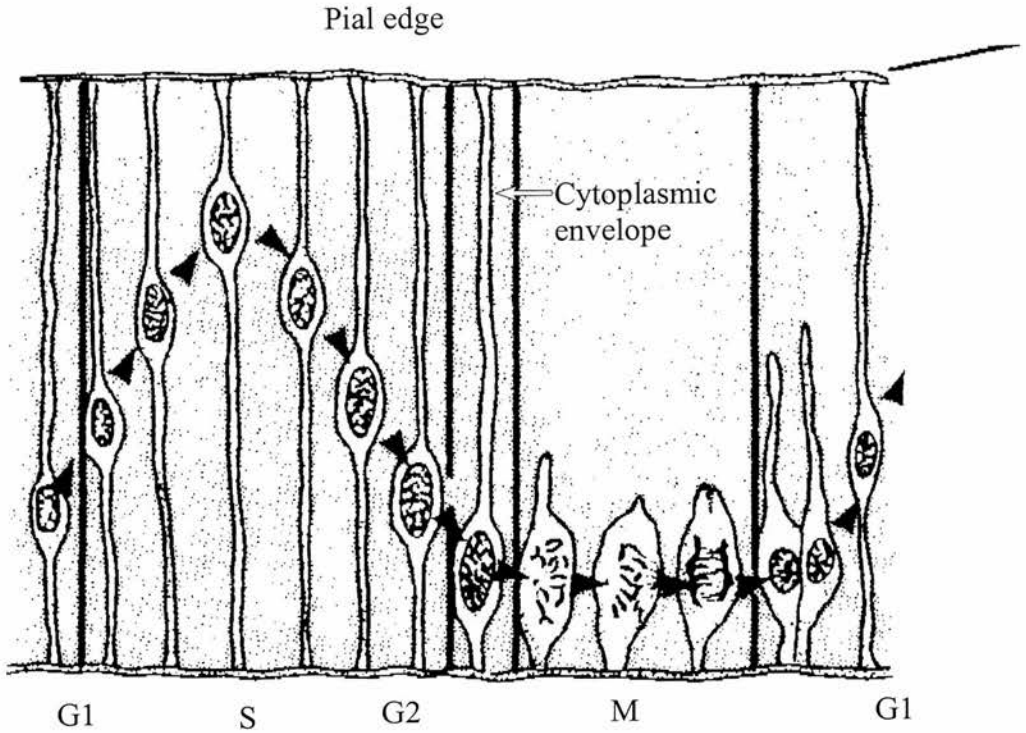


Figure 1 illustrates the interkinetic migration of nuclei in the neural tube during the stages of the cell cycle. Cells synthesise DNA in S-phase, near the pial edge and divide in M-phase, at the ventricular edge. G1: gap phase 1, S: synthetic phase, G2: gap phase 2, M: mitosis.

Migrating cells of the developing cortical plate reach the marginal zone and develop an apical dendrite which establishes contact with elements within it (Marin-Padilla, 1992). Soon after this happens they become detached from the radial glia, allowing later born cells to reach the marginal zone by the same route. Tritiated thymidine ([^{3}H] thymidine) labelling studies have shown that the later born cells of the cortical plate exhibit a strong correlation between their precise birthdate and their final laminar positions in the cortical plate (in the mouse, Angevine and Sidman, 1961; in the rat, Berry *et al.*, 1964; in the monkey Rakic, 1974; and the cat, Luskin and Shatz, 1985b). As the cortical plate thickens, the marginal zone and subplate become increasingly separated. However they retain both structural and functional interrelationships that persist during the formation of the cortical plate (Marin-Padilla, 1992). The development of the cortical wall in the mouse, as revealed by the study in Chapter 2 of this thesis, is illustrated diagrammatically in Figure 2.

When neurons reach their appropriate laminar position, they complete differentiation and form appropriate axonal connections with their target cells. For example, in the sensory cortex, neurons in the upper layers 2 and 3 send long-distance axons to other cortical areas, whereas neurons in the deep layers 5 and 6 extend axons to subcortical targets (Gilbert and Kelly, 1975; Lund *et al.,* 1975; Symonds and Rosenquist, 1984).

1.2.3 Cell proliferation in the ventricular zone during neurogenesis

A relatively small number of ventricular zone cells (the progenitor pool) give rise to a huge number of neurons and glial cells. Cell amplification is achieved through cell division. A mathematical model for the process by which the progenitor pool gives rise to all the neurons of the mature cortex has been proposed by Caviness *et al.* (1995). In this model, which is based on a comprehensive series of studies of cell

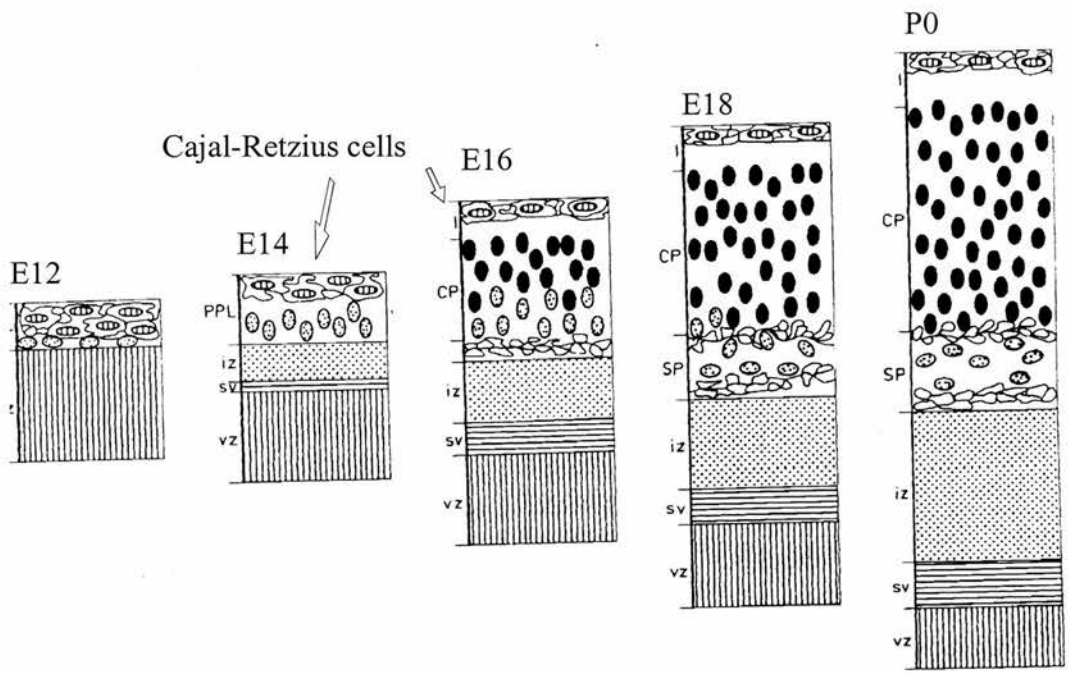


Figure 2. Diagramatic representation of how the cortical layers form. Neuroepithelial cells dividing on E12 give rise to cells of the preplate. The preplate is subsequently split into the marginal zone (layer I, which contains Cajal-Retzius cells) and the subplate by the insertion of later born cells of the cortical plate. The layers of the cortical plate form inside out as later born cells migrate through earlier born cells to take up position beneath the marginal zone. Adapted from Fig.5-5, Neocortical Development by Bayer and Altman, Raven Press (1991). VZ: ventricular zone, SV: subventricular zone, IZ: intermediate zone, CP: cortical plate, PPL: preplate, MZ: marginal zone

cycle parameters (Takahashi *et al.*, 1993; 1994; 1995), ventricular zone cells divide initially to produce two identical daughter cells that are thought to re-enter the cell cycle and contribute to expansion of the progenitor pool. After neurogenesis begins a fraction of these dividing cells become terminally postmitotic and form the neurons of the cortical plate. However, the progenitor pool continues to expand rapidly because the remaining fraction goes on cycling. With time, the proportion of cells that become terminally postmitotic increases and the proportion of cells that re-enter the cell cycle decreases, thus the progenitor pool diminishes. In this model, the final number of neurons in the cerebral cortex is predicted by the average output for a progenitor cell. Takahashi *et al.* (1996) suggest that, in the mouse, a single precursor marked on embryonic day 12.5 (E12.5) will give rise to approximately 140 neurons during the entire period of neurogenesis (E12 - E18 in the mouse neocortex). Interestingly, a very recent study by Tan *et al.* (1998), which marks members of the preneurogenetic founder population and their direct descendants, shows strong agreement with this estimate. This correlation is itself consistent with a ventricular zone in which 99% of proliferating cells have a similar cell cycle time (Cai *et al.* 1997, cited in Tan *et al.*, 1998). Thus, although others (Polleux *et al.*, 1997) have argued that the methods employed by T. Takahashi and colleagues to estimate the length of the cell cycle during neurogenesis are flawed, in that they don't take account of radial glial cells in the ventricular zone that are cycling during this time, these parameters have been referenced in this study with some degree of confidence.

In the model proposed by Caviness *et al.* (1995), the sparsely populous infragranular layers (layers V and VI) of the neocortex are produced early in neurogenesis during the period when neuronal output is relatively small. Conversely, the densely populated layers four, three and two are produced late in neurogenesis, when there is a huge increase in neuronal output.

1.2.4 Symmetric and asymmetric division in the ventricular zone

Rakic (1988) made the suggestion that neurogenesis involves asymmetric division, whereby a single precursor gives rise to a daughter cell that follows one fate and another daughter cell that follows a different fate, as first illustrated in *Drosophila* (reviewed in Huttner and Brand, 1997). Direct evidence for asymmetric division in the neocortex came from a study by Chenn and McConnell (1995) to investigate the possible relationship between cleavage plane orientation and cell fate in the neuroepithelium (an issue addressed almost thirty years earlier by Langman *et al.* 1966 and Martin, 1967, reviewed in Huttner and Brand, 1997). Chenn and McConnell (1995) used fluorescent dyes to label cells dividing in acute slices of ferret cortex. They employed time lapse confocal microscopy to image these cells and recorded the plane in which they divided. Cells that divided in a plane perpendicular to the ventricular surface (vertical division) gave rise to two cells that behaved identically and re-entered the cell cycle. Those that divided in a plane parallel to the ventricular surface (horizontal division) gave rise to one cell that re-divided and another that appeared to migrate out of the ventricular zone. They showed that in the period prior to the onset of neurogenesis, cell division was predominantly vertical or symmetrical. However, after the onset of neurogenesis there was an increase in the number of horizontal or asymmetric divisions that occurred. Thus, they concluded that cells dividing symmetrically contribute to expansion of the progenitor pool and those dividing asymmetrically give rise to both a progenitor and a migratory neuron. However, these authors also reported seeing a considerable number of cells that divided in a plane that was neither parallel or perpendicular to the ventricular surface, which they did not explain.

1.2.5 Cell cycle parameters

During neurogenesis, the length of the cell cycle is modulated over time. Waechter and Jaensch (1972) devised a method of estimating the length of the cell cycle based on cumulative labelling with [^{3}H] thymidine. T. Takahashi and colleagues have used a similar method, using 5-bromo-2-deoxyuridine (BrdU) as a marker of proliferating cells, in a comprehensive series of experiments designed to measure cell cycle

parameters in the mouse neocortex. Eleven cell cycles occur during the period of neurogenesis in the mouse (Takahashi *et al.*, 1995a). These increase in length from 8.1 hours at E11 to 18.4 hours at E16 as the result of an increase in the length of the G1 phase of the cycle (Takahashi *et al.*, 1995a). The cell cycle of the subventricular zone population (which gives rise to glial precursors) is approximately 15 hours during the period from E14-E16 (Takahashi *et al.*, 1995b).

1.3 Cell migration

Most neurons reach their laminar destination by migrating radially along the processes of radial glia (Rakic, 1972; 1988; 1990). However, a small population follows a tangential route through the developing cortical wall (O'Rourke *et al.*, 1995, Tamamaki *et al.*, 1997). The substrate upon which these cells migrate is not currently known. Radial glia are a transient population of cells that are among the first neuroepithelial cells to differentiate in the developing cortex (Misson *et al.*, 1988a, b). They extend long thin processes radially, from their cell bodies in the ventricular zone, to the external glial limiting membrane at the pia (Gadisseux *et al.*, 1989; Misson *et al.*, 1988a). Radial glia are transformed into astrocytes after neuronal migration is complete (Schmechel and Rakic, 1979; Levitt and Rakic, 1980; Voigt, 1989; Culican *et al.*, 1990; Takahashi *et al.*, 1990; Misson *et al.*, 1991).

Cell migration involves at least three steps; the first being that the young neuron must recognise its migratory pathway, then it must migrate along that pathway and finally it must cease migrating and detach from the radial glial cell (or other substrate). Rakic (1985) proposed that these three events could be mediated by differential cell adhesion. Thus, young neurons would attach to radial glia as they become postmitotic and migrate along them, by some unexplained mechanism that allows directional migration. Then they would detach from the radial glia either when cell adhesion molecules that enable them to attach to radial glia disappear or by the fact that they contact neurons that express a different cell adhesion molecule. Considerable evidence for the role of neuronal glial receptor systems in cell migration has since been published. For example Anton *et al.* (1997) showed that a

neuregulin, glial growth factor2 (GGF2), is produced by neurons in the developing cerebral cortex and its receptors, erbB2, erbB3 and erbB4, are expressed on both neurons and glia. Functional blocking antibodies were used to show that GGF2 promotes migration of cortical neurons. Similar studies on the neuronal protein astrotactin suggest that it too is a principal ligand for the directed migration of young neurons along glial fibres (Zheng *et al.*, 1996). Nadarajah *et al.* (1997) studied the expression of connexins in the developing cortex. They found that connexin 43 (which contributes to the formation of gap junctions) was localised in radial glial cells during neuronal migration suggesting that it is also involved in this process.

1.4 The thalamus

Like the cerebral cortex, the thalamus develops from cells dividing in the ventricular zone. In the early neural tube, the diencephalon is divided into three distinct regions or prosomeres (Puelles and Rubenstein, 1993) by strips of low cell density that coincide with borders of regulatory gene expression. Prosomere 1 (p1) gives rise to the pretectum, p2 to the dorsal thalamus and p3 to the ventral thalamus. The thalamus is the main relay station for information to the cortex. All sensory impulses (with the exception of the olfactory ones) and motor information reach the cortex via the thalamus. The thalamus is also responsible for maintaining and regulating the level of consciousness, alertness and attention and is also concerned with the emotional correlates that accompany most sensory experiences. The dorsal thalamus consists of relay nuclei, association nuclei, intralaminar and midline nuclei. It receives afferents from other thalamic nuclei or subcortical structures and relays information to the cortex via the thalamocortical pathway. The dorsal thalamus has a strong reciprocal innervation with the cortex and with the ventral thalamus which does not project to the cortex, but modulates the function of the dorsal thalamus.

1.4.2 How the thalamus develops

The neurons of the thalamus derive from the diencephalic ventricular zone. Unlike the cortex, the thalamus does not form inside out. Instead, the early born thalamic neurons give rise to the most lateral thalamic nuclei and the late born cells give rise

to the most medial nuclei (Altman and Bayer, 1979a, b,c; Angevine, 1970). In the mouse, the cells that will give rise to the thalamic nuclei are born between E10 and E15 (Angevine, 1970). From E15 to postnatal life the differentiation of functional thalamic nuclei becomes evident.

1.4.3 Thalamic innervation of the neocortex

Thalamic afferents terminate in the cortex in three tiers: an outer tier in layer I, a middle tier in layers IV and /or III and an inner tier in layer VI (Frost and Caviness, 1980). Axons grow into the developing cortex at the same time as neuronal precursors are migrating (reviewed by Molnar and Blakemore, 1995). At E15, axons from thalamic cells leave the thalamus and project through the primitive internal capsule, a large fibre bundle that contains the main axons running to and from cerebral hemispheres, to the telencephalon. Once through the internal capsule, thalamocortical afferents do not immediately grow into the cortical plate, but instead, invade the developing cortex via the subplate (Lund and Mustari, 1977; Miller *et al.*, 1993). Those axons destined for layer IV, which receives the principal thalamic innervation, wait in the subplate for a short time (Lund and Mustari, 1977; Blakemore and Molnar, 1990) before entering the cortex (which they do at around E18 in the mouse). Then they invade the differentiated layers VI and V before entering and stopping in layer IV once it also begins to differentiate, at around P0 (reviewed in Molnar and Blakemore, 1995).

The subplate is known to be involved in the formation and reshaping of thalamocortical connections during development, for example in the segregation of geniculate fibers of the ocular dominance columns (Ghosh *et al.*, 1990; Ghosh and Shatz, 1992). Subplate cells disappear, however, at later stages (Luskin and Shatz, 1985a; Price *et al.*, 1997). For a long time, it was not clear what role the marginal zone played in development. However, evidence is accruing that suggests a role for the marginal zone in lamination.

1.5.1. Studies from mutations in mice help in the understanding of the normal processes of migration and lamination

The molecular mechanisms that regulate migration and lamination are slowly being unraveled. The studies of mutant mice in which cortical migration and/or lamination are abnormal have been instrumental in this respect. In the mutant mouse *reeler,* the preplate is not split into marginal zone and subplate populations (by cortical plate neurons). The cortical plate neurons migrate apparently normally initially, but the later born cells fail to migrate through the earlier born cells (Caviness and Rakic, 1978; Caviness, 1982; Goffnet 1984), so the cortical layers are approximately inverted. Nonetheless, these neurons form normal connections with their subcortical structures (Caviness and Rakic, 1978), suggesting that it is not essential for a neuron to find its correct laminar position in order to be able to differentiate normally. The *reeler* gene is expressed in wild type (but not mutant) embryonic and postnatal neurons during periods of neuronal migration (D'Arcangelo *et al.,* 1995). A monoclonal antibody (CR-50) to the *reelin* gene product reacts specifically with Cajal-Retzius neurons in the marginal zone of wild-type and heterozygous, but not homozygous, cerebral cortex (Ogawa *et al.,* 1995). The cell surface location of the antigen, its temporal expression pattern and the ability of CR-50 antibody to disrupt reaggregation of dissociated wild-type cortical cells suggest that the antigen plays an important role in lamination in the neocortex. The predicted amino acid sequence (D'Arcangelo *et al.,* 1995) and expression studies (D'Arcangelo *et al.*, 1997) show that reelin is secreted. Its homology to F-spondin and the fact that it contains FGF repeats like that in tenascin C, tenascin X and restrictin and the integrin beta chain (D'Arcangelo *et al.,* 1995) have led these authors to suggest that it might act to mediate cell adhesion. Since, in the cerebral cortex, it is expressed specifically in the cortical marginal zone during neurogenesis, it might well play a role in enabling migrating cortical precursors to 'stop' beneath the marginal zone and form appropriate laminae. Reelin might also play a chemotrophic role. Since neuronal migration in dissociated cultures of cerebellar neurons and Bergmann glia (the cerebellar equivalent of radial glia) is bi-directional, something must be responsible

for the directional migration of neurons *in vivo*. Given its location at the top of the cortical wall, reelin is a prime candidate for this role.

Three other mouse mutants, with phenotypes identical to *reeler*, have been discovered. These are *scrambler* (Sweet *et al.,* 1996; Gonzalez *et al.*, 1997), *yotari* (Yonishema *et al.,* 1997, reviewed in Pearlman *et al.*, 1998) and *mdab1* (Howell *et al.* 1997). The *mdab1* mutant was produced by a targeted disruption of *mdab1*, one of the two murine homologues of the *Drosophila* gene *disabled*. *Scrambler* and *yotari* were found to be alleles of *mdab1* (Sheldon *et al.*, 1997 and Ware *et al.*, 1997). The *mdab1* gene encodes a cytoplasmic protein, mDab1 p80, that binds nonreceptor tyrosine kinases Src, Abl and Fyn (Howell *et al.* 1997). Since mDab1 is expressed on migrating neurons that will make contact with *reelin* at the cortical marginal zone, it has been proposed that mDab1 p80 is part of a signalling cascade that responds to *reelin* (Howell *et al.* 1997), although this remains to be tested.

Two other interesting mutants that have shed light on the factors that are important for normal migration and lamination are the serine/threonine kinase cdk5 knockout (Oshima *et al.*, 1996) and the p35 knockout (Chae, 1997 and Kwon and Tsai, 1998) mice. The p35 protein is a neuron specific activator of the cdk5 receptor, and Chae *et al.* (1997) proposed that the cdk5/p35 complex be integrally involved in neuronal migration. Kwon and Tsai (1998) show that in the p35 mutant, migration of early born neuronal precursors is normal and that they form a cortical plate between the marginal zone and subplate, but late born precursors form a second ectopic cortical plate beneath the cortical subplate. Thus, they conclude that the cdk5/p35 complex is important for enabling the late born neurons to navigate through the earlier born cells. A basis of support for their suggestion comes from the proposal that early born cortical cells, having only a short route to traverse from the marginal zone to the cortical plate, might not use radial glia as their method of travel (McConnell, 1988). McConnell (1988) suggests that these early precursors might traverse this distance by means of the cytoplasmic envelope that encloses them (see Fig. 1) and which extends, in the early embryonic wall, the distance from the

ventricular margin to the pial margin. Thus, the mode of migration may be different for early born and late born precursors.

Finally, the *pax-6* mutant mouse Small Eye (*Sey*) has abnormalities of cortical neuronal migration (Schmahl *et al.*, 1993). Like the cdk5 and p35 knockouts, the late born cortical precursors are aberrant in their migration (Caric *et al.*, 1997). However, in this mutant, the late born precursors do not form an ectopic cortical plate. Instead, they remain as postmitotic cells in the cortical subventricular zone. Thus, in the *Sey* telencephalon, the subventricular zone is enlarged and the cortical plate is much thinner than in the wild type. No mechanism has so far been found to account for the failure of these cells to migrate, though Caric *et al.* (1997) suggest that the defect is not a cell autonomous one. That is, the defect in migration is thought to be due to an abnormality in the environment in which the cells are attempting to migrate.

1.6.1 Glial cells

The major glial subtypes of the CNS, the astrocytes and oligodendrocytes, arise, like neurons, from the proliferating neuroepithelium of the embryonic cortical wall. The traditionally held view is that glial cells are generated after neurogenesis is complete, from cells in the subventricular zone, which themselves arise from the ventricular zone (Smart, 1972; Altman and Bayer, 1990; Halliday and Cepko, 1992 and Takahashi *et al.*, 1993). Lineage studies show that in many regions of the CNS, a common progenitor gives rise to both neurons and glial cells (reviewed in McConnell, 1995). In the cortex, on the other hand, it appears that neurons and glia derive from largely separate lineages (Luskin *et al.*, 1993 and reviewed in McConnell, 1995). However, as McConnell (1995) points out, the studies on lineage use spatial criteria to assign clustered cells to clones. Since little is know about the migration patterns of glia or their progenitors, it remains possible that distant clusters of neurons and clusters of glia could share a common progenitor cell if the daughters of the progenitor were to move apart and then subsequently produce subclones of purely neurons and purely glia. Therefore, the question arises: what tells a ventricular zone cell to form a neuron at a particular stage in its development and a

glial cell at a different stage? Very little is known about the molecular events that regulate the differentiation of neuroepithelial cells into neurons or glia. However, Hardy (1998) has identified a protein that might be involved in the process. The QKI protein, which is found in neuroepithelial cells that give rise to neurons and glia, is downregulated by neurons as they exit the ventricular zone. However, it is maintained by neuroepithelial cells that go on to differentiate into glial cells of both the oligodendrocytic and astrocytic lineage, suggesting a role for this protein in the neural/glial fate decision.

Potentially, the spatial restriction of neuronal and glial precursors (neurons in the ventricular zone and glial cells in the subventricular zone), could allow environmental factors to influence each population separately. However, Takahashi *et al.* (1995) showed that there is some overlap of precursors in the ventricular and subventricular zones during the embryonic period. The mechanism by which the neuronal/glial fate decision is made remains elusive. Part of the problem in identifying this mechanism has come from the fact that a lack of specific early glial cell markers has made it difficult to determine the spatial and temporal origins of glial cells during CNS development. Without reliable information, it is difficult to know at what stage (in terms of the sequential cell cycle, or embryonic day) or in what location (in terms of position within the cortical wall or within the ventricular zone itself) a cell decides to become a glial cell rather than a neuron. One of the aims of this thesis was to establish the temporal and spatial pattern of development of glial cells in the neocortex during the transition from neurogenesis to gliogenesis.

1.7.1 What determines a cell's fate?

The other interest of this study is what determines a cell's laminar fate. Integral to the process of laminar formation are the processes of proliferation, migration and 'stopping' (that is, finding an appropriate position and terminating migration). So although my main interest was in laminar fate cues, it was impossible to study these without reference to the mechanisms required for a cell to form its appropriate layer.

1.7.2 Intrinsic and extrinsic fate determinants

The study of cell fate determinants is vital to understanding how development occurs. Since all developing cells contain the same DNA, a cell's phenotype is regulated by the specific set of genes that it expresses. There are two ways in which gene expression is regulated and hence cell fate decisions are controlled. Intrinsic regulation refers to the particular sets of constraints on gene expression that a cell has inherited from its ancestors. That is, its fate is determined by its lineage. Extrinsic mechanisms are mediated by factors in the environment of the cell on which they act, and can be either secreted molecules or cell attached molecules. In the chick hindbrain, neuronal fate is restricted by cell lineage factors (Fraser *et al.*, 1990), whereas in the *Drosophila* eye, contact mediated interactions between 'boss' and 'sevenless' are required for the development of the full complement of photoreceptors (Hafen *et al.*, 1987 and Cagan *et al.*, 1992). During its development, a cell will be influenced by both mechanisms. For example, in the developing leech ganglia, a cell's lineage determines that it becomes a particular type of neuron, but the pattern of its axonal projections is influenced by neighbouring cells (Stuart *et al.*, 1989; Torrence *et al.*, 1989). In the developing neocortex, lineage is thought to be important in determining a cell's neuronal phenotype (Parnavelas *et al.*, 1991; Luskin *et al.*, 1993; Tan *et al.*,1998) but environmental cues are thought to specify neurons to a particular laminar fate (McConnell and Kaznowski, 1991).

1.7.3 When do cells become specified to a particular fate?

Central to the question of what determines a cell's fate is the question of when a cell becomes specified to a particular fate. In terms of regionalisation in the neocortex, two different theories prevail. Rakic (1988) proposed the theory of the 'protomap' in which cells in the ventricular zone are specified to form a particular cortical region before they begin their migration. Support for this hypothesis comes from at least two observations (1) the strict radial migration of neurons (from their site of proliferation to their ultimate position in the mature cortex) as reported in the primate cortex (Rakic, 1972) and (2) the correlation between the rates of cortical neurogenesis within the ventricular zone and regional differences in neuronal number

in the cerebral cortex (Dehay *et al.,* 1993; Polleux *et al*, 1997). The alternative theory of a 'protocortex' holds that cortical precursors are equipotential and neurons acquire their positional specification after they have migrated out of the ventricular zone from, for example, cortical afferents (O' Leary, 1989). Support for this theory comes from transplant studies. For example if occipital cortical neurons are transplanted to rostral cortex they alter their pattern of long-distance projections to innervate targets appropriate for their new environment (reviewed in McConnell, 1995). Likewise, if cells from either the dorsal or ventral telencephalon are transplanted into the ventricular zone they will incorporate into the telencephalon, diencephalon or mesencephalon and acquire phenotypic features appropriate for their final location (Brustle *et al.*, 1995). These authors suggest that neuronal migration and differentiation are regulated by a non-cell autonomous signal.

However, the interpretation of results of cell fate studies must be met with some caution. For example, Ferri and Levitt (1993) used a transplant study to show that most cortical progenitors are fated to a limbic or nonlimbic phenotype early in their development, prior to the completion of neurogenesis. Thus they argued for a role for cell lineage in the specification of a cell to a particular cortical region. Their result is quite unlike that of Brustle *et al.* (1995). These different results can be reconciled by the fact that the methods of cell dissociation (for transplantation) were different. Where Ferri and Levitt employed a method that allowed cells to retain the expression of certain cell surface molecules, the method used by Brustle *et al.* (1995) did not. Therefore, although cells from the different regions might be determined by their lineage to a particular phenotype, if the factors that constrict these cells to that fate (in this case, cell surface molecules) are removed, the cell can regain a degree of plasticity. These different findings show that apparently stringent tests to determine lineage versus environmental influence can reveal very contrary results.

1.7.4 Laminar fate cues

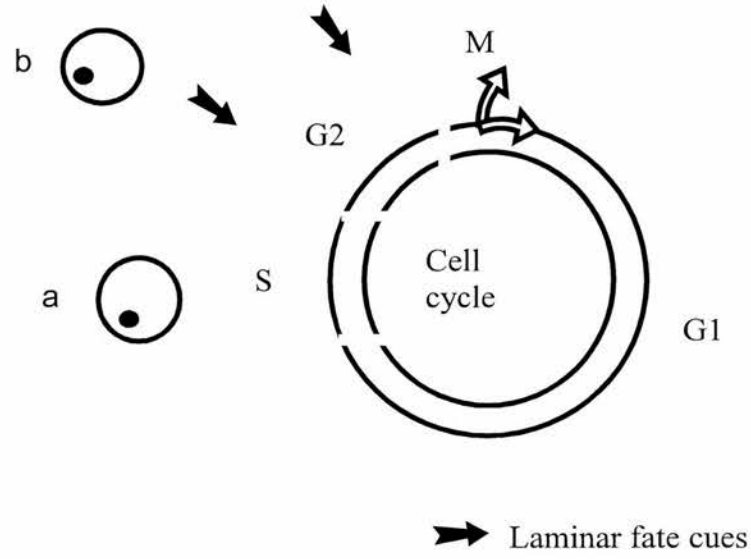
In terms of laminar identity, lineage studies have shown that a single cortical precursor can give rise to neurons that will populate many cortical layers (Luskin *et*

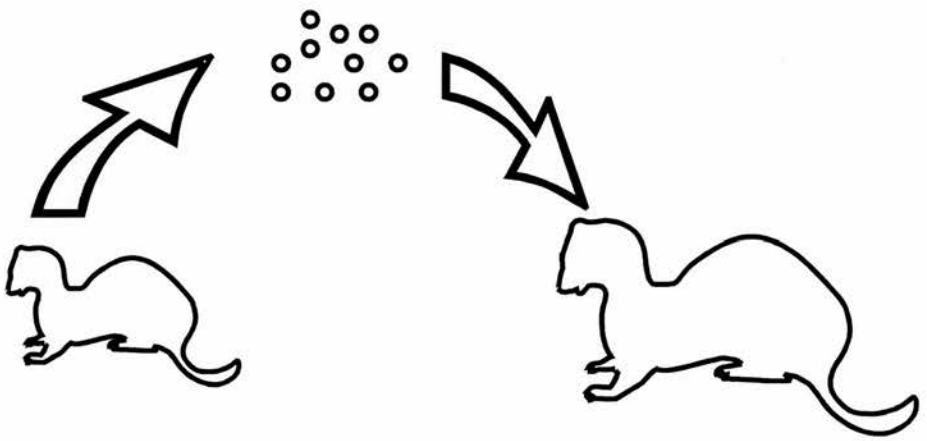
al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Price *et al.*, 1991; Mione *et al.*, 1997; Tan and Breen, 1998). This suggests that precursors are not intrinsically restricted to form only one cortical layer. McConnell and Kaznowski (1991) showed that environmental factors specify cortical neurons to a deep layer fate. These authors used a transplant technique to show that environmental factors act on precursors in the ventricular zone during the final stages of the cell cycle, between the end of S-phase and mitosis (Fig. 3). They showed that if cells were removed from the brain before exposure to these factors, and transplanted into a new environment in which later born cell were dividing, they would take on the laminar fate of cells dividing in the new environment. However if the cells were transplanted after exposure, they retained their deep layer fate. But where do laminar fate cues come from? Laminar fate can be altered by selectively destroying earlier born cortical cells (Gillies and Price, 1993). Thus, these experiments suggest that cortical laminar fate cues may come from the earlier born cells of the cortical plate. However, fate cues could potentially come from any source including glial cells, subcortical structures and the cerebrospinal fluid.

1.8.1 Tissue culture

The purpose of this study was to localise cortical laminar fate cues. A tissue culture technique was employed to do this.

Tissue culture was created by Ross G. Harrison (Harrison, 1907, 1910) in an attempt to determine how axons are established. Harrison put pieces of neural crest from frog embryos into drops of lymph and observed axonal extension under the microscope. This technique provided the first direct evidence that growth cones are the sites of axonal extension. Tissue culture techniques have evolved dramatically since that time. To date, four relatively distinct techniques have been employed to study developmental processes in slices of brain tissue. These include (1) the





Cell a → layer 2/3
Cell b → layer 6

Figure 3. McConnell and Kaznowski (1991) used heterochronic transplants to show that laminar fate cues act during late S-phase or G2 of the cell cycle. They showed that if cells were removed from the brain during S-phase, and transplanted into a new environment in which later born cells were dividing, they would take on the laminar fate of cells dividing in the new environment (cell a, in this illustration). However, if cells were transplanted between S-phase and mitosis, they retained their deep layer fate (cell b).

Maximow depression-slide technique in which brain slices are maintained in a drop of culture medium in a small glass-enclosed chamber, (2) the coverslip/roller-tube method in which explants are enclosed in a plasma clot or vitrogen gel on a coverslip, and alternately exposed to medium and air by means of rotation in a roller-drum (3) the polyamide gauze/rocker method, in which several explants are placed on a piece of gauze seated in a medium bath and intermittently exposed to air by slowly rocking the cultures (1-3 reviewed in Humphreys *et al.*, 1996) and (4) the air/medium interface method, in which explants are placed on a porous membrane at the interface between medium below and air above, without rocking (Stoppini *et al.*, 1991).

Tissue culture techniques provide a system that is highly amenable to manipulation and can therefore be used in ways that *in vivo* techniques cannot. For example, regions of the brain can be separated and grown in isolation or in combination with a variety of other tissues. Such techniques have shed light on a variety of developmental mechanisms. For example, Molnar and Blakemore (1991) used organotypic corticothalamic co-cultures to show that the topographic distribution of thalamic fibres does not depend upon regional chemoattractants. Price and Lotto (1996) used a similar technique to show that the thalamus influences the survival of cortical subplate cells in the developing mouse cortex. Del Rio *et al.* (1995) also used a co-culture system to demonstrate a role of entorhinal afferents in hippocampal Cajal-Retzius (CR) cell death. Lavdas *et al.* (1997) showed that serotonin promotes the differentiation of glutamate neurons in organotypic slice cultures of the developing cerebral cortex.

Tissue culture has also been used to study cell migration. The migration of cells that had divided *in vivo* in cortical explants have been studied using the coverslip/roller-tube method in which explants were enclosed in a plasma clot (Gotz and Bolz, 1992) or vitrogen gel (Roberts *et al.*, 1993). One of the problems with the use of the roller tube method is that it causes extreme flattening of the tissue (to a single cell layer) and therefore the 3-dimensional aspect of the cortical architecture is

lost. This might explain why migration terminated prematurely (Gotz and Bolz, 1992) or why neurons appeared to 'slip' back to the ventricular part of the slice after reaching the top of the cortical wall (Roberts *et al.*, 1993). Gillies and Price (1993) used a different system (developed by Stoppini *et al.*, 1991, see above), to study cell migration in cortical slice cultures. They found that in this system, in which slices were cultured on a collagen filter, the slices flattened by only 20%, even after 14 days in culture. This technique, with appropriate modifications (see Chapter 4), was employed for the studies undertaken here.

1.9.1 Standardisation of embryonic ages

The studies carried out in this thesis were done mainly on embryonic mouse brains. Two different methods are used to designate embryonic age. The method used in this thesis refers to the day on which a vaginal plug is seen as embryonic day 1 (E1). For simplicity, when referencing work in which the day of the plug was deemed E0, the ages referred to have been adjusted appropriately to match the system used here.

1.10.1 Aims and objectives of this study

The aims and objectives of this study were to investigate the factors that are important for the normal formation of cortical layers, and particularly to attempt to localise laminar fate cues, the nature of which are largely unknown. Although the formation of cortical layers from cells dividing in the ventricular zone is already well characterised, I felt it was necessary to examine this process (1) in the mouse strain that I intended using for the *in vitro* studies and (2) using BrdU (which I used in the subsequent *in vitro* studies) as a marker of cell proliferation, since most of the earlier studies have been done using [3H] thymidine. Therefore I began this study by investigating the migration and/or location of E12, E14, E16 and E18 born precursor cells in the developing cortical wall in BALB/c mice. The second stage in this process was to ask if cells that are born *in vitro* (in a defined environment) would mimic their *in vivo* behaviour and migrate and form normal cortical laminae. This required that I develop a tissue culture technique that was suitable for this study. I employed a technique already in use in the lab and modified it considerably to suit

this study. The results of the tissue culture study showed that, under certain culture conditions, *in vitro* born cells will migrate relatively normally, but they will not form new cortical layers. Therefore, I investigated the numbers and location of Cajal-Retzius cells (which play an important role in normal lamination) in the cultured tissue. Previously, this has only been done on postnatal cortical slices. Based on the results of my *in vitro* study, which showed that the thalamus was important for the normal process of migration (and possibly lamination), I used an antibody to calretinin (which labels specific thalamic nuclei and thalamocortical axons) to study the thalamus and its axonal projections and Cajal-Retzius cells in the marginal zone of *Pax-6* mutant mice in which cortical migration is aberrant. Although gene expression has been studied in the diencephalon of the Pax-6 mutant, later markers of its differentiated state have not been used. Neither have Cajal-Retzius cells been studied in the mutant.

The initial *in vivo* study showed that when animals were injected with BrdU on E18, two populations of cells were present. Investigation of the literature led me to realise that both neurons and glial cells are born at around this time. Since most studies on gliogenesis have been done in the postnatal animal, I decided to investigate the development of the cells born during the transition between neurogenesis and gliogenesis, in the late embryo and newborn mouse. For this study I also developed a double labelling protocol to help me identify the nature of these cells.

CHAPTER 2: CELL MIGRATION IN THE DEVELOPING MOUSE NEOCORTEX: AN *IN VIVO* STUDY

2.1 INTRODUCTION

As described in the introduction to this thesis, the cortical layers form from cells that divide in the ventricular zone and migrate to the cortical plate. I decided to investigate this process in the mouse strain and using the marker of proliferating cells that I intended to use in the *in vitro* experiments described in Chapter 4 of this thesis.

Most of the early studies on neuronal migration used tritiated thymidine ([^{3}H] thymidine) to label dividing cells (Angevine and Sidman, 1961; Berry *et al.*, 1964; Rakic, 1974 and Luskin and Shatz, 1985b). However 5-bromo-2-deoxyuridine (BrdU) is a useful alternative that can be employed to study proliferation, migration and time of origin of cells in the developing central nervous system (Miller and Nowakowski, 1988). Like [^{3}H] thymidine, BrdU is incorporated into the DNA of proliferating cells during S-phase of the cell cycle. BrdU has two main advantages over [^{3}H] thymidine. Firstly it does not require the lengthy exposure times that are necessary to reveal [^{3}H] thymidine labelling. Secondly, it can be visualised in relatively thick tissue sections whereas limited emission of radiation means that only cells on the surface of a tissue section can be visualised when [^{3}H] thymidine is used (Rogers, 1973 cited in Miller and Nowakowski, 1988). Takahashi *et al.* (1992) showed that a single injection of 50 $\mu g\ g^{-1}$ BrdU will label 100% of nuclei in S-phase over a period of between 15 minutes and at least two a half hours in E14 mouse cerebral cortex. Mione *et al.* (1997) found that BrdU was still detectable in cells that had undergone at least four divisions after incorporating it, thus it is a useful marker for labelling cells of several consecutive generations. Like [^{3}H] thymidine, it can provide strict quantitative information about the mitotic history of the neurons that incorporate it (Thomaidou *et al.*, 1997). However, in this study, the density of the immunocytochemical detection product (diaminobenzidine, in this case) was used to distinguish first and subsequent generations of cells only.

The aim of this study was to provide a direct *in vivo* correlate for the *in vitro* experiments to follow. As mentioned above, migration and lamination in the mouse cortex is well-characterised (Angevine and Sidman, 1961, Caviness, 1982; Gillies and Price 1993). However, the early studies (Angevine and Sidman, 1961, Caviness, 1982) used [^{3}H] thymidine as the marker of dividing cells and did not investigate the lightly labelled cell population (Caviness, 1982) or did not differentiate quantitatively between densely and lightly labelled populations (Angevine and Sidman, 1961). In addition, although BrdU has some advantages over [^{3}H] thymidine, it has been suggested that, at high concentrations, it may interfere with mitosis in cortical neurons *in utero* (Kolb *et al.*, 1997). The concentrations used in this study were unlikely to produce such an effect. However it was important not to rely on these earlier studies using [^{3}H] thymidine, as direct correlates for the *in vitro* work to follow. Although Gillies and Price (1993) used BrdU investigate the ultimate laminar position of BrdU labelled cells in the mouse, they did not investigate the pattern of migration of these cells. Thus, this study differs in some or all respects from previous studies in that it uses BrdU as a marker of proliferating cells, differentiates between darkly and lightly labelled populations and was done at embryonic ages and early postnatally to reveal the early events in the formation of cortical layers.

2.2 MATERIALS AND METHODS

Animals and BrdU administration

BALB/c mice from an isolated colony were mated overnight and checked for a vaginal plug at 9 a.m. the following day. The day of plug was considered embryonic day one (E1). Mice were injected (i.p.) with BrdU in saline solution (70μg g^{-1}) at midday on E12, E14 or E16. For each injection protocol, three to five brains from two or more litters were reacted immunohistochemically and analysed at E16, E18 and P1 (see Table 1).

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Dissections and fixation

Embryonic mice brains were obtained as follows: the mother was anaesthetised using 0.35 ml 25% urethane in saline (i.p.) and the embryos were removed by Caesarian section. The brains were removed from the skulls and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 1/2 hours. They were then rinsed thoroughly and stored overnight (or longer) in PBS at 4^0 C.

For pup brains, the animals were killed using 0.05 ml sodium pentobarbitone (60 mg/ml; intramuscular). They were then perfused with cold 0.9% saline followed by 4% paraformaldehyde in PBS. The brains were removed from the skulls and fixed in 4% paraformaldehyde for 1 ½ - 3 hours then rinsed and stored in PBS as described above.

Tissue processing

All brains were dehydrated in a series of alcohols (75% ethanol for 30 minutes x 2, 95% ethanol for 30 minutes x 2, 100% ethanol for 30 minutes x 3) then cleared in chloroform for 90 minutes. The brains were then immersed in paraffin wax (60^o C) for three hours then positioned for sectioning in wax pots. Ten μm parasagittal sections were cut using a wax microtome and mounted on poly-L-lysine coated slides and left overnight (or longer) in an incubator at 37°C to dry. The slides were dewaxed in xylene (12 minutes) then the tissue was rehydrated in a series of alcohols (100% alcohol for three minutes, 95% alcohol, three minutes, 75% alcohol, three

minutes and tap water, three minutes). The slides were then reacted immunohistochemically to reveal BrdU labelling.

BrdU immunohistochemistry

Slides were immersed in 0.1% trypsin in 0.1% $CaCl_2$ at 37° C for 25 minutes to permeabilise the cell membranes. They were then washed in tris buffered saline (TBS at pH 7.6, for five minutes) then immersed in 1N HCl (for eight minutes at 60° C) to denature the double stranded DNA. The slides were then rinsed and washed in tap water (for five minutes), rinsed in TBS and coated with blocking serum (normal rabbit serum in TBS, 1 in 5, for 40 minutes). This was replaced with primary anti-BrdU (Becton Dickinson; diluted 1 in 200 in blocking solution, for 30 minutes). A negative control was done (1) by omitting the primary antibody from the blocking solution or (2) on tissue that had not been exposed to BrdU. The slides were rinsed and washed twice (5 minutes per wash) in TBS and then treated with secondary antibody (Dako, biotinylated rabbit anti-mouse; diluted 1 in 200, for 30 minutes). The slides were then rinsed and washed twice as before. ABC complex (Vector) was diluted according to the manufacturer's instructions and applied for thirty minutes. The slides were washed as before then reacted with 3,3'-diaminobenzidine (DAB, Sigma) solution (0.05% DAB, 750 µl tris buffer, 20 µl 0.1% H_2O_2 and one flake of imidazole) for eight minutes. They were then washed twice in tap water, stained in 0.1% cresyl-violet (for twenty seconds to 1 minute) and differentiated in 70% alcohol and 95% alcohol (acidified with a few drops of acetic acid) and rehydrated in 70% alcohol (2 minutes in each) then rinsed in water and allowed to air dry overnight. They were then coverslipped with DPX mounding medium (BDH).

Measuring cell distribution

Camera Lucida drawings were made of densely and lightly BrdU labelled cell profiles (subsequently referred to as cells) throughout the depth of the cortical wall, in a 0.5mm wide section of the cortex. Drawings were made at x25 magnification using a Leica Laborlux S microscope and drawing tube. Following the protocol of Gillies and Price (1993) and Caric *et al.* (1997) the analysis was made in the dorsal

cortex, one third of the way between the medial and lateral parts of the brain, directly above the lateral ventricle, corresponding approximately to Figure GD 18 SAG. 8 (Schambra *et al.*, 1992; see Appendix to this thesis). Four drawings were made of non-consecutive sections. The drawings were divided into ten equal sized bins, from ventricular edge to pial edge, and the average number of cells in each bin was plotted against bin number.

To correlate bins with cortical zones, camera Lucida drawings were made of the cortical wall at all ages studied. Its zones were identified and the depth of each was measured. This is described in detail in Chapter 3.

TABLE 1

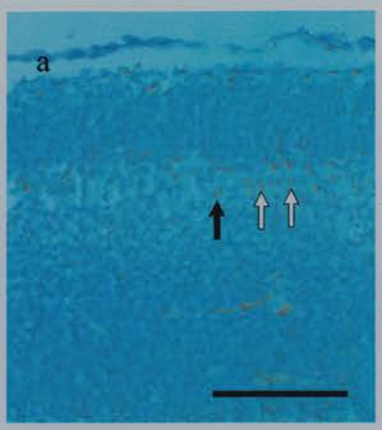
Time of Injection of BrdU	Time of fixation	No. of brains	No. of litters
Midday E12	Midday E16	3	2
Midday E12	Midday E18	5	2
Midday E12	Midday P1	4	2
Midday E14	Midday E16	3	2
Midday E14	Midday E18	4	2
Midday E14	Midday P1	4	2
Midday E16	1.00 p.m. E16	5	3
Midday E16	Midday E18	5	3
Midday E16	Midday P1	4	2

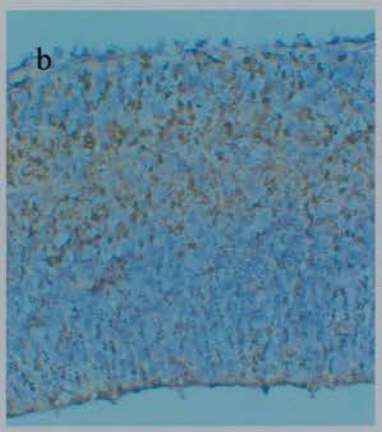
Table 1. Summary of the experimental protocol indicating the time at which BrdU was injected, the subsequent times at which the brains were fixed and processed and the number of brains that were used and litters from which they came.

2.3 RESULTS

BrdU labelled cells were seen in cresyl-violet stained sections of the mouse neocortex (Figures 1, 2 and 3). The brown DAB reaction product was usually clearly visible against the cresyl-violet stain. However, in older brains it was usually necessary to use a very light counter stain to be able to distinguish the BrdU labelled cells. They were often only clearly visible at high magnification (x25 and above). This is probably due to changes in the chromosome packing in the older brains that might lead to a dilution of the BrdU. As demonstrated previously (Del Rio and Soriano, 1989; Gillies and Price, 1993) BrdU labelled cells fall into one of two categories: (1) densely labelled cells and (2) lightly labelled cells. Camera Lucida drawings were made of both densely and lightly labelled cell nuclei. Classification of cells as densely or lightly labelled was done by eye. Only cells in which the nucleus was completely labelled (no matter the intensity of the reaction product) were considered densely labelled (filled arrows Figures 1, 2 and 3). Cells in which the nuclei were partly labelled were considered to be lightly labelled (open arrows Figures 1,2 and 3). Densely labelled cells were assumed to have become postmitotic at the end of the cycle in which they took up BrdU. Lightly labelled cells were considered to represent at least three populations (1) cells that may or may not have redivided after they incorporate BrdU but were lightly labelled because they were entering or leaving S-phase during the peak of the BrdU pulse, (2) cells that may or may not have redivided and were lightly labelled because they incorporated BrdU during the period before or after BrdU reached saturating levels, (3) cells that underwent at least one more round of the cell cycle after the cycle in which they incorporated BrdU. Nuclei of endothelial cells were easily identified by their characteristic shape (they are long narrow cells, associated with blood vessels) and were not included in the analysis.

Figure 1 shows photographs of sections through the cortical wall at E16 labelled with BrdU (a) at E12 (b) at E14 and (c) at E16. At E16, E12 labelled cells were seen mainly in the subplate and marginal zone (Fig. 1a). E14 labelled cells were seen mainly in the subplate and cortical plate (Fig.1b) although many very





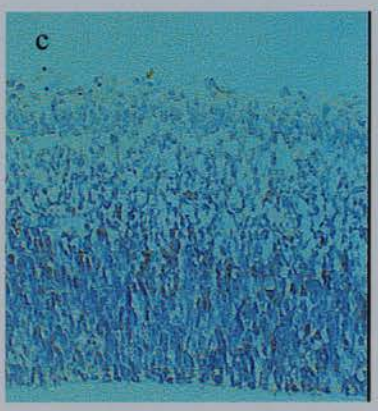


Figure 1 shows the cortical wall at E16 after BrdU injection at (a) E12 (b) E14 and (c) E16. The filled arrow in (a) indicates a densely labelled cell. The open arrows indicate lightly labelled cells. The cortical zones are indicated. PZ: proliferative zone, IZ: intermediate zone, SP: subplate, CP: cortical plate, MZ: marginal zone. Scale bar: 100 μm

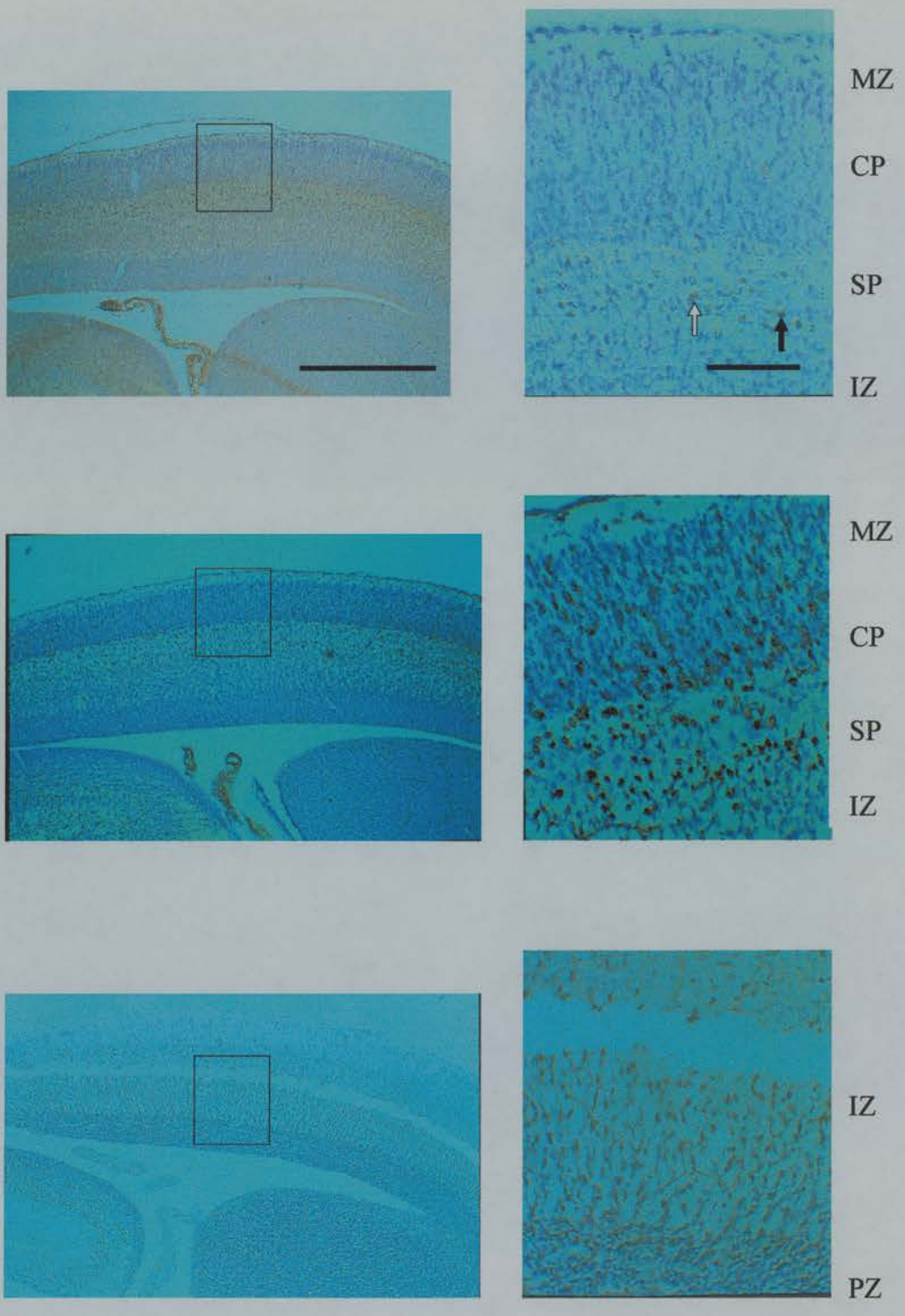


Figure 2 shows the cortical wall at E18 after BrdU injection at (a and b) E12 (c and d) E14 and (e and g) E16. The boxes in a, c, and e represent the regions from which the higher magnification views (b, d and f) are taken. The filled arrow in (b) indicates a densely labelled cell. The open arrow indicates a lightly labelled cell. Scale bar in (a): 500 μm. Scale bar in (b): 100 μm

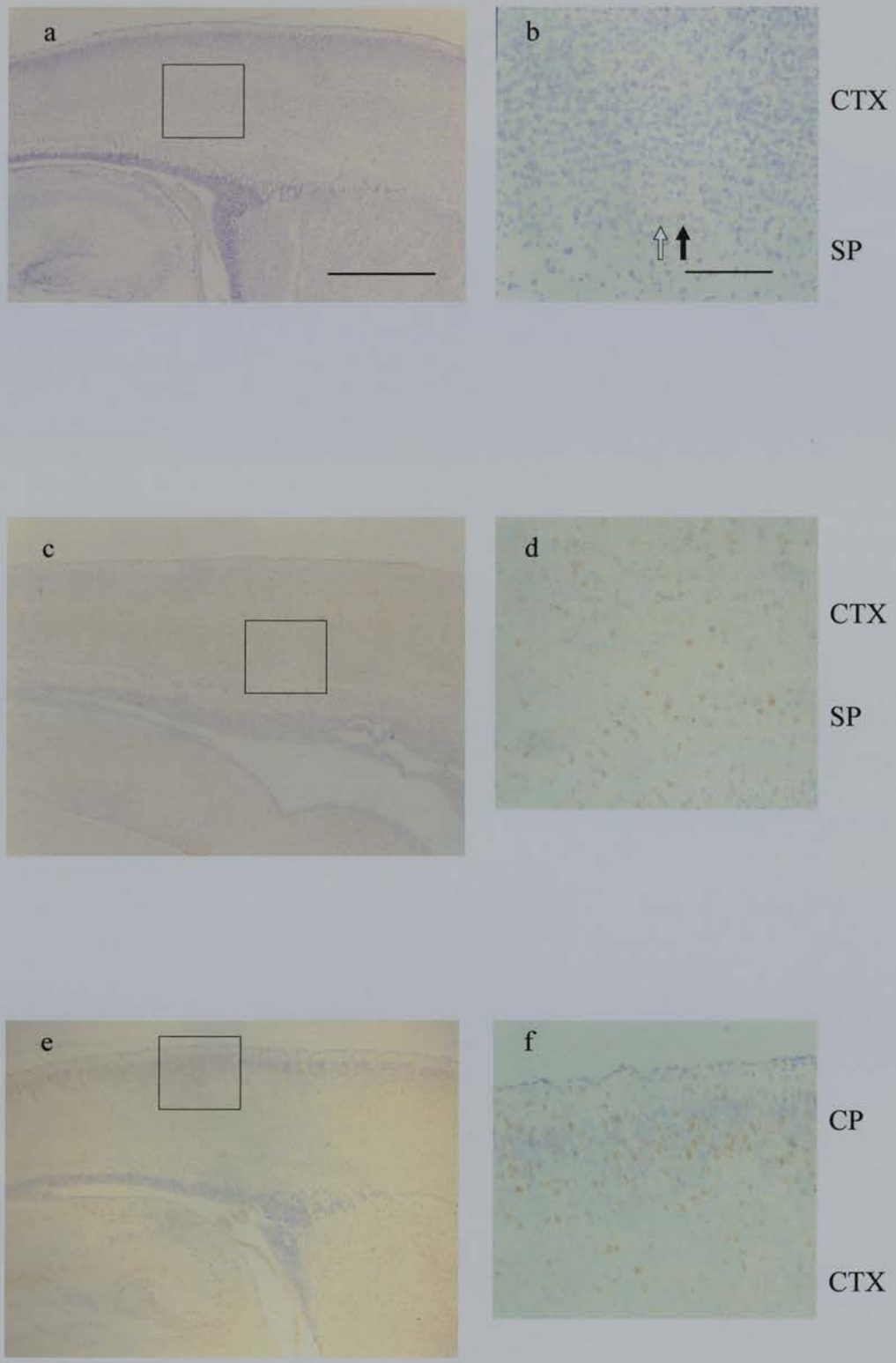


Figure 3 shows the cortical wall at P1 after BrdU injection at (a and b) E12 (c and d) E14 and (e and f) E16. The boxes in a, c and e represent the regions from which the higher magnification views (b, d, and f) are taken. The filled arrow in (b) indicates a densely labelled cell. The open arrow indicates a lightly labelled cell. CTX: cortex, SP: subplate. CP: cortical plate. Scale bar in (a): 500 μm. Scale bar in (b): 100 μm

lightly labelled cells were are also visible in the intermediate zone and proliferative zone. E16 labelled cells were seen exclusively in the proliferative zone, mainly at a distance from the ventricular edge, in a band just below the intermediate zone (Fig. 1c). Figure 2 shows photographs of sections through the neocortex at E18 after labelling with BrdU (a and b) at E12 (c and d) E14 and (d and e) E16. At E18, E12 labelled cells were found mainly in the subplate (Fig. 2b), though a few cells were also seen in the marginal zone. E14 labelled cells were found mainly in the cortical plate and subplate (Fig. 2d). E16 labelled cells were found mainly in the intermediate zone (Fig. 2f). Figure 3 shows photographs of sections through the neocortex at P1 after labelling with BrdU (a and b) at E12 (c and d) E14 and (d and e) E16. At P1, the E12 labelled cells were found exclusively in the subplate (Fig. 3b). E14 labelled cells were found in the deep layers of the cortex, just above the cell sparse intermediate zone and subplate (Fig. 3d). E16 labelled cells were found mainly in the cell dense cortical plate, just below the marginal zone/layer I (Fig. 3f).

The zones of the cortical wall were identified on the basis of location and cell density. The proliferative zone is the cell dense region right above the lateral ventricle. The intermediate zone is the cell sparse region above the proliferative zone. The subplate is also cell sparse and lies between the intermediate zone and the cortical plate. It was less easy to distinguish than the other zones, but it was identified in this study by utilising the location of E12 labelled cells within it (Figs 1a, 2b, 3b). The cortical plate is the cell dense region above the intermediate zone and subplate. As the cortical plate expands radially due to migration of cells from the ventricular zone, the cells in its lower layers begin to differentiate and form the cortex. The cortex is slightly less cell dense than the cortical plate. The marginal zone/layer I is the thin cell sparse region above the cortical plate.

Movement of labelled cells

For the purpose of describing the movements of labelled cells I assigned nuclei to bins according to their positions in the developing cortical wall. The cortical wall was divided into ten equal sized bins, numbered 1-10, from ventricular edge to pial

edge. The number of cells in each bin was plotted against bin number. Measurements of the average width of the cortical wall and its zones were used to correlate bins with cortical zones. The following analysis reveals the position of E12, E14 and E16 labelled cells at E16, E18 and P1. The position of a cohort of cells (that is, a densely or lightly labelled population, injected at either E12, E14 or E16) is described in terms of (1) its relative position in the cortical wall (2) its association with the cortical zones and (3) its relationship to the other cohorts. For the purposes of description, the cortical wall is described in terms of upper and lower halves: bins 1 - 5 constituting the lower or ventricular half and bins 6 - 10 constituting the upper or pial half.

E12 labelled cells

Figure 4 illustrates the relative positions of cells labelled with BrdU on E12 (a) at E16 (b) at E18 and (c) at P1. At E16, E12 labelled cells were found in the upper half of the cortical wall (Fig. 4a). Both densely and lightly labelled cells had a bimodal distribution, with peaks in bin 7, corresponding to the intermediate zone and subplate, and bin 8, corresponding to the subplate, and bin10, corresponding to the marginal zone (see also Fig. 1a). At E16 there was approximately two times as many lightly labelled cells as densely labelled ones.

At E18, both densely and lightly E12 labelled cells continued to exhibit a bimodal distribution (Fig.4b). The lower peaks were seen in bins 6 and 7, corresponding to the subplate (bin 6) and subplate and lower cortical plate (bin 7) at this age. The higher peak remained in bin 10, corresponding to the marginal zone and upper cortical plate. As illustrated in Figures 2 a and b the cells were actually found almost exclusively in the subplate and marginal zone. In comparison to their E16 distribution, the subplate population had become further separated from their marginal zone counterparts (compare Figs. 4 a and b). At this age there was approximately two and a half times as many lightly labelled cells as densely labelled ones.

At P1, both the densely and lightly E12 labelled cells demonstrated a peak in bins 5 and 6. Bin 5 represents the subplate and lower margin of the cortical plate and bin 6 represents the lower cortical plate. The subplate population had become further separated from the marginal zone/layer I compared to E16 and E18 (compare Figs. 4a and b with Fig. 4c). The E12 marginal zone/layer I cells were no longer seen at P1. The lightly labelled subplate population was approximately three times the size of the densely labelled population.

E14 labelled cells

Figure 5 shows the distribution of cells labelled with BrdU on E14 at (a) at E16 (b) at E18 and (c) at P1. Figure 5a shows that at E16, the densely E14 labelled cells were found throughout the cortical wall, but especially in the upper half of the wall, in bins 8, 9 and 10, corresponding to the cortical plate and marginal zone (see also Fig. 1b). Thus, the densely E14 labelled cells were approximately sandwiched between the two peaks in the distribution of the E12 labelled cells (compare Fig. 4a with Fig. 5a). Figure 5a shows that the lightly E14 labelled cells were found throughout bins 1-10, in regions corresponding to the proliferative zone, intermediate zone, subplate and cortical plate and marginal zone (see also Fig. 1b). There was no obvious peak in their distribution. Thus the lightly labelled cells coincided with the densely labelled cells and were also found in regions of the cortical wall beneath the densely labelled cells. There was approximately six times more lightly labelled cells than densely labelled ones.

At E18, the densely E14 labelled cells were found throughout the width of the cortical wall (Fig. 5b). However, there was a noticeable peak in their distribution in bins 6 and 7 which corresponds to the subplate and lower cortical plate (see also Figs. 2c and d). Thus they had also been displaced 'downwards' relative to their position at E16 and relative to the marginal zone E12 population (compare Fig. 5a with 5b). The lightly labelled cells were found predominantly in the outer half of the cortical wall, especially in bins 7, 8 and 9. These bins correspond to the subplate and cortical plate at this age (see also Fig. 2c and d). Thus, they were found both in the

same regions as the lightly labelled cells and in regions above the peak distribution of densely labelled cells. As at E16, there were more lightly labelled cells than densely labelled cells. However the lightly labelled population was now only three times the size of the densely labelled population.

Figure 5c shows that at P1, densely E14 labelled cells were found throughout the width of the cortex with a peak in their distribution in bins 4 and 5 which correspond to the subplate and deep layers of the developing cortical plate at this age (see also Fig. 3b and c). This population had also become further separated from the marginal zone/layer I, compared to their distributions at E16 and E18 (compare Figs. 5a and b with Fig. 5c). The lightly labelled cells were found in the upper two thirds of the cortical wall, especially in bins 5, 6, 7 and 8. These correspond to the subplate, lower and middle cortical plate/cortex at this age (see also Fig. 4c and d). As at E18, the peak in the distribution of lightly labelled cells lies above that of the densely labelled cells. Again, there was approximately three times as many lightly labelled cells as densely labelled cells.

E16 labelled cells

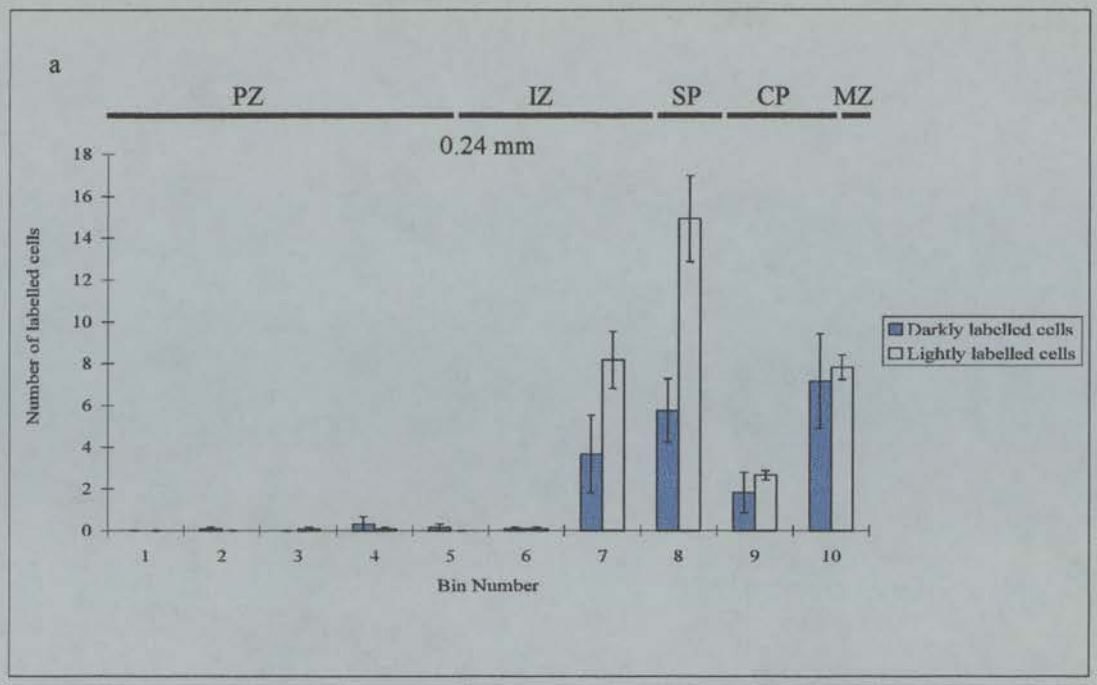
Figure 6 shows the distribution of cells labelled with BrdU on E16 at (a) at E16 (b) at E18 and (c) at P1. Figure 6a shows that one hour after injecting BrdU on E16, labelled cells (densely and lightly labelled cells) were found almost exclusively in the lower half the cortex, in bins 1 to 5, which correspond to the ventricular and subventricular zones (see also Fig. 1c). There was a peak in their distribution in bins 3 and 4. The E16 labelled cells therefore lay below the earlier born E12 and E14 populations (compare Figs. 4a and 5a with Fig. 6a). For reasons described in the discussion, these acutely labelled cells were not distinguished on the basis of the density of the their label.

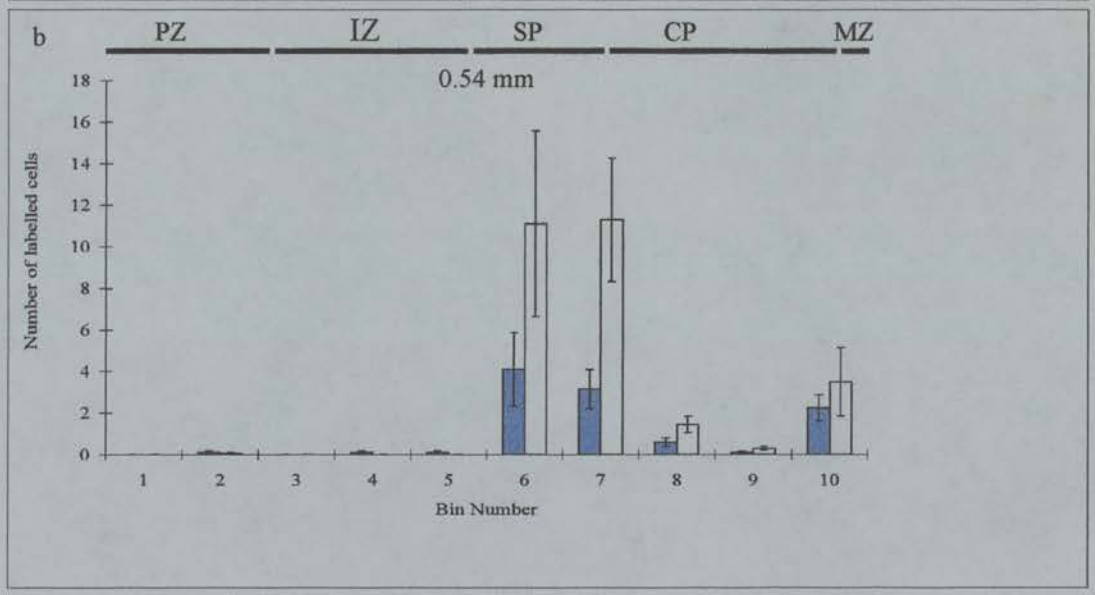
At E18 the E16 labelled cells were found throughout the width of the cortex (Fig. 6b). A few cells were seen in the cortical plate region (bins 7, 8, 9 and 10), but the majority were found in the middle of the cortical wall, in the subplate and

intermediate zones (bins 4, 5 and 6). The distribution of the lightly labelled cells was very like that of the densely labelled cells in terms of location (Fig. 6b). The peak in their distribution was in bins 4 and 5. This corresponds to the intermediate zone and subplate (See Figs. 7b, Fig.3e and f). Thus, densely and lightly labelled E16 populations had moved outward relative to their position at E16 and coincided with the E12 and E14 populations (compare Figs. 4b and 5b with Fig. 6b). There was approximately one and a quarter times more lightly labelled cells than densely labelled ones.

At P1, the densely E16 labelled cells were found in the upper third of the cortical wall, although a few were found throughout its entire width (Fig. 6c). There was a peak in their distribution in bin number 9, corresponding to the upper cortical plate (see also Figs. 3 d and e). Thus the E16 labelled cells had moved outward relative to their positions at E16 and E18 and had taken up a position above the E12 subplate and E14 populations (compare Figs. 4c and 5c with Fig. 6c). The lightly labelled cells had a very similar distribution to the densely labelled cells with a peak in bin number 9 (Fig. 6c). There was approximately one and three quarter times more lightly labelled cells than densely labelled ones.

Figure 4 illustrates the relative distribution of densely and lightly BrdU labelled cells after injection at E12, at (a) E16, (b) E18 and (c) P1. The cortical wall was divided into ten equal sized bins, numbered 1-10 from ventricular edge to pial edge. The blue bars represent the number of densely labelled cells and the open bars represent the number of lightly labelled cells. The approximate positions of the cortical zones and the average cortical width are indicated above each graph. PZ: proliferative zone; IZ: intermediate zone; SP: subplate; CP: cortical plate; MZ: marginal zone


a
PZ
IZ
SP
CP
MZ
0.24 mm
Number of labelled cells
Darkly labelled cells
Lightly labelled cells
Bin Number


b
PZ
IZ
SP
CP
MZ
0.54 mm
Number of labelled cells
Bin Number

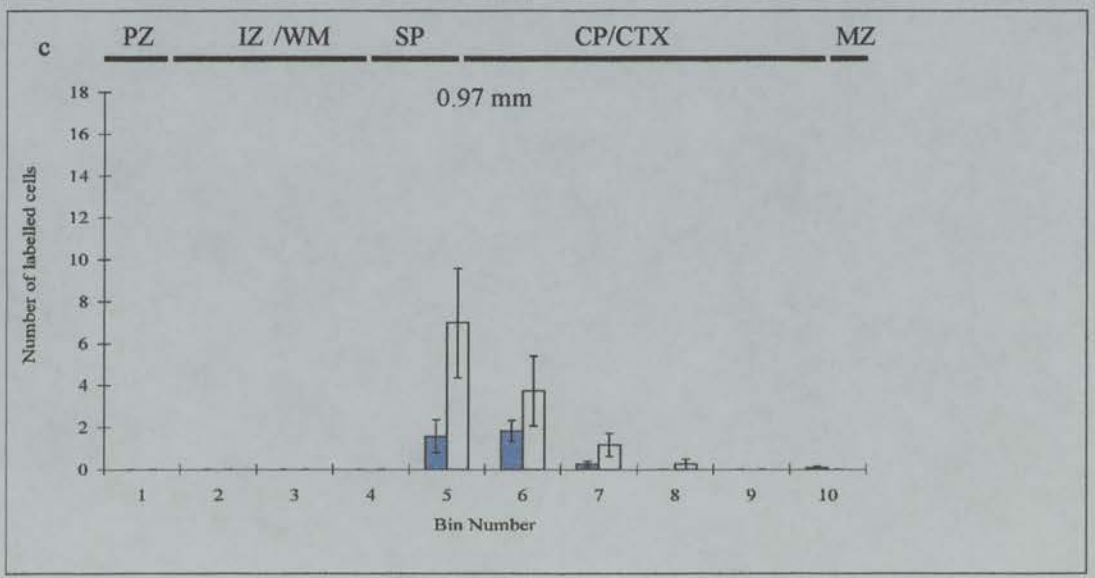
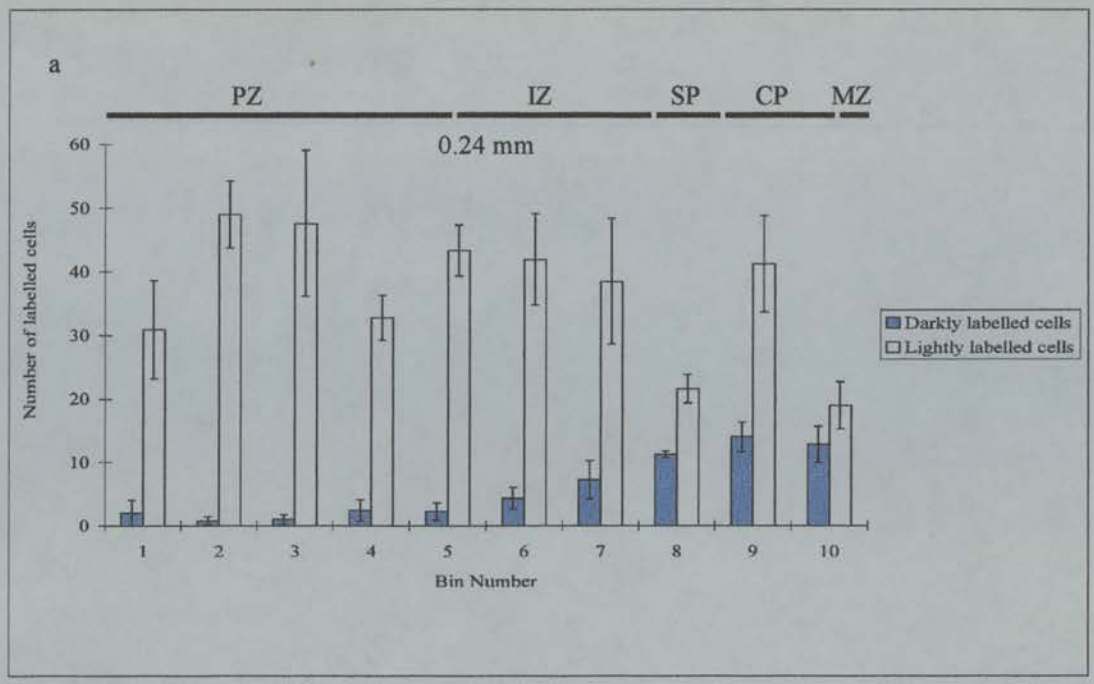
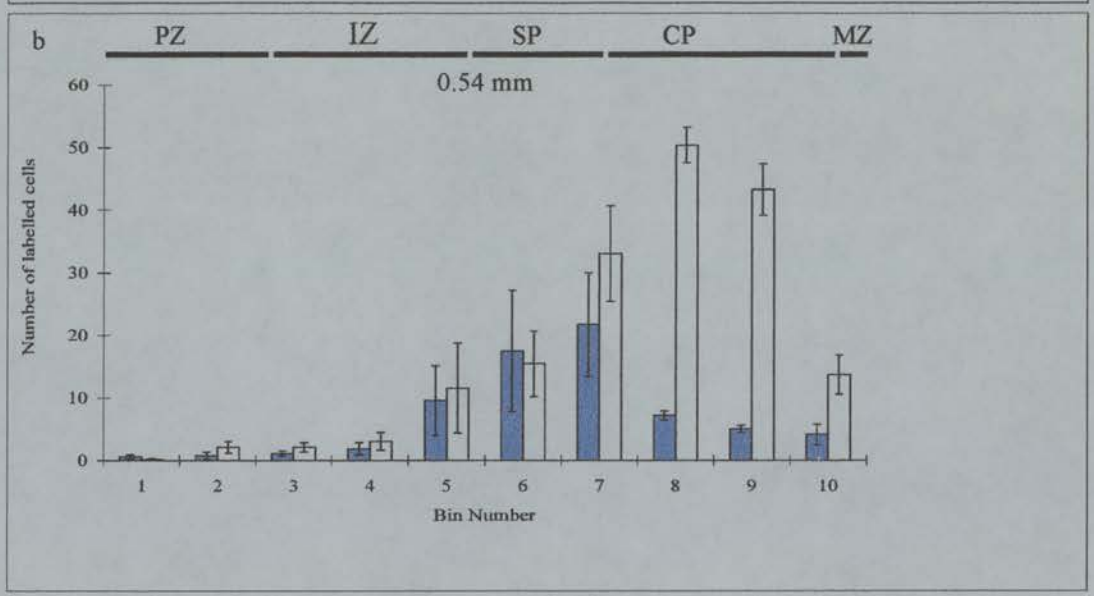

c
PZ
IZ /WM
SP
CP/CTX
MZ
0.97 mm
Number of labelled cells
Bin Number

Figure 5 illustrates the relative distribution of densely and lightly BrdU labelled cells after injection at E14, at (a) E16, (b) E18 and (c) P1. The cortical wall was divided into ten equal sized bins, numbered 1-10 from ventricular edge to pial edge. The blue bars represent the number of densely labelled cells and the open bars represent the number of lightly labelled cells. The approximate positions of the cortical zones and the average cortical width are indicated above each graph. PZ: proliferative zone; IZ: intermediate zone; SP: subplate; CP: cortical plate; MZ: marginal zone


a
PZ
IZ
SP
CP
MZ
0.24 mm
Number of labelled cells
Bin Number
Darkly labelled cells
Lightly labelled cells


b
PZ
IZ
SP
CP
MZ
0.54 mm
Number of labelled cells
Bin Number

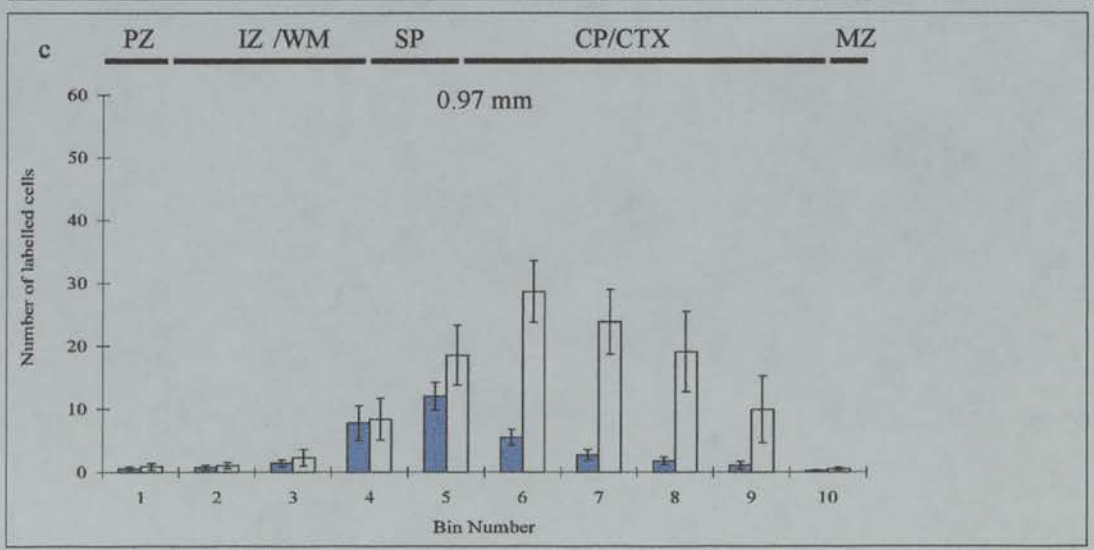
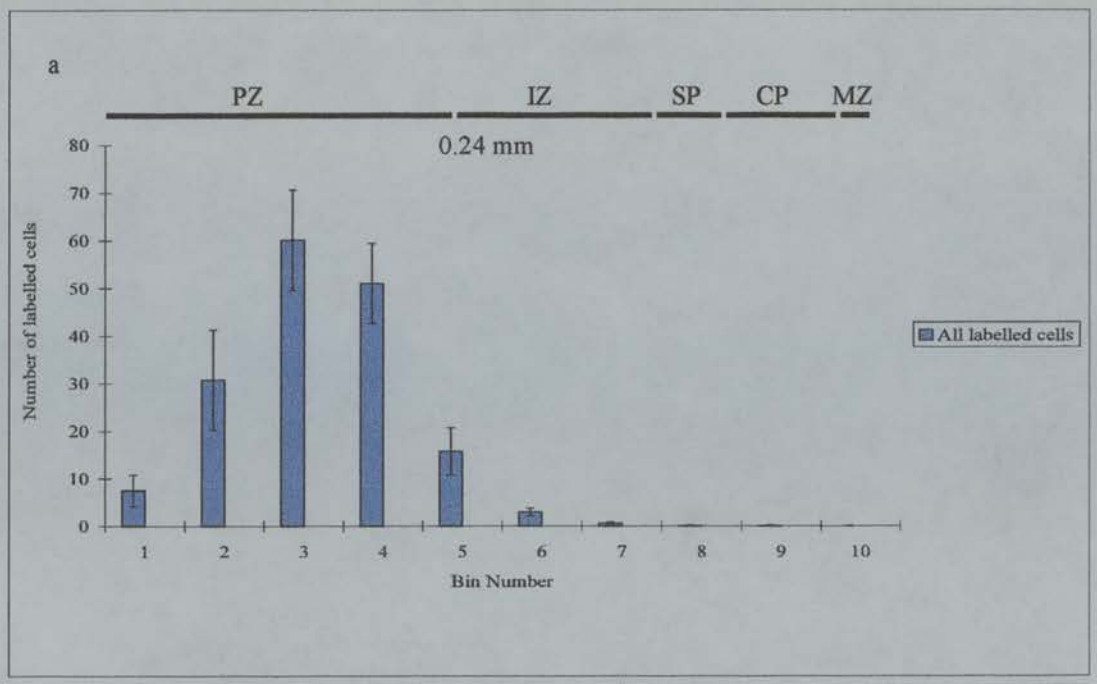
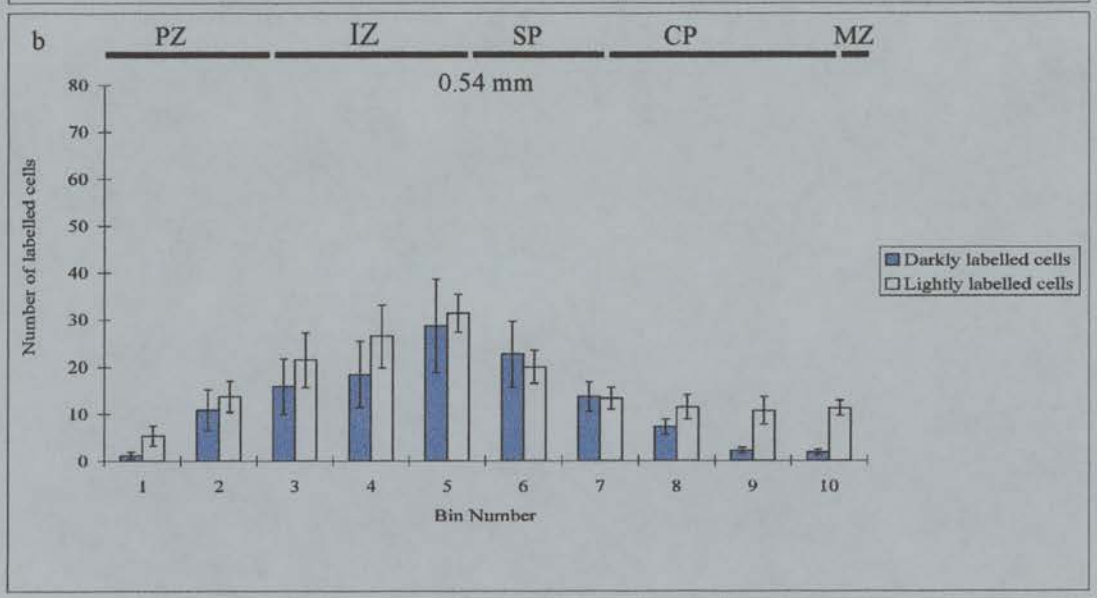
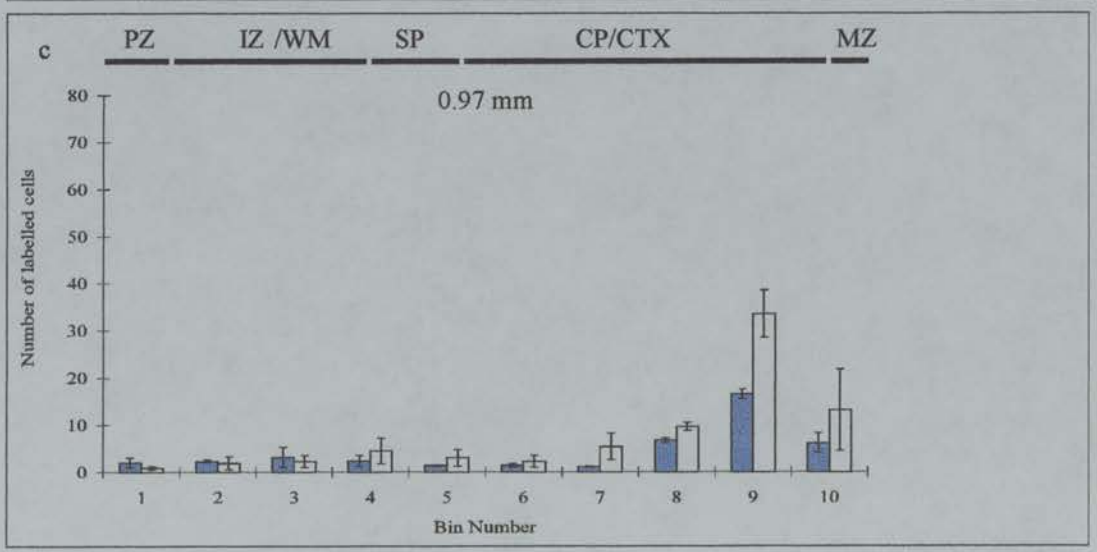

c
PZ
IZ /WM
SP
CP/CTX
MZ
0.97 mm
Number of labelled cells
Bin Number

Figure 6 illustrates the relative distribution of densely and lightly BrdU labelled cells after injection at E16, at (a) E16, (b) E18 and (c) P1. The cortical wall was divided into ten equal sized bins, numbered 1-10 from ventricular edge to pial edge. The blue bars represent the number of densely labelled cells and the open bars represent the number of lightly labelled cells. The approximate positions of the cortical zones and the average cortical width are indicated above each graph. PZ: proliferative zone; IZ/: intermediate zone/white matter; SP: subplate; CP/CTX; cortical plate/cortex; MZ: marginal zone


a
PZ
IZ
SP
CP
MZ
0.24 mm
Number of labelled cells
All labelled cells
Bin Number


b
PZ
IZ
SP
CP
MZ
0.54 mm
Number of labelled cells
Darkly labelled cells
Lightly labelled cells
Bin Number


c
PZ
IZ /WM
SP
CP/CTX
MZ
0.97 mm
Number of labelled cells
Bin Number

2.4 DISCUSSION

In this project I studied the locations of E12, E14 and E16 labelled cells in terms of (1) their relative positions within the cortical wall (2) their locations in the cortical zones and (3) their positions relative to each other. I described the locations of cells in terms of their relative positions within the cortical wall because, in the *in vitro* studies to follow, this was the most appropriate way to track cell movement. However, because of the changing thickness of the cortical wall *in vivo*, this method does not demonstrate the actual distances travelled by the cells. Therefore, the thickness of the cortical wall, at each of the ages investigated, was shown above each graph. The movement of E16 labelled cells, in terms of distances travelled, is described in more detail in Chapter 4.

The position of BrdU labelled cells at E16

At E16, the E12 labelled cells formed two populations: those of the marginal zone and those of the subplate. Luskin and Shatz (1985a) showed that the subplate and marginal zone populations begin their lives as a single layer of cells that is subsequently split in two by the insertion of cells forming the cortical plate. Indeed, I found that many of the E14 labelled cells (and, at later ages, E16 labelled cells) were approximately sandwiched between the two early born layers. The marginal zone and subplate were readily identified in E12 labelled sections by their location (above and below the cortical plate respectively) and their low cell densities (Boulder Committee, 1970). At E16 many E14 born cells were found subjacent to the marginal zone, in the cortical plate. Presumably, these cells had migrated there from the ventricular zone in the two days between E14 and E16. The cortical plate was identified by its high cell density compared to the overlying marginal zone and underlying subventricular and intermediate zones.

Cells labelled with BrdU for one hour on E16 were found mainly in the ventricular zone of the developing cortical wall. This is agreement with the pioneering studies of Angevine and Sidman (1961) who showed that the source of most cortical cells in the mouse is the primitive ependyma (ventricular zone; Boulder

Committee, 1970). The E16 born cells formed a normal distribution curve that peaked in bins 3 and 4. These bins probably correspond to the ventricular S-phase zone, where neuronal precursors replicate their DNA before undergoing mitosis. Cells located in bins 1 and 2 were probably in late S-phase at the start of the BrdU pulse and had progressed into G2 and M-phase of the cell cycle during the one hour period between the time the BrdU injection was given and the animals were sacrificed. This temporal progression of nuclei from the S-phase to the M-phase zone has been described previously (Takahashi *et al.*, 1992) for E14 labelled cells. Those cells that were found in bins in 5 and 6 are probably glial cell precursors in the subventricular zone that do not undergo interkinetic movement during their cell cycle (Takahashi *et al.*, 1992). The subventricular zone lies immediately above the S-phase region of the ventricular zone at this age.

In my analysis, I counted the group of cells that were labelled with BrdU for one hour and fixed immediately (acutely labelled cells) as a single population and did not differentiate into densely and lightly labelled populations. This is because the lightly labelled cells in this population do not represent cells that have undergone subsequent round(s) of division after the BrdU pulse. Takahashi *et al.* (1992) suggested that a distinct partial BrdU staining pattern is associated with acutely labelled cells that were either entering or leaving S-phase during the peak of the BrdU pulse. For all other analyses, I did distinguish densely labelled cells from lightly labelled cells because many of the lightly labelled cells do represent cells that re-divide after the BrdU pulse. Thus their birthdate is subsequent to the BrdU pulse.

BrdU labelled cell populations at E18

By E18, E12 born subplate cells and the E14 born cells and had been displaced away from the pial edge. This is due to the radial expansion of the cortical plate, as illustrated by the increasing thickness of the cortical plate (compare Figs. 1a and 2b and Figs. 1b and 2d) and has been described many times previously (for example, Marin-Padilla, 1992). At this age the E16 born cells were found mainly in the

intermediate zone, subplate and lower cortical plate suggesting that they had begun to migrate out of the ventricular zone.

BrdU labelled cell populations at P1

By P1, the E12 subplate and E14 labelled cells had been displaced further from the pial edge and very few E12 marginal zone/layer I cells remained. Although the fate of marginal zone cells is controversial, it has been suggested that Cajal-Retzius (CR) cells of the marginal zone/layer I die during early postnatal life (Del Rio *et al.*, 1995). Assuming that at least some of the E12 labelled cells are CR cells, cell death (which peaks at around P5, Del Rio *et al.*, 1995) and the considerable expansion of the cortex between E16 and P1, probably account for the dilution of this already sparse population. The E14 population, which forms the deep cortical layers, had probably begun to differentiate at this age.

At P1 the majority of E16 born cells were found in the outer (pial) region of the cortical plate, subjacent to the marginal zone/layer I (Fig.4e and f). This is in agreement with Marin Padilla's observation that all migrating neurons reach layer I (Marin Padilla, 1992). Unlike the E14 born cells that had presumably migrated from the ventricular edge to the marginal zone between E14 and E16, the E16 born cells took more than two days to reach the marginal zone (at E18, the cells were found mainly in the intermediate zone). Presumably this is because these cells have a greater distance to traverse, given that the cortical wall is much thinner between E14 and E16 than between E16 and E18, as illustrated by the thickness of the cortical wall, which is indicated above each distribution graph. At P1, a few E16 born cells were found in lower regions of the cortical wall. These cells may be neuronal precursors that are migrating at a much slower rate than their contemporaries. Alternatively, they might be glial precursors that do not follow the same route of migration as the neuronal precursors or stem cells that remain in adulthood.

The inside out formation of the cortical plate

The positional relationship between E14 and E16 densely labelled cells, and between the densely and lightly labelled populations (from a single BrdU pulse) at all ages of fixation, illustrate the well documented inside out formation of the cortical plate (Angevine and Sidman, 1961; Berry *et al.*, 1964; Berry and Eayres, 1965; Caviness, 1982). This is illustrated particularly well by the E14 densely and lightly labelled populations (see Figs. 5a, b and c). At E16 (Fig. 5a), many lightly labelled cells were seen in the lower regions of the cortical wall, beneath the densely labelled cells. Presumably many of these represent cells that have re-entered the cell cycle after the densely labelled ones have become terminally postmitotic. Thus, they were probably born after the densely labelled ones. This would account for their position in the cortical wall, since it would suggest that they are still dividing in the ventricular zone or only just beginning migration. By E18, however (Fig.5b), most of these cells had bypassed the densely labelled population and had come to lie above them. By P1 (Fig. 5c) the lightly labelled population had themselves been displaced from the upper cortical plate, presumably by E16 (and possibly E17 labelled cells), as illustrated by Figure 6c.

A quantitative analysis of BrdU labelled cell populations

There was a much smaller population of E12 born cells than E14 and E16 born cells at all ages studied. This can be explained by the relatively small size of the founder population at the start of neurogenesis and the fact that the output fraction (i.e. the fraction of dividing cells that become terminally postmitotic) is very small at this time (Caviness *et al.*, 1995). The much larger E14 and E16 born populations arise at a time in neurogenesis when both the founder population and the output fraction are large (Caviness *et al.*, 1995). At all ages of injection, there were more lightly labelled than densely labelled cells. This is particularly noticeable for the E14 labelled population. This result is not surprising in light of the fact that cells dividing on this day give rise to (1) cells that will become terminally postmitotic (that is, E14 born cells) and (2) cells that will re-enter the cell cycle and undergo up to six more

rounds of the cell cycle before neurogenesis ends (Caviness *et al.*, 1995). On the contrary, cells labelled on E16 that do not become terminally postmitotic will only undergo a further one or two rounds of cell division before neurogenesis is complete (Caviness *et al.*, 1995). Thus the large proportion of lightly labelled cells derived from BrdU injection on E14 is readily explained by the fact that they probably arise from several contiguous rounds of cell division. There was proportionally more lightly labelled cells at E16 than at E18 and P1. This is contrary to what might be expected given that more cell divisions will occur with increased time after BrdU injection. Presumably this is due to the fact that the BrdU is diluted out during sequential cell divisions.

Surprisingly, given that the E12 labelled cells that do not become terminally postmitotic after the BrdU pulse will undergo up to ten more rounds of the cell cycle after they are labelled, the proportion of lightly labelled cells was not much greater than densely labelled cells. Although the dilution of BrdU accounts for loss of some labelling, given the proportional size of the E14 lightly labelled population at E16, I would have expected to see more lightly E12 labelled cells. This could mean that a disproportionately large number of the E12 lightly labelled cells die before E16. However, I think it is probably more likely that the immunohistochemical technique used in this study was not sufficiently sensitive or consistent to reveal all the lightly labelled cells that were present. I found that the protocol that I used in the next chapter, to look at BrdU labelled cells with a fluorescent marker, was more sensitive. The main difference between that protocol and the one used here is the method used to denature the double stranded DNA and the concentrations of the antibodies used.

In summary, these results illustrate (as earlier studies have done) the inside out formation of the cortical plate, confirm the suitability of BrdU as a marker for the study of cell migration and lamination and provide a quantitative analysis of neocortical cell output that is in line with that predicted by Caviness *et al.* (1995). For the purposes of this thesis they provide a correlate for the *in vitro* studies to follow.

CHAPTER 3: THE TRANSITION BETWEEN NEUROGENESIS AND GLIOGENESIS IN THE DEVELOPING MOUSE NEOCORTEX: AN *IN VIVO* STUDY

INTRODUCTION

Following the investigation of cells born in the neocortical proliferative zone between E12 and E16 (Chapter 2), I decided to investigate the proliferation, migration and antigenic properties of cells born in the period between E18 and P0. The purpose of this study was to provide a clear picture of the events occurring during the period between the end of neurogenesis and beginning of gliogenesis, since there is very little literature on the events occurring during this transition period.

As described in the introduction to this thesis, the neurons and glial cells that populate the cerebral neocortex and underlying cerebral wall originate in the proliferative region that lines the ventricles of the developing cerebral cortex (Schaper, 1897, and His, 1889, 1904; both cited in Takahashi *et al.*, 1995; Boulder Committee, 1970; Schmechel and Rakic, 1979; Sidman and Rakic, 1982; Levinson an d Goldman, 1993; Takahashi *et al.*, 1993, 1994). This proliferative region consists of two distinct proliferative populations which, for the purposes of this study, are termed the pseudostratified ventricular epithelium (PVE) and the secondary proliferative population (SPP; Takahashi *et al.*, 1995a). The PVE approximates to the region designated the ventricular zone (Boulder Committee, 1970), whereas, at least in the midgestation mouse embryo, the SPP extends from the interface of the ventricular zone and subventricular zone, through the intermediate zone/white matter to the base of the developing cortical layers (Takahashi *et al.*, 1995a). Both the neuronal precursors and radial glial cells of the cerebral cortex derive from the PVE (Levitt *et al.*, 1981 and Misson *et al.*, 1988a; Takahashi *et al.*, 1992). The SPP, which gives rise to many of the late born glial cells of the cerebral cortex (Smart, 1961; Smart and Leblond, 1961; Patterson *et al.*, 1973; Privat, 1975; Mares and Bruckner, 1978; Todd and Smart, 1982; LeVine and Goldman, 1988a and b;

Levinson and Goldman, 1993), also develops out of the PVE (Smart, 1972; Altman and Bayer, 1990; Halliday and Cepko, 1992 and Takahashi *et al.*, 1993). PVE cells undergo an interkinetic movement in the ventricular zone as they progress through the different phases of the cell cycle (Sauer, 1935, 1936, cited in Sturrock and Smart, 1980; Takahashi *et al.*, 1992) whereas cells of the SSP do not (Takahashi *et al.*, 1992).

In the mouse, the SPP arises out of the PVE by E13 (Sturrock and Smart, 1980; Takahashi *et al.*, 1995a) and expands massively by cell division during the period prior to the end of neurogenesis (Sturrock, 1979 and Smart, 1976, cited in Sturrock and Smart, 1980; Takahashi *et al.*, 1995a). The PVE on the other hand is diminishing in size at this time and its cells undergo their ultimate division on E18 (Caviness *et al.*, 1995, Polleux *et al.*, 1997). This is reflected in the disappearance of the VZ at E18 (Takahashi *et al.*, 1995b). Thus, the period around the end of neurogenesis and beginning of gliogenesis is one of great dynamic change in the proliferative population. And yet, although the neurons that are born in this period are well characterised, with few exceptions (Gressens *et al.*, 1992; Kamel *et al.*, 1998), there is very little literature on the other cells that are dividing during this time. Thus, although there are several studies on postnatal gliogenesis in the mouse (Smart, 1961) and rat (Altman, 1966; Levison and Goldman, 1993; Lewis, 1968; Paterson *et al.*, 1973; Privat and Lebond, 1972), embryonic gliogenesis is not well characterised. This is due in part to the fact that the number of glial cells dividing in the embryonic brain is much less than that of neurons (Altman, 1966), therefore the mitotic glia are concealed by the larger neuronal population (Kamel *et al.*, 1998). Additionally, although the invasion of the cortical wall by neurons is well documented (Angevine and Sidman, 1961; Berry *et al.*, 1964; Caviness and Sidman, 1973; Luskin and Shatz, 1985b), much less is known about the migration of glial precursors (Levison and Goldman, 1993; and reviewed in McConnell, 1995). Therefore by studying cells dividing during this transitory period when the PVE is diminishing, I hoped to be able to discern the two populations and examine the behavior of each. This information is important for studies designed to investigate

the factors that guide neuronal/glial fate mechanisms, because little is known of the molecular events that promote the differentiation of progenitor cells into neurons or glia. Similarly, precise descriptions of patterns of cell division and migration are important for understanding the molecular basis of these processes.

I decided, therefore, to characterise the population of cells that are undergoing cell division in the period between E18 and P0 in the mouse. I used BrdU to label these populations. I compared the distribution of E18, E19 and P0 dividing cells with that of E16 and E17 born cells, one hour after BrdU injection. Next, I looked at the numbers and radial migration of the E18, E19 and P0 labelled cells at early postnatal stages. Finally I investigated the antigenic properties of these cells. I used antibodies to (1) glial fibrillary acidic protein (GFAP), which is a marker of cells of the astroglial lineage (radial glia and astrocytes) in mice (Choi, 1988) (2) 2',3'-cyclic nucleotide 3'-phophohydrolase (CNPase), which is a marker of the second stage of oligodendrocyte cell maturation (Cameron and Rakic, 1991) and (3) anti B-tubulin III which is a neuronal cell specific cytoskeletal component first expressed by neuronal precursors at their penultimate or terminal mitotic division (Cameron and Rakic, 1991).

It was found that the distribution of cells dividing on E18, E19 and P0 was quite different from that of cells dividing on E16 and E17. At later stages, the distribution of E18 labelled cells was different from that of the E19 and P0 labelled cells. Double labelling techniques revealed that only a small proportion of cells that labelled with BrdU on E18, E19 and P0 were positive for GFAP. Although many of the BrdU labelled cells coincided with regions strongly expressing CNPase or B-tubulin III, the nature of the labelling made it difficult to show if individual cells were double labelled. Thus the phenotypic nature of many of the cells remained unclear.

MATERIALS AND METHODS

Animals and BrdU injection

C3H mice from an inbred laboratory colony were mated overnight and the following day deemed E1. Pregnant mice were injected (i.p.) with BrdU in saline (50μg g^{-1}) at midday on E16, E17, E18 and E19 to label proliferating cells. P0 pups were also injected with BrdU (50μg g^{-1}; subcutaneously) at midday. Brains were fixed and analysed at one hour, two (+ 2d), five (+ 5d), nine (+9d) and twenty days (+ 20d) post-injection. At each time point, two to six brains from at least two separate litters were reacted immunohistochemically to reveal BrdU labelling. Adjacent sections or similar sections from the opposite hemisphere were also reacted for BrdU and GFAP, BrdU and CNPase or BrdU and B-tubulin III. The number of animals used in this study and the time points at which they were injected with BrdU and subsequently fixed for processing is listed in Table 1.

Dissections and fixation

Embryonic mice were obtained by Caesarian section from pregnant females injected (i.p.) with 0.35 ml 25% urethane in 0.9% saline. The brains were removed from the skulls and fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) for 3 hours. For postnatal brains, animals were injected with 0.05ml sodium pentobarbitone (60 mg ml^{-1} i.p) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in PBS, then postfixed for 3 hours. Brains were rinsed and stored overnight at 4^0 C in PBS.

Tissue processing

Embryonic and early postnatal brains were processed and wax embedded as described in Chapter 2. Older brains (P5 and older) were processed using an automated tissue processor. This protocol was the same as for the embryonic brains, except that they were first dehydrated in 50% alcohol instead of 70% alcohol and they were immersed in each alcohol for two to three hours instead of one hour.

The zones of the cortical wall

The developing cortical wall and its zones change over time. Camera Lucida drawings were made of the cortical wall and its zones using a Leitz Laborlux S light microscope and camera Lucida drawing tube at x 10 magnification. Drawings were made at embryonic and postnatal ages from sections from which the cell distribution analyses were obtained (see below for description). These sections were de-coverslipped (after the BrdU analysis was done) and heavily counter stained with cresyl-violet, then dehydrated in alcohols and cleared as described in Chapter 2.

The cortical zones (proliferative zone, intermediate zone/white matter, subplate, cortical plate/cortex and marginal zone/layer I) were identified by their location and cell density. The depth of each was measured. Three measurements were taken from one section of each of three brains, at each age studied. Three lines, 250 μm apart, were drawn at a 90° angle to the ventricular edge and the upper limit of each cortical zone was marked on each line. The first line was taken at the junction between the cortex and the striatum, the next was taken 250 μm caudally and the next a further 250 μm caudally. Measurements of the width of each zone were made using UTHSCASA Image Tool for Windows Version 1.27. The average width of each zone was taken to be the average from three animals. These measurements were plotted against age to reveal the changing stratification of the cortical wall and the average width of each zone.

Cell distribution

To investigate the distribution of BrdU labelled cells, camera Lucida drawings were made using a Leitz Laborlux S light microscope and camera Lucida drawing tube at x 25 magnification. Drawings were made of densely and lightly BrdU labelled cell profiles (subsequently referred to as cells) in a 0.5mm wide, 10 μm thick parasagittal section extending the width of the cortical wall (Fig.1a). This was done on E16, E17, E18, E19 and P0, one hour post-injection and for E18, E19 and P0 injected animals at +2d, +5d, +9d and +20d post-injection. Sections approximately one third of the distance from the midline to the lateral edge of the brain, corresponding to Fig.GD18

SAG.7 Shambra *et al.* (1992) in Atlas of the Prenatal Mouse Brain and sagittal section 133 Sidman *et al.* (1971) in Atlas of the Mouse Brain and Spinal Cord, were used. Copies of these figures are contained in the Appendix to this thesis. These sections could be identified by the presence of the lateral ventricle, hippocampus, fimbria of the hippocamus, choroid plexus and striatum (globus pallidus and caudate putamen) immediately ventral to the cortical wall. Four non-consecutive parasagittal sections were drawn from each brain. Where tissue preservation allowed, every fourth section was drawn. Where a damaged section prohibited counting, its neighbour was used. The average number of cells per cortical zone was plotted against cortical zone.

Cell numbers and Expansion factor

The average total number of BrdU labelled cells within the 500 μm wide bin at +2d post-injection was compared with that at +5d, +9d and +20d post-injection. Cell profile counts were corrected according to Abercombie's (1946) formula. The length of BrdU labelled cell nuclei was measured at x100 magnification, using a micrometer eyepiece, in a bin approximately 100 μm wide, extending the width of the cortical wall. For each age of injection, approximately 400 cells were measured. At a particular age, the average length of the cells was similar in all zones. The only exception was the neuronal population labelled on E18. These cells were significantly larger than the other cells after +5d post-injection, and were therefore dealt with separately. Since total cell counts were made in a bin of constant width (500 μm) and thickness (10 μm), the rostrocaudal and mediolateral expansion of the cortex that occurs over time had to be accounted for. To do this, the expansion in these directions was calculated and the + 2d counts were divided by the expansion factor to predict an expected value for the + 5d, + 9d and + 20d counts. For all of the ages concerned, four equally spaced parasagittal sections and 4 equally spaced coronal sections were measured (as illustrated in Fig. 1c). Measurements from 4 brains (2 sectioned coronally and 2 sectioned parasagittally, from each age) were used for this calculation. The distances along the pial edge and the ventricular edge (as indicated in the Fig. 1) were measured and the expansion factor was taken to be

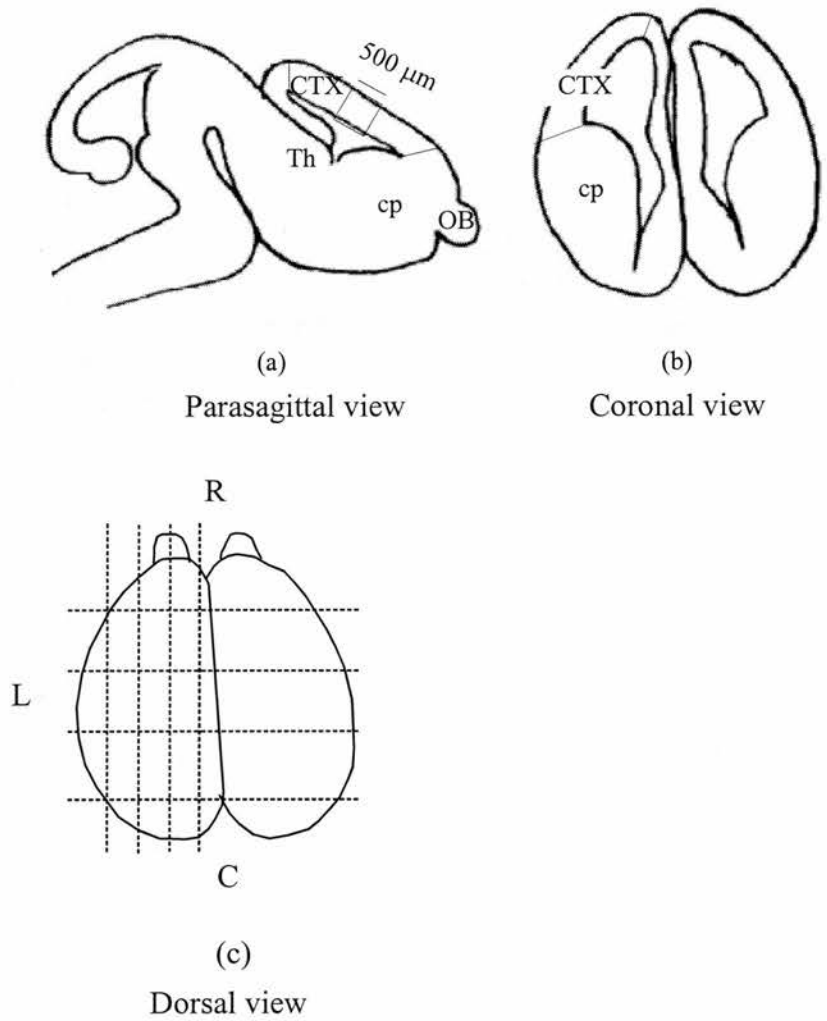


Figure 1. Schematic representation of how measurements of cell distribution and cortical expansion were made. (a) Cell distribution was measured in parasagittal sections in a 500 μm wide bin, immediately above the lateral ventricle, extending the entire width of the cortical wall. Cortical expansion was estimated by taking measurements of the extent of the ventricular and pial surfaces of the cortex (between the lines marked) in (a) and (b) in four equally spaced parasagittal and four equally spaced coronal sections (c) of which the 2nd most medial (a) and 2nd most rostral (b) are represented. CTX: cortex; cp: caudate putamen; Th: thalamus; OB: olfactory bulb; R: rostral C: caudal; L: lateral

the average of changes in both directions. A similar method was used previously (Price *et al.*, 1997).

Immunocytochemical procedures

Following fixation, and wax processing, 10 μm sections were cut in the parasagittal or coronal plane and mounted on poly-L-lysine coated slides. For the cell distribution study, BrdU labelled cells were revealed on parasagittal sections using DAB as the chromagen. For the double label studies, fluorochromes were used to reveal the antigens on parasagittal sections.

For the distribution studies, BrdU labelling was revealed using a method similar to that used in Chapter 2. The only difference being that 0.2% triton x 100 was added to the TBS for the incubations and washes. The TBS/triton was then thoroughly rinsed from the slides using tris buffer (pH 7.6) prior to incubation in DAB. Sections were counterstained and coverslipped as described in Chapter 2.

Combined anti-BrdU and anti-GFAP, CNPase or B-tubulin III immunocytochemistry

For the double labelling study, slides were dewaxed and treated with trypsin, as described above, then washed twice in PBS/0.2% triton (PBS-T). As a control, all antigens were first labelled individually with fluorochromes, before the double labelling study was carried out. They were then immersed in 50% HCl containing 1% triton X for 12 minutes at room temperature, rinsed in PBS-TX and incubated in blocking solution (10% swine serum in PBS-TX) for 40 minutes. The sections were then incubated in a solution of rabbit anti-GFAP (Sigma) and mouse anti-BrdU, both diluted 1:100 in blocking solution, overnight at 4° C. Sections were washed thoroughly in PBS-TX and incubated in a solution containing biotinylated swine anti-rabbit antibody (DAKO) 1:100 and Texas red anti-mouse IgG (Vector) 1 in 100 in PBS with 2% swine serum for 2 hours at room temperature. Sections were washed and treated with fluorescein avidin D (Vector) diluted 30 μg/ml in 0.1M sodium

bicarbonate, pH8.5, 0.15M NaCl, then rinsed, mounted with Vectashield anti-fade mounting medium (Vector) and coverslipped.

For double labelling with BrdU and CNPase or B-tubulin III, the sections were trypsinated as described in Chapter 2, washed and incubated with blocking solution (10% rabbit serum in PBS-TX) for 40 minutes. They were treated with mouse anti-CNPase (Chemicon) diluted 1 in 20 in blocking solution or anti-B-tubulin III (Sigma) diluted 1 in 100 in blocking solution overnight at 4° C, washed and reacted with biotinylated rabbit anti-mouse secondary (Dako) diluted 1 in 100 in blocking solution. They were visualised with fluorescein avidin D (Vector) diluted 30 g/ml in 0.1M sodium bicarbonate, pH8.5, 0.15M NaCl. The slides were then fixed in 50% ethanol/50% acetic acid, rinsed, immersed in 50% HCl containing 1% triton X for 12 minutes at room temperature then treated with anti-BrdU as described above.

Identification of double-labelled cells

To reveal the spatiotemporal pattern of expression of the fluorescent markers of GFAP, CNPase and B tubulin III, sections were viewed on a Leica DMR microscope. For the purposes of identifying double-labelled cells, a Leica TCS NT confocal microscope and Leica TCS NT v 1.6.551b software package were used. Double-labelled cells were studied at +20d. Ten μm thick sections were optically sectioned (to 0.5 to 1 μm) and optical sections were superimposed. Cells that were double labelled with BrdU and GFAP were identified. When there was doubt about their double-labelled nature, a 3D reconstruction was made of them using the Leica TCS NT v 1.6.551b software package, and only cells in which the nucleus (BrdU labelled) was clearly seen to be contained within the GFAP +ve processes were considered to be double labelled.

TABLE 1

Time of Injection of BrdU	Time of fixation	No. of brains	No. of litters
Midday E16	1.00 p.m. E16	5	3
Midday E17	1.00 p.m. E17	3	2
Midday E18	1.00 p.m. E18	6	3
Midday E18	Midday E18 + 2d	4	3
Midday E18	Midday E18 + 5d	4	3
Midday E18	Midday E18 + 9d	4	3
Midday E18	Midday E18 + 20d	4	3
Midday E19	1.00 p.m. E19	4	3
Midday E19	Midday E19 + 2d	4	3
Midday E19	Midday E19 + 5d	5	3
Midday E19	Midday E19 + 9d	4	2
Midday E19	Midday E19 + 20d	4	3
Midday P0	1.00 p.m. P0	4	3
Midday P0	Midday p0 + 2d	4	3
Midday P0	Midday P0 + 5d	4	3
Midday P0	Midday P0 + 9d	2	1
Midday P0	Midday P0 + 20d	3	3

Table 1. Summary of the experimental protocol indicating the time at which BrdU was injected, the subsequent time at which the brains were fixed and processed and the number of brains that were used and litters from which they came.

RESULTS

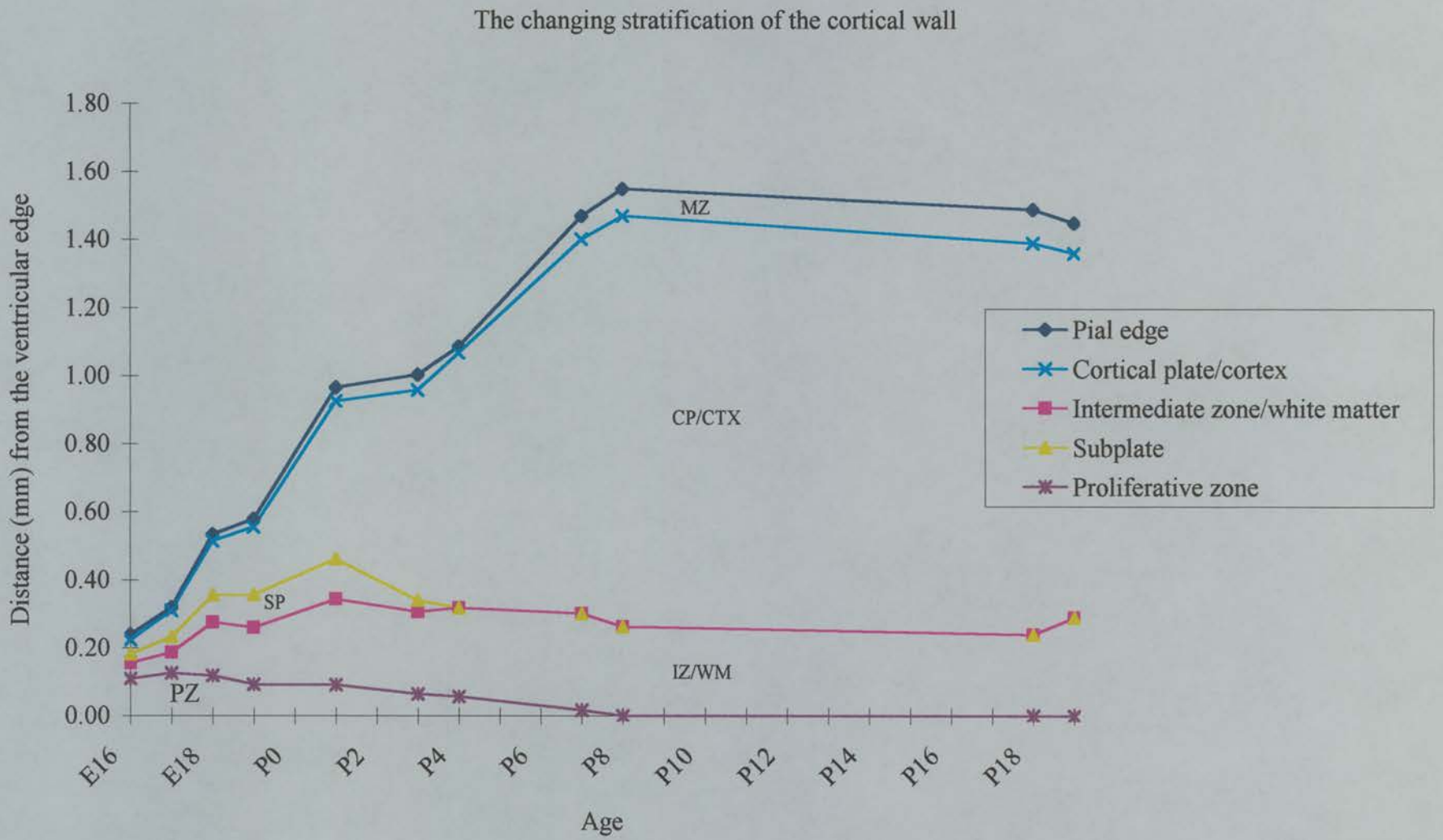
In this study I investigated the changing stratification of the cortical wall over the period of analysis. Then I investigated the change in the number of cells within a defined region of the cortical wall over time. Next I compared the distribution of cells dividing in the neocortex on E16, E17, E18, E19 and P0. Finally I studied the distribution and antigenic properties of E18, E19 and P0 labelled cells during the early postnatal period.

In the BrdU labelled sections it was found that densely and lightly labelled cells could be distinguished. The criteria for distinguishing densely and lightly labelled cells were described in Chapter 2. As described in Chapter 2, the amount of BrdU that a cell contains reveals something of the mitotic history of the cell. Therefore, when studying cells labelled on E18, E19 and P0, densely and lightly labeled cells were generally considered separately. However an exception was made for the data obtained from animals sacrificed one hour after BrdU injection (subsequently referred to as acutely labelled). In this case densely and lightly labelled populations were counted together. Since the cell cycle of E14-E16 born SPP cells is approximately 15 hours (Takahashi *et al.,* 1995a) it is extremely unlikely that the cell cycle of these cells is less than one hour, and so the lightly labelled cells cannot represent the product of a subsequent division. Therefore, for acutely labelled brains, it was assumed that both densely and lightly labelled cells were the products of a single cell cycle.

Development of the neocortical wall

Figure 2 shows the changing stratification of the neocortical wall during the period from E16 to P19. At E16 and E17 the proliferative zone, consisting of the ventricular and subventricular zones, accounted for almost half of the width of the cortical wall. The narrow intermediate zone lay just above. At these ages the cortical plate was just beginning to form and was still relatively thin. The marginal zone and subplate sandwiched the cortical plate between them. From E18 onward there was quite a dramatic change in the structure of the wall. The proliferative zone

Figure 2. The changing stratification of the developing cortical wall from E16 to P18. The points plotted represent the average distances of the outer limit of the cortical zones from the ventricular edge at the ages indicated on the x axis. PZ: proliferative zone; IZ/WM: intermediate zone/white matter; SP: subplate; CP/CTX: cortical plate/cortex; MZ: marginal zone.


The changing stratification of the cortical wall
Distance (mm) from the ventricular edge
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
E16
E18
P0
P2
P4
P6
P8
P10
P12
P14
P16
P18
Age
MZ
CP/CTX
SP
IZ/WM
PZ
Pial edge
Cortical plate/cortex
Intermediate zone/white matter
Subplate
Proliferative zone

began to decrease in width. This reduction continued until P7 or P8 when a single layer of cells was all that remained of this zone (see white arrow Fig. 10). As the proliferative zone diminished, the intermediate zone/white matter, subplate and cortical plate expanded. The subplate expanded only transiently and subsequently disappeared at around P4 or P5. The intermediate zone/white matter reached its maximum thickness at around P1. The cortical plate (cortex) expanded rapidly from about P0 onwards and reached its maximum width at about P9. At this stage the cortex accounted for almost 90% of the width of the cortical wall. Although Figure 3 suggests that the overall thickness of the cortical wall reduced slightly after P9, this was probably due to the processing method used in this study.

Cell numbers at postnatal age

I compared the number of labelled cells within a defined region of the cortical wall (see Fig. 1) at two days post-injection with that at five, nine and twenty days post-injection. Cell numbers were obtained from cell profile counts after correcting according to the method of Abercrombie (1946). Cell counts from the acutely labelled animals were not used in this comparison because the periods of exposure to BrdU are not comparable. In acutely labelled brains the exposure to BrdU lasts no more than one hour. In animals allowed to survive for longer, the BrdU is available for incorporation until it is metabolised, a period of greater than 3.5 hours (Takahashi *et al.*, 1992). Since cortical expansion in the rostrocaudal and mediolateral directions will affect the cell counts, I calculated the amount by which the cortex expanded in these directions between two days post-injection and five, nine and twenty days post-injection. I divided the number of cells present at two days post-injection by the expansion factor at each age. This revealed the number of cells that would be expected to be found if cortical expansion alone affected cell number.

For E18 labelled cells there was a general decrease in cell number over time, from +2d to 20d (Fig. 3 grey and pink lines). However, for both densely and lightly labelled populations, this was similar to that predicted from the expansion factor

(Fig. 3 yellow and blue lines). The number of E19 labelled cells also decreased over time (Fig.4 purple and pink lines). However, the number of cells at 9 days post-injection was less than 20% and 34 % of that predicted for densely and lightly labelled populations respectively (Fig. 4 yellow and blue lines). There was a subsequent small increase in cell number at 20 days post-injection, but the number still remained at 51% and 57% of that predicted by the expansion factor. The number of P0 labelled cells also decreased with time (Fig. 5 grey and pink lines). However, for this population the decrease was much greater than that predicted by the expansion factor at all ages (Fig. 5 yellow and blue lines). At +5d the number was 27% and 46% of the predicted value for densely and lightly labelled populations respectively. At + 9 d it was less than 5% and 25% of that predicted and at +20d it was less that 30% and 37% of that predicted. The smaller than predicted number of cells at twenty days post injection could be more than completely accounted for by the earlier decrease. For all ages, the number of cells as a proportion of the predicted value is given in Table 2.

This analysis suggested that after E19 and P0 injections, some BrdU labelled cells disappear (at least from within the section analysed). I examined the proportions of densely and lightly labelled cells after BrdU injection at E18, E19 and P0, after +2d, +5, +9d and +20d. Figure 6 shows that the proportions of densely and lightly labelled cells are similar for the E18 and E19 injected animals, but the proportion of densely labelled cells is smaller (and conversely the proportion of lightly labelled cells is greater) for the P0 labelled animals, at all ages investigated. There was no significant change in the proportions of densely and lightly labelled cells at any of the ages investigated (Student t-test).

Figure 3. Graph of the number of BrdU labelled cells within a defined region of the cortical wall at E18 +2d, +5d, +9d and +20d, versus expected.

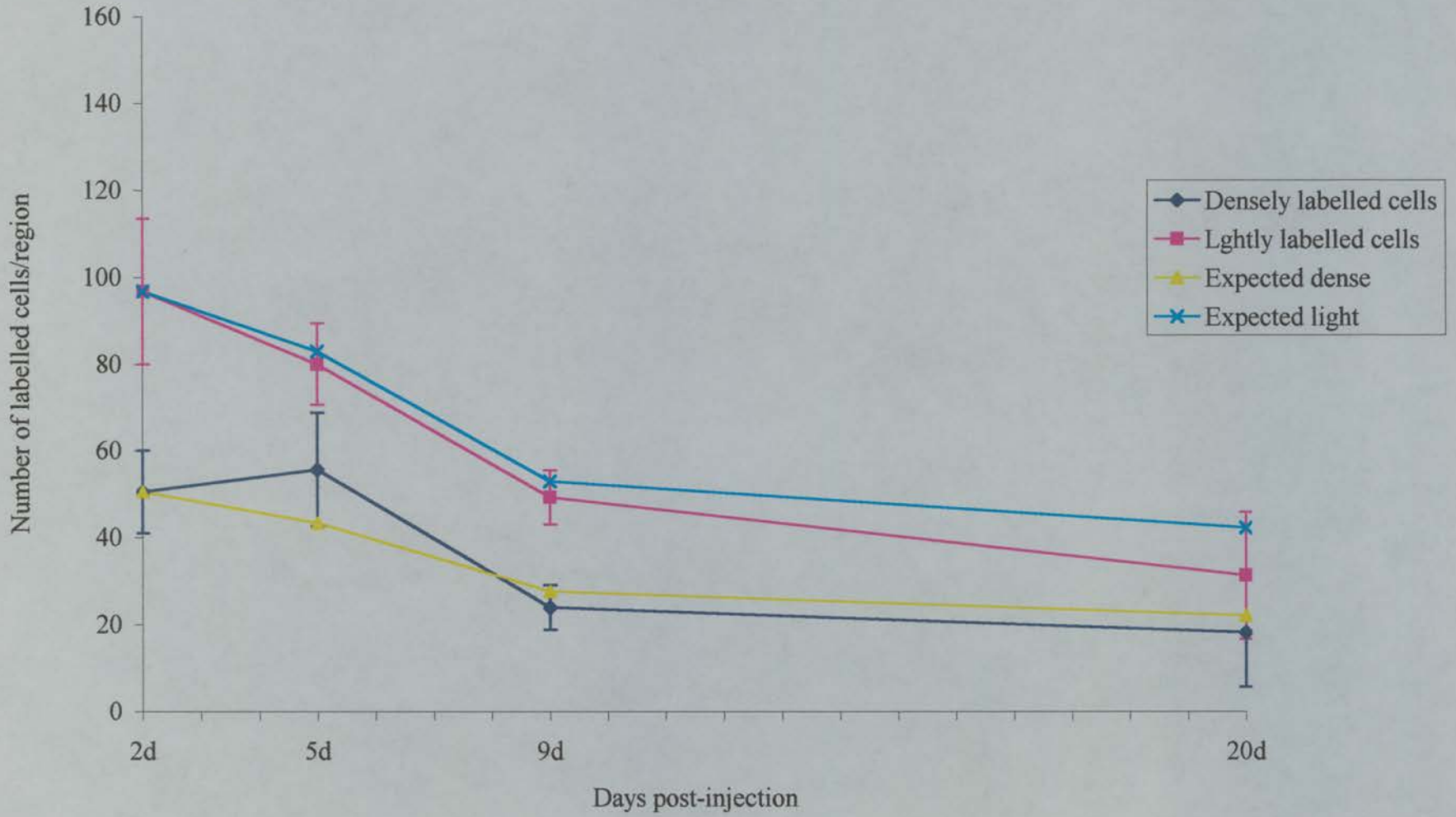


Figure 4. Graph of the number of BrdU labelled cells within a defined region of the cortical wall at E19 +2d, +5d, +9d an +20d, versus expected.

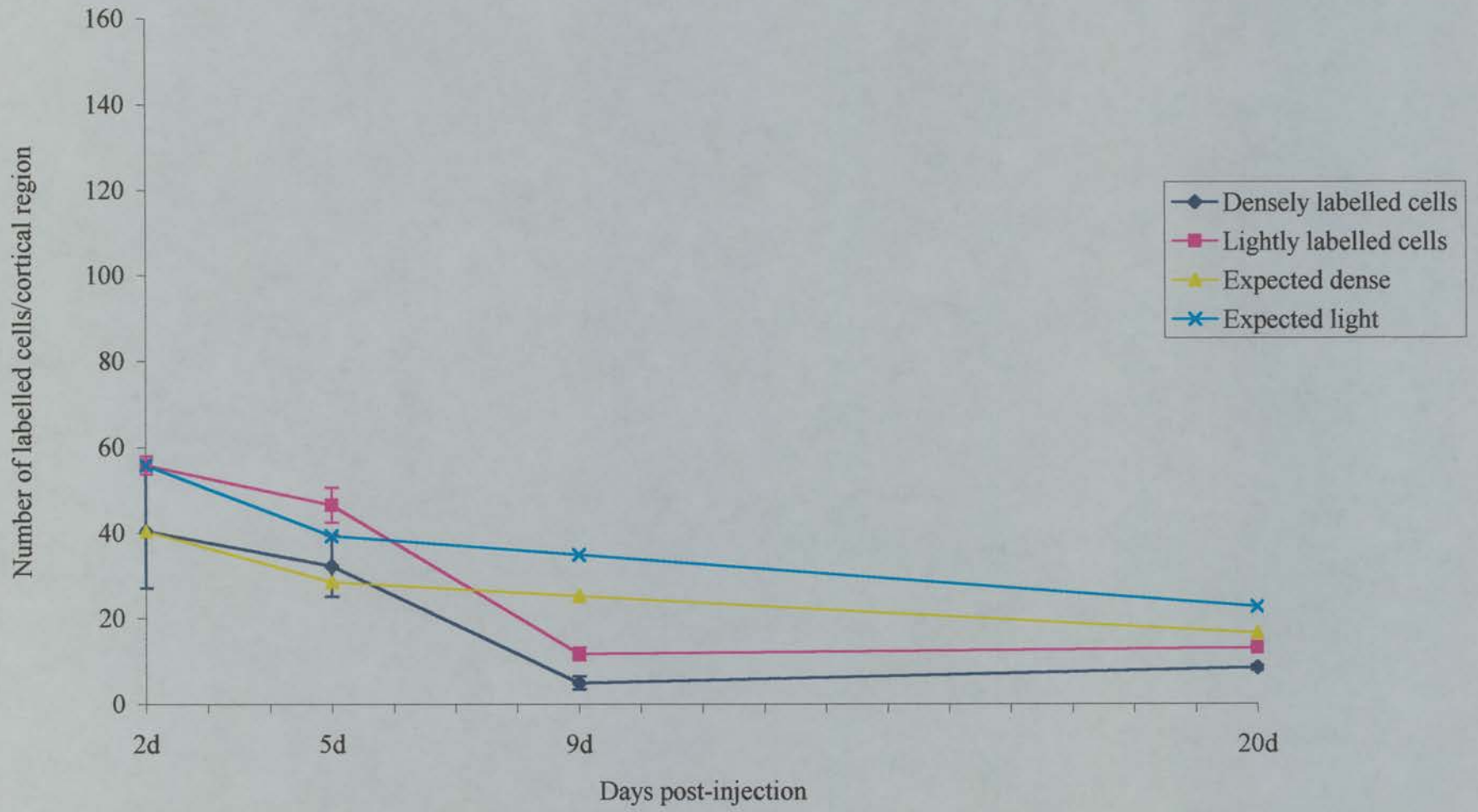


Figure 5. Graph of the number of BrdU labelled cells within a defined region of the cortical wall at P0 +2d, +5d, +9d and +20d, versus expected.

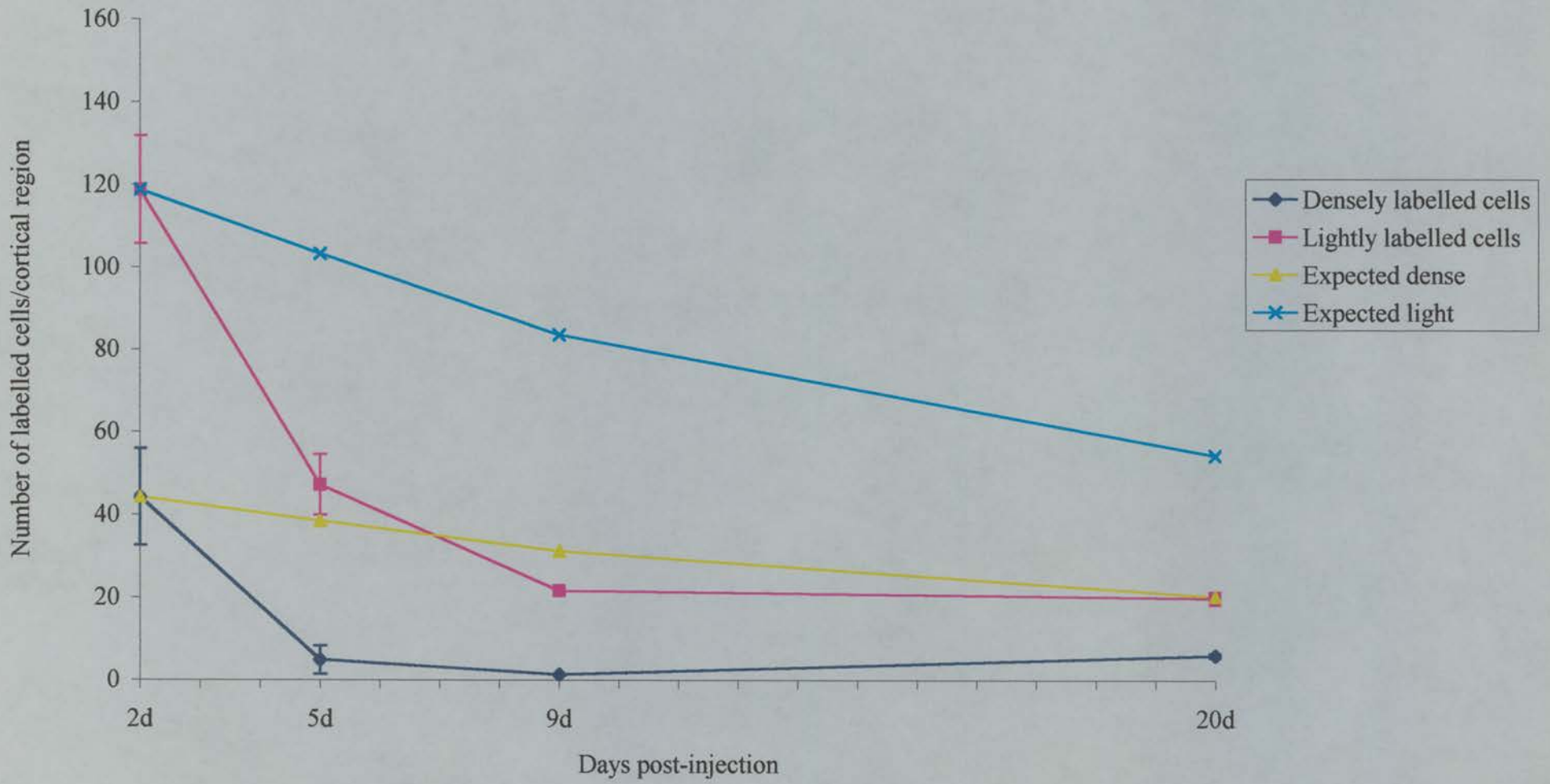


Figure 6. Proportions of densely and lightly labelled cells after BrdU injection at E18, E19 and P0, over time

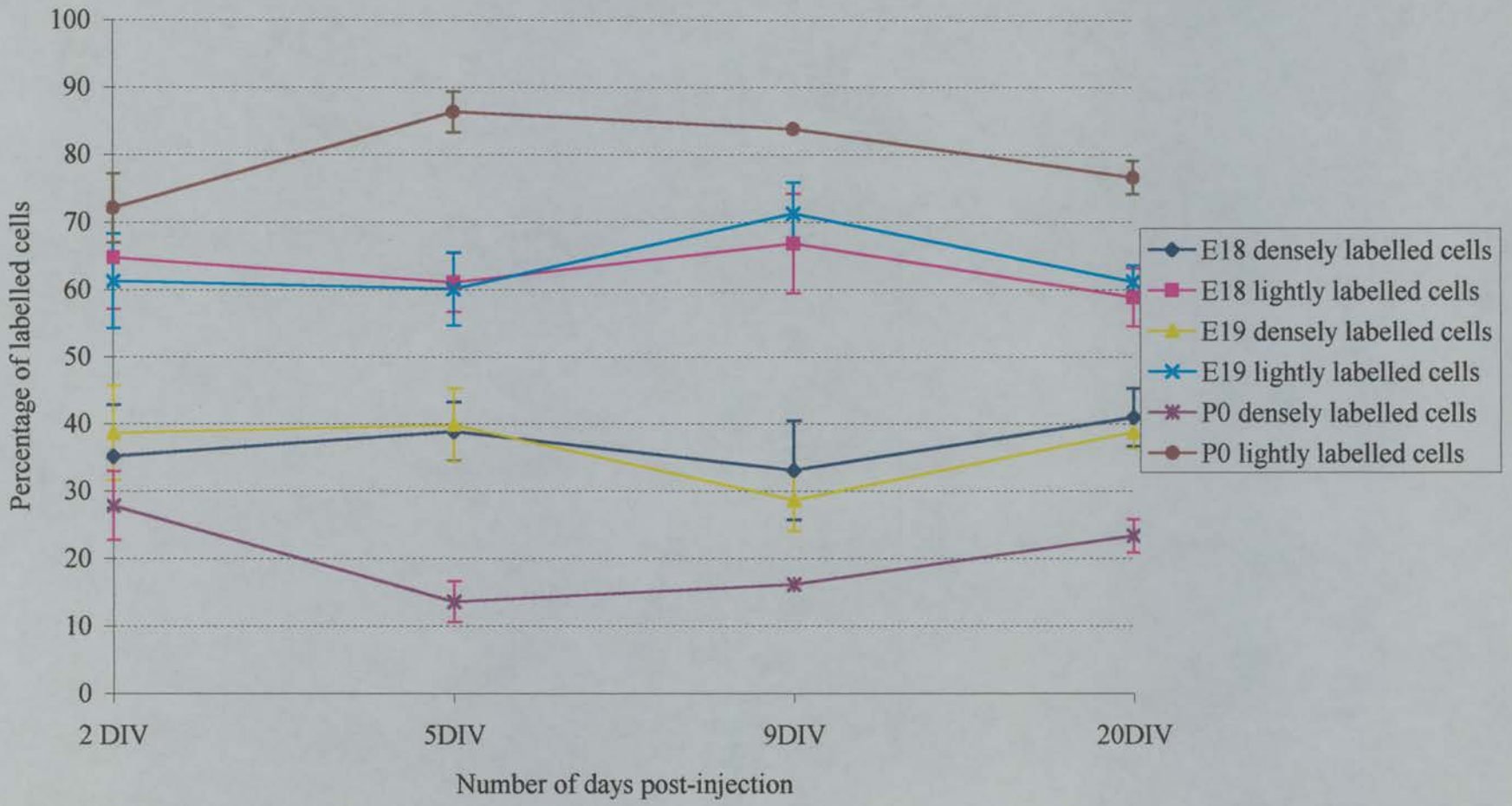


TABLE 2

	Age at injection		
	E18	E19	P0
+2d	100%	100%	100%
+5d dense label	128%	113%	28%
+5d light label	96%	118%	46%
+9d dense label	86%	20%	5%
+9d light label	93%	34%	25%
+20 d dense label	82%	51%	30%
+20d light label	74%	57%	37%

Table 2 illustrates the actual number of BrdU labelled cells at the ages investigated as a proportion of the number predicted from the two +2d data divided by the expansion factor.

Distribution of acutely labelled cells

I used BrdU to label cells dividing at E16, E17, E18, E19 and P0. One hour after BrdU injection, the brains were fixed and reacted to reveal the location of BrdU labelled cells. I compared the location and distribution of these acutely labelled cells. At E16 and E17, dividing cells were found almost exclusively in the proliferative zone. At E18 the majority of cells were seen in the proliferative zone, but a few cells were also seen in the intermediate zone/white matter and occasionally in the subplate and cortical plate. At E19 and P0 the majority of dividing cells were found in the proliferative zone and intermediate zone/white matter, but cells were also seen in the subplate, cortical plate and marginal zone. This is illustrated for the E16, E18 and P0 populations in Figure 7 which shows representative camera Lucida drawings of BrdU labelled cells in the cortical wall one hour after injection. The pattern of distribution within the proliferative zone also differed. At E16 and E17 the cells formed a relatively tight band at a short distance from the ventricular edge (illustrated for the E16 population in Fig. 7a) whereas at E18, E19 and P0, labelled cells were seen throughout the width of the proliferative zone (illustrated for the E18 and P0 populations in Fig. 7b and c).

Distribution of labelled cells at two, five, nine and twenty days post-injection: E18 labelled cells

The distribution of cells dividing on E18, E19 and P0 was investigated. Although the distributions of densely and lightly labelled cells were analysed separately, there was no obvious difference in their behaviour. Therefore, for simplicity, the two populations were combined in this analysis. Figure 8 illustrates the proportional distribution of BrdU labelled cells within the cortical wall at the time of injection and at +2d, +5d, +9d and +20d post-injection. Figure 9 illustrates the number of BrdU labelled cell profiles in each of the cortical zones at the same time points. The E18 labelled population (Fig.8a) divided mainly in the proliferative and intermediate zone/white matter, though a very small proportion of labelled cells were also seen in the subplate and cortical plate (light purple bars) at E18 + 1h. By + 2d post-injection there was a small significant increase in the proportion of labelled

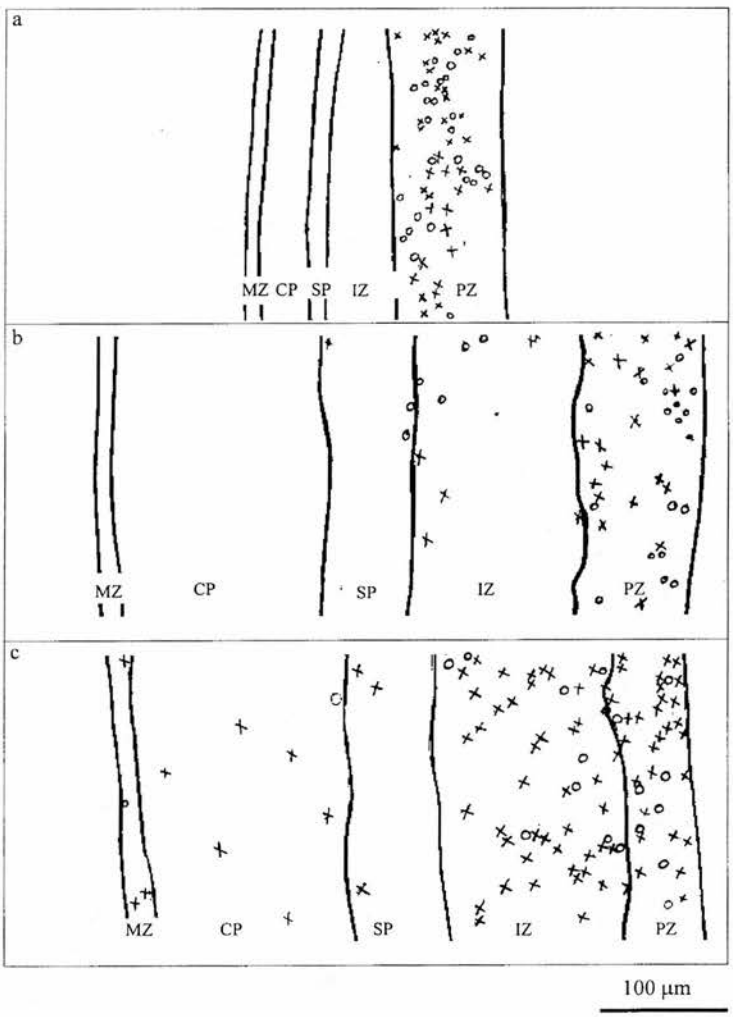
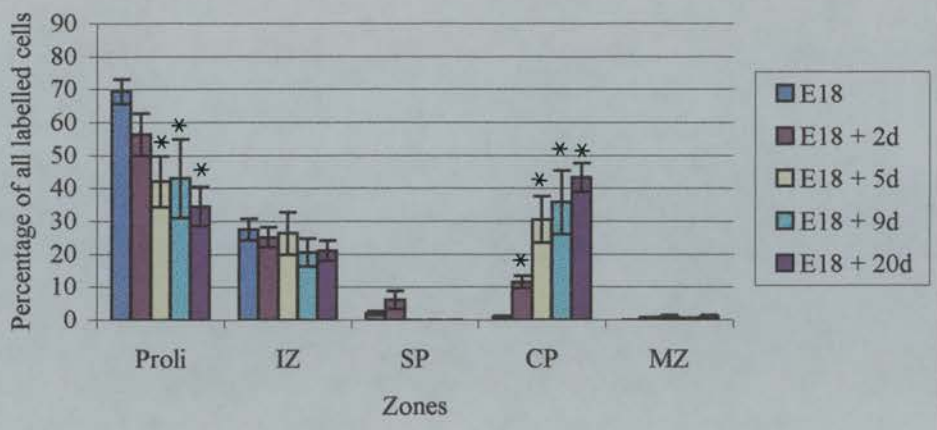


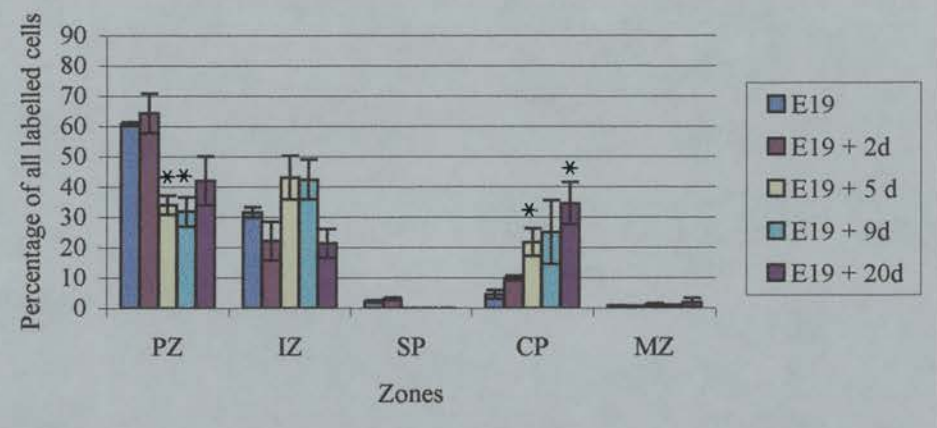
Figure 7. Camera lucida drawings of the cortical wall at (a) E16 (b) E18 and (c) P0 showing the location of BrdU labelled cells one hour after injection. Crosses represent densely labelled cells and circles represent lighly labelled cells. PZ: proliferative zone, IZ: intermediate zone, SP: subplate, CP: cortical plate, MZ: marginal zone

Figure 8 illustrates the proportional distribution of all cells labelled with BrdU (a) on E18 (b) E19 and (c) P0 one hour after BrdU injection (purple bars), +2d (red bars, +5d (yellow bars), + 9d (light blue bars) and +20 d (dark purple bars). The asterisks indicate a significant change in the proportion of cells within a particular zone, from that at the time of BrdU injection. Prolif: proliferative zone IZ: intermediate zone SP: subplate CP: cortical plate MZ: marginal zone.

a


Change in the distribution of all cells labelled with BrdU on E18
Percentage of all labelled cells
90
80
70
60
50
40
30
20
10
0
Proli
IZ
SP
CP
MZ
Zones
E18
E18 + 2d
E18 + 5d
E18 + 9d
E18 + 20d

b


Change in the distribution of all cells labelled with BrdU on E19
Percentage of all labelled cells
90
80
70
60
50
40
30
20
10
0
PZ
IZ
SP
CP
MZ
Zones
E19
E19 + 2d
E19 + 5 d
E19 + 9d
E19 + 20d

c

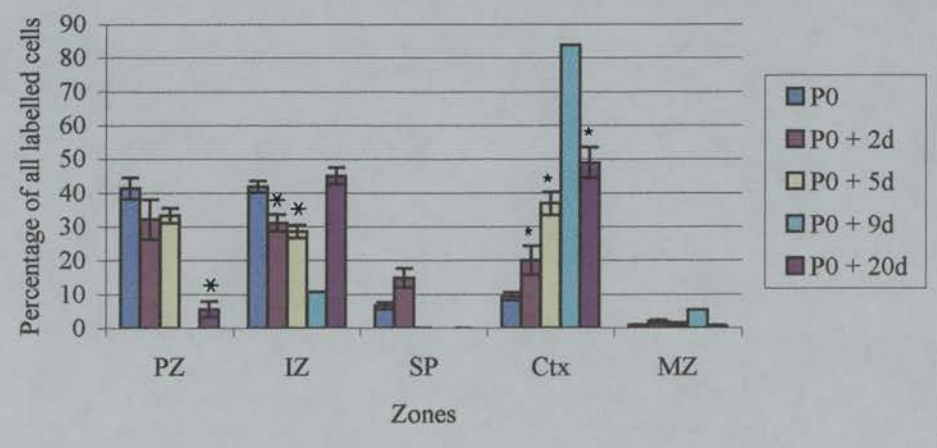
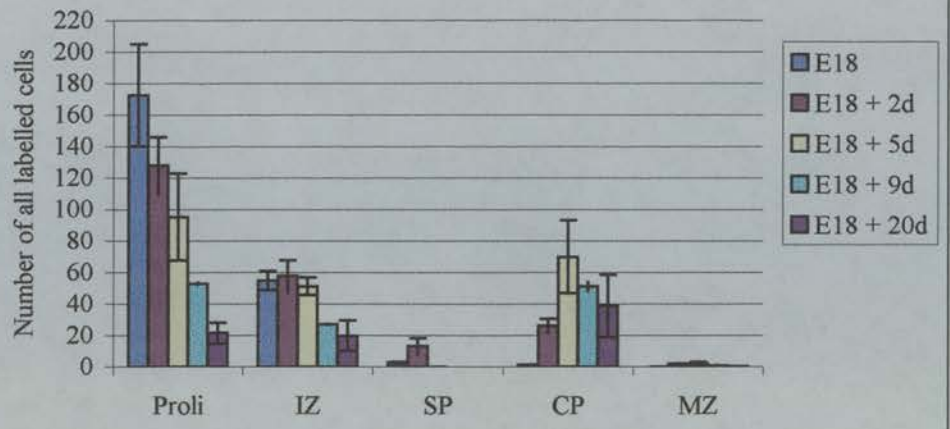

Change in the distribution of all cells labelled with BrdU on P0
Percentage of all labelled cells
90
80
70
60
50
40
30
20
10
0
PZ
IZ
SP
Ctx
MZ
Zones
P0
P0 + 2d
P0 + 5d
P0 + 9d
P0 + 20d

Figure 9 illustrates the numerical distribution of BrdU labelled cells at one hour after BrdU injection (purple bars), +2d (red bars, +5d (yellow bars), + 9d (light blue bars) and +20 d (dark purple bars) after labelling with BrdU (a) on E18 (b) E19 and (c) P0),. Prolif: proliferative zone IZ: intermediate zone SP: subplate CP: cortical plate MZ: marginal zone

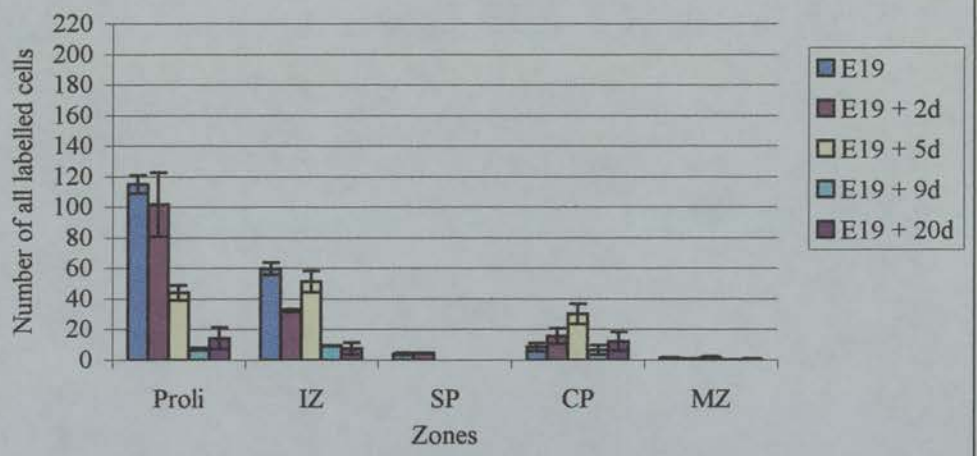
a

Change in the distribution of all cells labelled with BrdU on E18



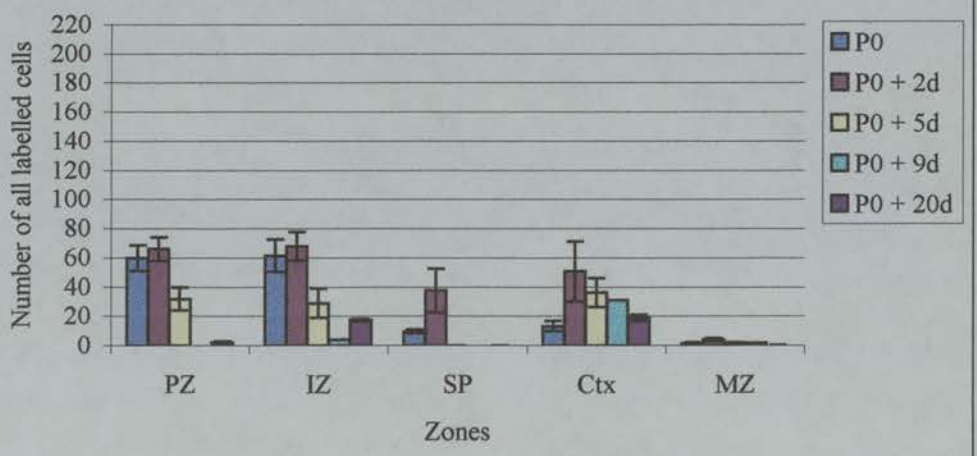
b

Change in the distribution of all cells labelled with BrdU on E19



c

Change in the distribution of all cells labelled with BrdU on P0



cells in the cortical plate (red bars; Student t test $p < 0.05$) compared to that at the start. The proportion of cells in the cortical plate (the subplate disappears around P4/5) increased over time (yellow bars +5d, blue bars +9d) until +20d post-injection (dark purple bars) when the cortical plate population accounted for more than 40% of the total number of labelled cells. The proportion of cells in the cortical plate was significantly different from that at the start at all ages investigated ($p < 0.05$). Most of the cortical plate population was found in a tight band just beneath the marginal zone (Fig. 16). The increase in the proportion of cells in the cortical plate was accompanied by a decrease in the proportion of cells in the proliferative zone. The proportion of cells in the proliferative zone at +2d post-injection was not significantly different from that at the start. However, the proportion in the proliferative zone at +5d, +9d and +20 post-injection was significantly less than that at the start ($p < 0.05$). In terms of the numbers of BrdU labelled cell profiles, these proportional changes were real, up to five days post-injection (Fig.8a). The decrease in numbers in the cortical plate/cortex between five days and twenty days was almost exactly that predicted by the expansion factor (Fig. 9a). The intermediate zone/white matter population changed little in terms of proportional distribution during the period of the study (Fig.8a) but there was a real decrease in cell profile number between +5d and + 9d post-injection (Fig. 9a). The proliferative zone and cortical plate changes were accompanied by corresponding changes in the size of the respective zones (Fig. 2). In Figure 8, the asterisks indicate a significant difference between the proportion of cells in a particular zone at the start of the investigation and the proportion at the time point indicated.

E19 labelled cells

Fig.8b shows the proportional distribution of cells labelled with BrdU on E19. The E19 population divided in the proliferative and intermediate zone/white matter, and to a small extent the subplate and cortical plate (light purple bars, Fig. 8b). At + 2d post-injection there was a small increase in the proportion of cells in the cortical plate/cortex (red bars). The proportion of cells in the cortical plate/cortex increased over time (yellow bars +5d, blue bars +9d) until +20d post-injection (dark purple

bars). The proportion of cells in the cortical plate was significantly different from that at the start at +2d ($p < 0.01$) and +20 d. These proportional increases were accompanied by decreases in the proportion of cells in the proliferative zone. The proportion of cells in the proliferative zone at +2d post-injection was similar to that at the start. However, there was a significant difference in the proportion of cells in the proliferative zone between 1h post-injection and + 5d, + 9d and + 20d ($p < 0.05$). There was no significant change in the proportion of cells in the intermediate zone/white matter compared to that at the start, at any of the ages investigated. In terms of cell profile numbers, there was a small increase in cell numbers in the cortical plate/cortex between the start and +5d post-injection. The numbers of cells in the proliferative and intermediate zone/white matters fell dramatically after +5 d post-injection. In summary there was an increase in the proportion and number of cells in the cortical plate in the first five days. This was accompanied by a reduction in the number and proportion of cells in the proliferative zone. After +5d the number of cells in all zones fell dramatically so that there were very few cells in any of the zones at + 9d and +20 d post-injection.

P0 labelled cells

The P0 population (Fig.8c) divided in the proliferative and intermediate zone/white matters, and to a small extent, the subplate and the cortical plate (light purple bars). Between P0 and +20 d post-injection there was an increase in the proportion of cells in the subplate and cortical plate. The proportion of cells in the cortical plate/cortex was significantly different from that at the start at all ages studied ($p < 0.05$), except at +9d when the number of samples was too small for a comparison. The proportion of cells in the proliferative zone was similar to that at the start at +2d and +5d but fell dramatically at +9d and +20d. The proportion of cells in the proliferative zone was significantly different from that at the start at +20d ($p < 0.05$). In the intermediate zone/white matter, there was a small significant decrease in the proportion of cells at +2d and +5d, however, there was no significant difference in this population at +20d. In terms of cell numbers, there were large decreases in the numbers of cells in the

proliferative and intermediate zone/white matters from +5d post-injection. The decrease in numbers in the cortical plate/cortex was small (Fig.9c).

In summary, for the E18 labelled population, there was a general increase with time in the proportion of labelled cells in the cortical plate/cortex that was accompanied by a decrease in the proportion of cells in the proliferative zone. For the E19 labelled population, there was a similar, but slightly smaller increase in the proportion of cells in the cortical plate/cortex that was also accompanied by a decrease in the proportion of cells in the proliferative zone. For the P0 population, the increase in the cortical plate/cortex was slightly larger than at E18, but the decrease in the proliferative zone after + 5d post-injection was more dramatic.

Identification of BrdU labelled cells

I used antibodies to GFAP, CNPase and B-tubulin III as astrocyte, oligodendrocyte and neuronal markers respectively, to identify BrdU labelled cells. GFAP expression was first seen at P3 and was found within the proliferative and intermediate zone/white matters, and directly below the pial membrane at all postnatal ages (Fig. 10). Figure 10 shows a narrow band of GFAP positive fibres along the ventricular edge of the cortical wall, in what remains of the proliferative zone. This strip of fibres resembles the glia tube described by Peretto *et al.* (1997) through which tangentially migrating subependymal cells of the rostral migratory stream of the adult rat and mouse are though to pass. CNPase expression was also first seen at P3. It was restricted to the intermediate zone/white matter at all ages (Fig. 11). B–tubulin III expression was weak at all ages investigated. It was most obvious in the upper cortex at P7 and later. In the younger brains there was a general faint staining in all cortical zones.

Double labelled cells were identified using 3D confocal imaging in +20d brains. The 30-40 % of E18 labelled cells that lay beneath the marginal zone were associated with B–tubulin III expression. Approximately 10% of the remaining E18 labelled cells and 10% of all E19 BrdU labelled cells were also GFAP +ve at 20 days

post-injection. These cells were located in the proliferative zone, intermediate zone/white matter and lower, and occasionally, upper cortical plate/cortex (Fig.10). The population of E18 labelled cells that lay in a band beneath the marginal zone were never GFAP +ve (Fig.12). Approximatley 15% of P0 labelled cells were also GFAP +ve. A high magnification view of a double labelled cell in the lower cortex is shown in Figure 13. These too were found in the proliferative zone, intermediate zone/white matter, lower cortical plate and upper cortical plate.

It was more difficult to identify cells double labelled with CNPase and B-tubulin III. Although CNPase expression coincided with the location of BrdU labelled cells in the intermediate zone/white matter, the CNPase expression was too homogenous to say categorically that individual BrdU +ve cells were double labelled (Fig. 14). The same problem was associated with B-tubulin labelling, which in any case, was not a particularly efficient marker of neurons in wax processed sections of mouse brain.

Figure 10 shows a section of the cortical wall above the lateral ventricle at P9, labelled with antibodies to GFAP and BrdU, after BrdU injection at E19. The ventricle is to the left and lower cortex to the right in this pictue. Rhodamine was used to visualise BrdU labelled cells and fluorescein to label GFAP positive cells. Most GFAP positive cells are seen within the white matter and lower cortical plate and there is a strong band of GFAP positive fibres along the ventricular edge. Scale bar 500 μm.

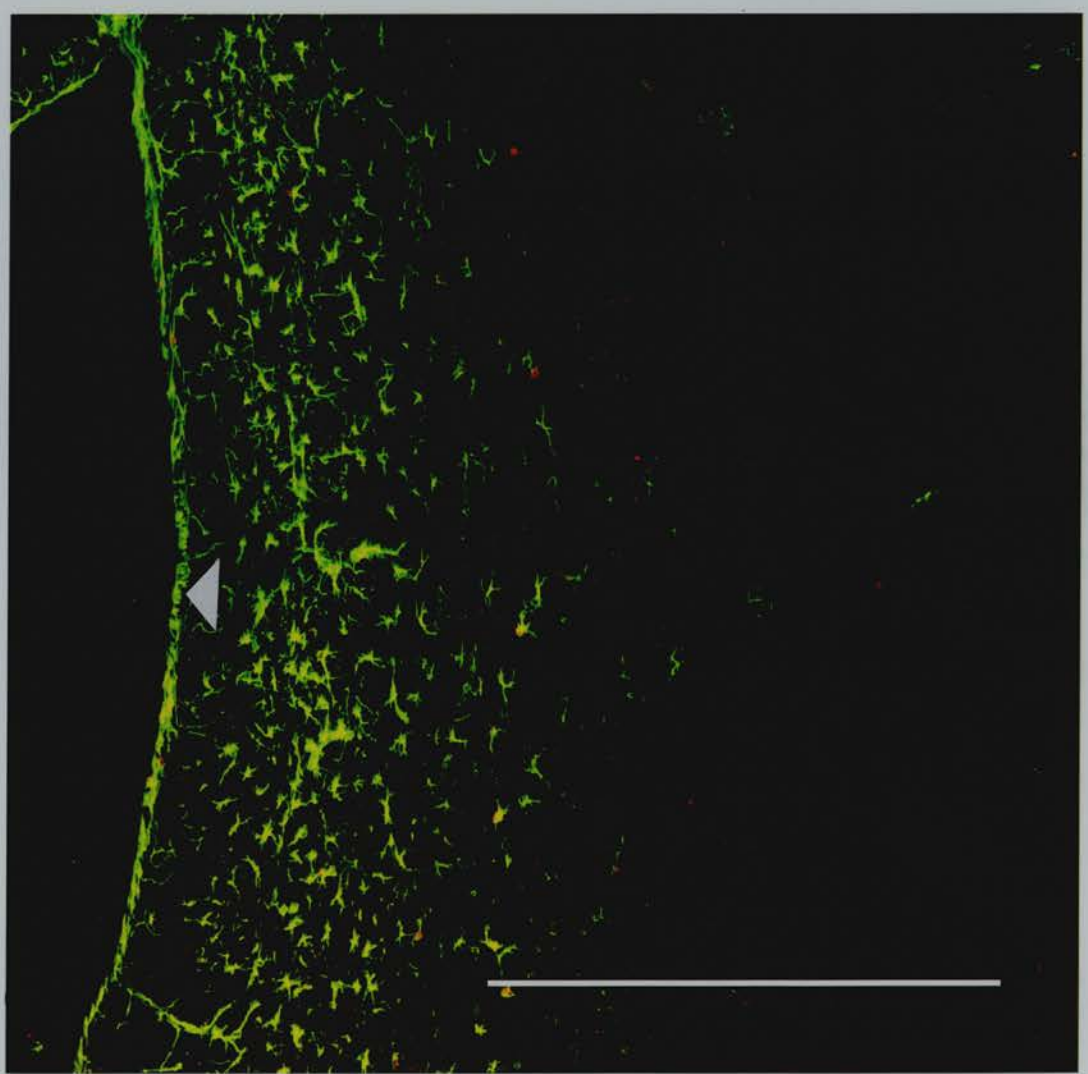


Figure 11 shows a section of the cortical wall at P20 labelled with an antibody to CNPase. Strong CNPase expression was seen in the white matter (to the left in this figure). Scale bar 500 μm.

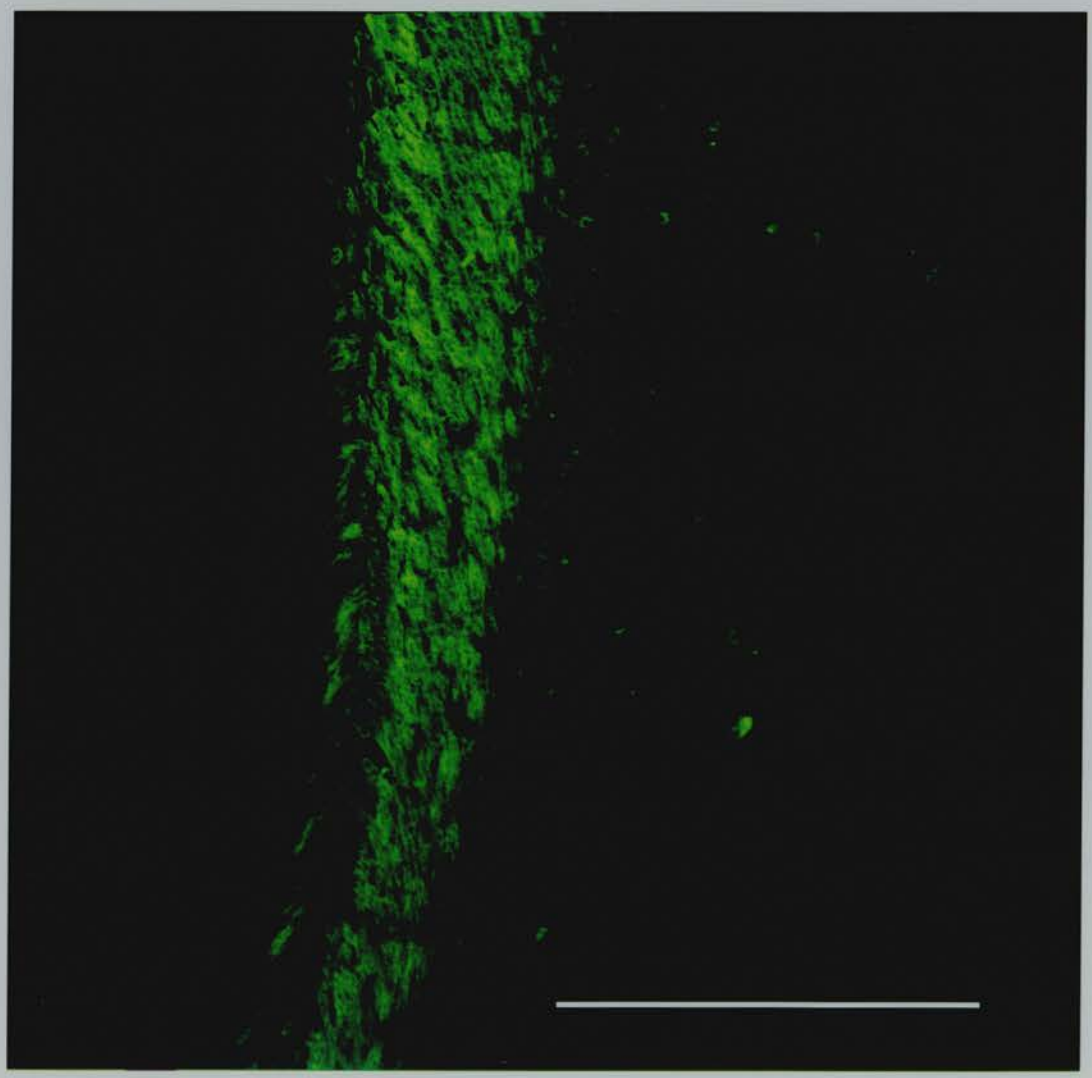


Figure 12 shows a section of the pial edge of the cortical wall at P18 labelled with antibodies to GFAP and BrdU, after BrdU injection at E18. The BrdU llabelled cells (red) are not double labelled with GFAP. A few GFAP positive fibres are seen along the pial edge (green). Scale bar 50 μm.

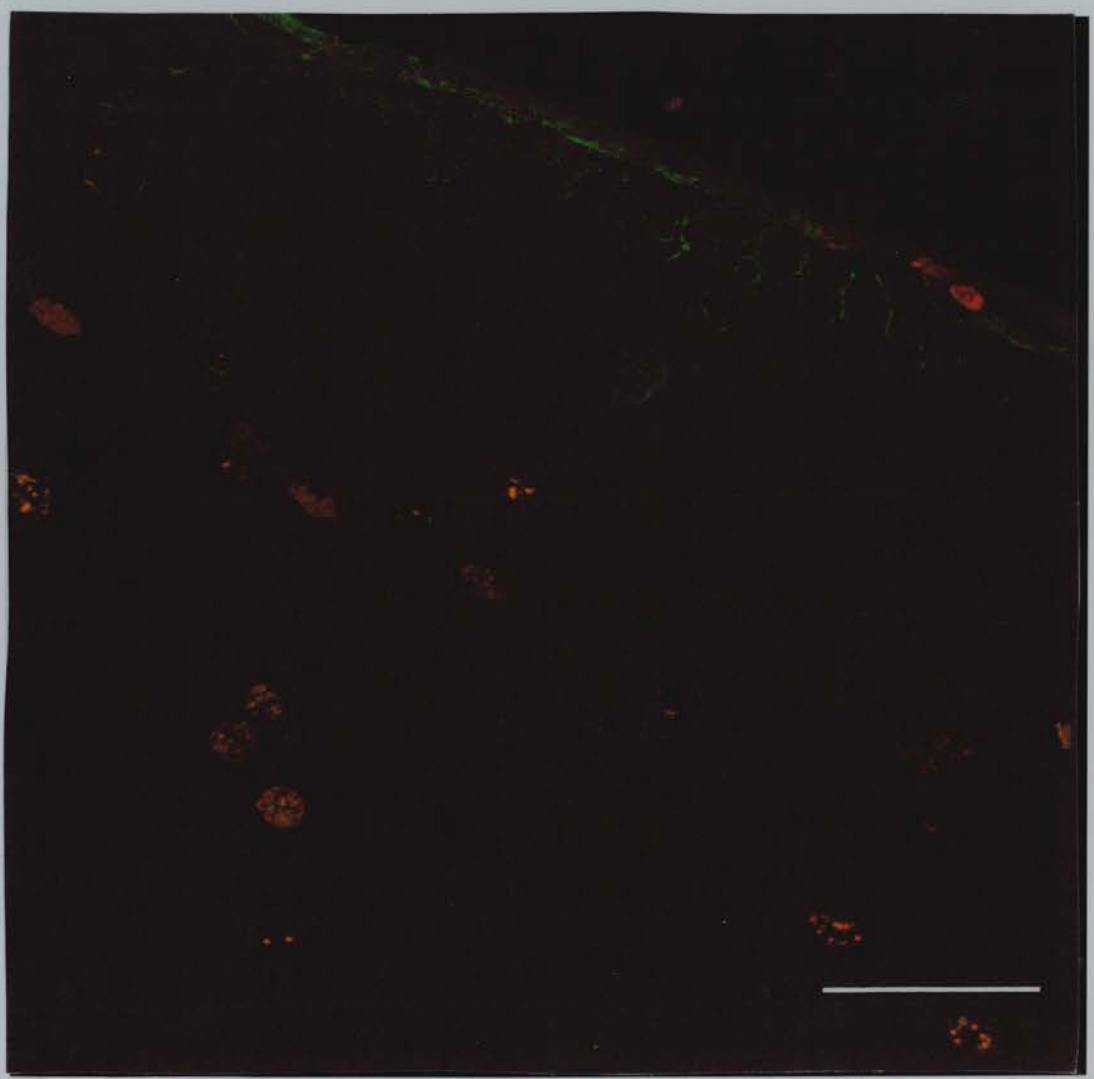


Figure 13 shows a high magnification view of a cell lin the lower cortex labelled with an antibody to BrdU (red) and GFAP (yellow) after BrdU injection on P0. A 3-D reconstruction of this cell was made to confirm its double labelled nature. Scale bar: 10 μm

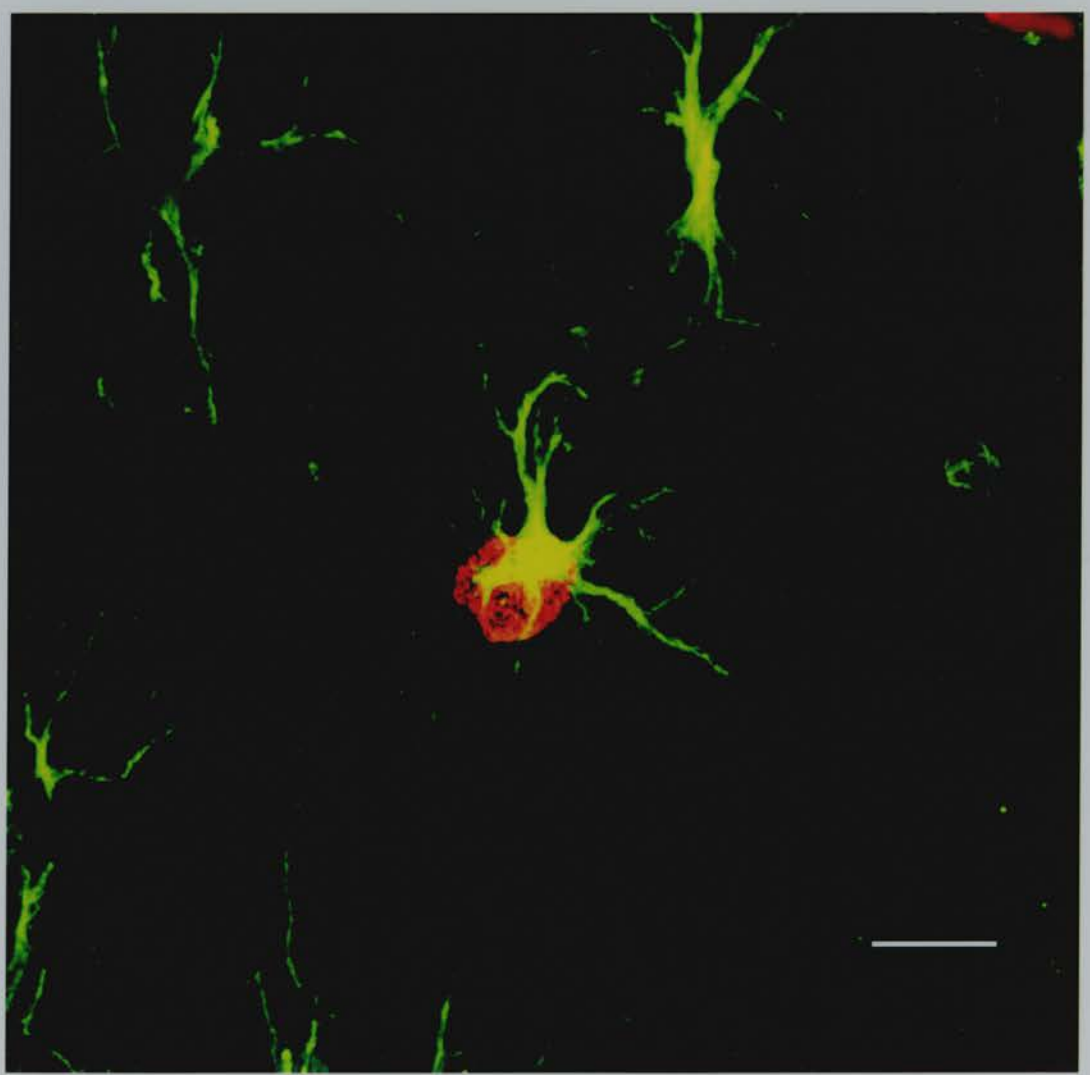
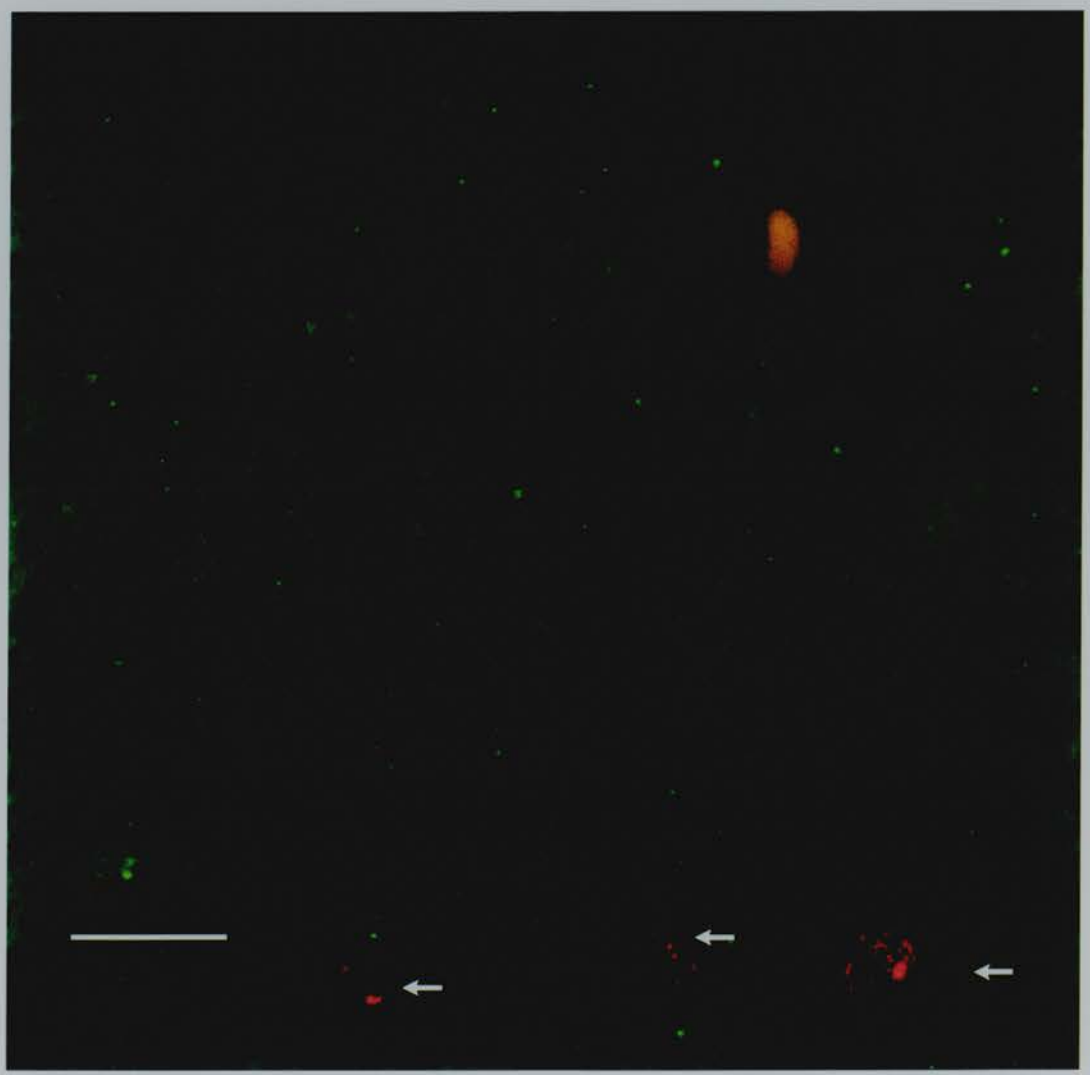


Figure 14 shows a high magnification view of BrdU positive cells (white arrows) among CNPase positive white matter tissue. The homogenous nature of the CNPase labelling makes it impossible to tell if the BrdU labelled cells are double labelled. Scale bar 10 μm.



DISCUSSION

The purpose of this study was to characterise the proliferative behavior, radial migration and antigenic properties of cells born during the transition between neurogenesis and gliogenesis.

It is traditionally held that cells of the ventricular zone gave rise to neurons and those of the subventricular zone gave rise to glial cells after neurogenesis is complete. The neuronal population is well characterised, as described in Chapters 1 and 2 of this thesis. Less is known about the development of glial cells arising during embryonic life (Levinson and Goldman, 1993). This is due in part to the fact that these glial cells are notoriously difficult to study. There are a number of reasons for this. The first is that a comprehensive range of stage-specific and cell type-specific markers is not available for early precursors (Goyne *et al.,* 1994). The second reason is that glial precursors divide many times throughout life (Sturrock, 1979) therefore markers of proliferating cells (BrdU and [^{3}H]-thymidine) get diluted out (Levison *et al.,* 1993). I hoped that by examining cells at short time intervals after BrdU injection I could overcome this problem. Thirdly, the embryonic glia constitute a small population compared to neurons (Takahashi *et al.*, 1995) and are therefore difficult to discern in the embryonic brain. To attempt to overcome this difficulty, I decided to study the glial cells arising during the transition between neurogenesis and gliogenesis when the neuronal precursor population is diminishing. In addition to these well-documented obstacles, I encountered problems associated with double labelling these cells. This was due in part to the fact that many of the glial cell markers belong to the immunoglobulin G (IgG) family, like the BrdU antibody used in this study. This creates a problem of cross-reaction of secondary antibodies with the two primary antibodies of the same immunoglobulin family. Additionally, many of the glial cell markers do not work on wax sections, on which this study was carried out. Finally both CNPase and B-tubulin III are expressed so homogeneously that it was impossible to discern individual cells. Ways of overcoming these problems are discussed at the end of this discussion.

Stratification of the neocortical wall

In order to examine cell distribution, I described the locations of cells according to the cortical zones in which they were found. I began therefore by looking at how the stratification of the cortical wall changed over time. Though this has been done previously it was important to show how the tissue processing method that I used affected these changes. I showed that the thickening of the cortical plate/cortex was accompanied by a reduction in the thickness of the proliferative zone. These changes are due largely to neurons of the proliferative zone moving out to populate the rest of the cortical wall. The intermediate zone/white matter (white matter) expanded initially and then achieved a stable thickness. Although the subplate has been shown to be a transitory structure in a number of species, there was much debate as to its fate in the rodent forebrain (reviewed in Price *et al.*, 1997). However, a recent study by Price *et al.* (1997) shows that murine subplate cells disappear in early postnatal life (Price *et al.*, 1997). In this study I found the subplate could not be discerned after approximately P5. The cortex reached its maximum thickness at P8. However it is unlikely that this is actually the case since, on dissection, the P18 and P19 brains are larger than the P8 brains. Therefore it seems likely that the neuropil, which contributes most to expansion of the cortex once neurogenesis is complete (Angevine, 1965), preferentially shrinks due to the processing used for paraffin wax embedding.

The distribution of dividing cells

Whereas neuronal precursors undergo interkinetic movement in the proliferative zone when they divide, glial precursors arising around E14, do not (Takahashi *et al.*, 1995). I asked if cells dividing on E18, E19 and P0 would behave differently in terms of their proliferative behaviour, from cells dividing on E16 and E17 that are known to give rise mainly to neurons (Angevine and Sidman, 1961; Caviness and Sidman, 1973; Caviness, 1982). It was found that cells labelled on E16 and E17 formed a band at a distance from the ventricular edge that presumably represents the regions of the ventricular zone were cells are in S-phase. Similar results for E14 labeled cells have been reported previously (Takahashi *et al.*, 1993). A few cells lay

above and below this prominent band. These probably represent PVE and/or SPP cells of the subventricular zone and PVE cells leaving S-phase and entering G2 of the cell cycle respectively (Takahashi *et al.*, 1992). By E18, the ventricular zone largely disappears and the proliferative zone consists mainly of the subventricular zone (Takahashi *et al.*, 1995a). This coincides with the decrease in the PVE population (Caviness *et al.*, 1995). When I labelled cells with BrdU on E18, E19 and P0, they did not form a tight S-phase band, suggesting that most or all of these cells did not undergo interkinetic movement. Furthermore, there was a difference in the locations of dividing cells. Cells acutely labelled with BrdU on E16 and E17 were only found in the proliferative zone, whereas cells acutely labelled between E18 to P0 were found in the intermediate zone/white matter, subplate and cortical plate and occasionally, the marginal zone. Since neuronal precursors do not normally divide in these regions it is most likely that these cells are glial precursors or glial cells that were undergoing further rounds of cell division after they had left the proliferative zone. Previous studies have also identified the intermediate as a proliferative region for glial cells (Bermann *et al.*, 1997) and it has been known for some time that glial cells are capable of redividing after they have invaded the cortical plate (Altman, 1966).

The proportion of proliferating cells that belong to the SPP

To various extents, the E16, E17 and E18 labelled populations consist of both neuronal and glial precursors, which can be discerned by (i) utilising two different proliferative markers and exploiting the difference in their proliferative behaviour (Takahashi, 1995) or (ii) by characterising the cells at postnatal stages as I did in this study. The SPP accounts for about 10% of the proliferative population at E14 (Takahashi *et al.*, 1993) and about 35% at E16 (Takahashi *et al.*, 1995a). By examining the fates of cells at postnatal stages, I showed that 30-40% of the E18 labelled cells behaved like neurons (presumably derived from the PVE), therefore I speculate that the remaining 60-70% of the proliferating population belongs to cells derived from the SPP. This supposition assumes that there is not a disproportionate loss/proliferation of one or other proliferative population between injection and

postnatal stages. As discussed later, I think not. According to Takahashi *et al.* (1995a) cells of the PVE have largely disappeared by E19. However, a number of radial glial cells (that belong to the PVE) may still be dividing at this time (Misson *et al.*, 1988). Nonetheless, this population is probably small (Tan and Breen 1998) and therefore it is likely that the majority of E19 and P0 labelled cells are derived from the SPP and that at these ages the SPP constitutes almost 100% of the proliferative population.

Cell numbers

I compared the number of cells within a 500 μm wide bin at +2d post injection with that at +5d, +9d and +20d. To correct counts for cell dilution due to cortical growth, I calculated the expansion of the cortex in both the rostrocaudal and mediolateral directions (expansion dorsoventrally was accounted for by taking counts from the entire depth of the cortex). Although the sample size used for the estimation of expansion was small, measurements were made independently by two people and found to be very similar. In addition, the results were similar to those obtained by Del Rio *et al.* (1995) who used a similar method to determine expansion in the rostrocaudal direction. I used the expansion factor to calculate, from the two day counts, the expected numbers of cells at five, nine and twenty days post-injection.

For E18 labelled cells, there was little change either in cell numbers (except to the extent predicted by cortical expansion) or in the proportion of densely and lightly labelled cells, over time. This suggests that this is a stable population that does not disappear between two and twenty days post-injection. Given that the cells of the PVE are dividing for the last time on E18 (Caviness *et al.*, 1995; Polleux *et al.*, 1997), I would expect the PVE proportion of the population to be stable. The stability of the remaining 60-70% of the population could be due to (1) the cells not redividing, referred to as a stable equilibrium, or (2) the rate of cell division being equal to the rate of either cell death or migration of the dividing cell to other regions of the brain, referred to as a dynamic equilibrium. I prefer the former explanation because it is the one most in accordance with the fact that there was no real decrease

in the densely labelled population. If cell death/migration occurred at the same rate as cell division, the total number of labelled cells could potentially remain constant but the numbers and proportion of densely labelled cells would decrease due to BrdU dilution.

Although I did not directly test for changes in cell density between E18 and two days post-injection (because the populations are not directly comparable given the different exposures to BrdU) my results suggest that they do not. If the cells had divided between these two points (assuming a cell cycle length of about 15-20 hours) they would have given rise to E19 and P0 'born' cells. Therefore, I would expect a proportion of the lightly labelled cells to have behaved like E19 or P0 labelled cells. That is, I would expect them to disappear as these populations did at postnatal ages. The fact that they did not suggests that these cells were not labelled by the E18 injection or they were such a small proportion of the total number that their behaviour was concealed.

The number of E19 labelled cells at five days post-injection was similar to that predicted from expansion of the cortex, though there were slightly fewer densely labelled cells and slightly more lightly labelled cells than predicted. This suggests that a small proportion of the densely labelled cells had divided between +2d and +5d post-injection. Nonetheless, for the reasons given above, I suggest that most of these cells were in a state of stable equilibrium. However, by nine days post-injection, the number of both densely and lightly labelled cells had fallen to considerably less than predicted. This result implies that these cells are diluting out, dying or moving elsewhere in the brain, sometime between five days post-injection and nine days post-injection. The small increase in cell numbers at twenty days post injection is most likely the result of cells migrating tangentially into the region. Tangential migration of olfactory bulb interneurons generated postnatally in the anterior subventricular zone of the rat and mouse has been reported previously (Luskin *et al.*, 1994; Luskin *et al.*, 1995). This might be a similar population, not previously reported in the literature.

The P0 labelled cells behaved differently from either the E18 or E19 labelled populations. There was a large decrease in numbers of both densely and lightly labelled cells between +2d and +5d and between +5d and +9d. There is some evidence to suggest that this might be through cell division. The evidence comes from the fact the lightly labelled cells (many of which depict the product of one or more subsequent round of cell division after labelling) represent a larger proportion of total cell numbers than they do for the E18 and E19 populations (see Fig. 6). It is interesting to note that by far the largest decrease in number occurred between two and five days post-injection. It has been shown previously that cells proliferating in the adult mouse subependymal layer dilute out BrdU within eight days of labelling (Morshead and van der Kooy, 1992). If proliferation accounts for the disappearance of the P0 labelled cells, then these cells must have a relatively short cell cycle since they begin to dilute out the BrdU at least as early as five day post-injection. However, I cannot rule out the possibility that cell death or migration to other brain region account for the decrease. For the P0 population, the reduction occurs in the PZ and IZ and to a lesser extent in the CP. As for the E19 population, there was a small increase in the number of cells between nine and twenty days post-injection suggesting that cells are migrating tangentially along these zones, from other regions of the brain.

Thus, the E18 labelled cells are an apparently non-dividing population that retain a strict radial array. The E19 labelled cells begin to disappear between +5d and +9d post-injection, possibly due to dilution of BrdU through continuous division, migration to other regions of the brain or cell death. The P0 labelled cells begin to disappear between +2d and +5d post injection possibly by one or all of the same mechanisms as proposed for the E19 population. It would be interesting to use a double or triple labelling technique with BrdU, [^{3}H] thymidine and acute markers of proliferating cells to show if cells labelled at each of these ages really are rediviging during the period of the study. For example, cells could be labelled with BrdU at E18, E19 or PO, then with [^{3}H] thymidine at +2d post-infection and then with an

acute marker of dividing cells such the antibody to Cdc2 kinase-phosphorylated vimentin used by Kamel *et al.* (1998) to specifically label dividing cells of the astroglia lineage. If combined with a marker of cell death, such as TUNEL staining, this would provide a clearer picture of the fate of these cells that disappeared during the period of study.

The distribution of BrdU labelled cells

Cell distribution was illustrated in terms of cell profile numbers and proportions. Only the changes in the proportional distribution of cells was analysed statistically because the loss of cells after BrdU injection at E19 and P0 made it impossible to derive any meaningful results (in terms of cell distribution) from the analyses of cell profile numbers. For the distribution studies, cell profile counts were used and these were not corrected to cell densities using Abercombie's (1946) formula. The reason for this is that there was very little difference in cell size between cortical zones. For the E18 labelled population at +5d (in which there was the greatest range of sizes), the average length of the cells of the upper cortical plate (neurons) was 7.1 μm and the average length of the remaining cells was 5.5 μm. It is clear from Abercrombie's formula that this small difference in cell profile length has very little effect on cell density. Using Abercrombie's formula:

Cell density = profile number x cell length/(cell length + section thickness)

Therefore, for every 100 profiles of average length 7.1 μm, the cell density is 41 cells/unit volume whereas for every 100 profiles of average length 5.5 μm, the cell density is 35 cells/unit volume – a difference of 6 cells per hundred profile counts.

Since, to accurately determine cell density within each cortical zone would have required measuring thousands of BrdU +ve cell profiles, I felt that given the small effect it was likely to make, it was more efficient to demonstrate cell distribution in terms of profile numbers.

The distribution of E18 labelled cells

The results of the distribution study showed that a proportion (30-40%) of the E18 labelled cells behaved like neuronal precursors in that they migrated from the proliferative zone to the upper cortical plate just under the marginal zone. However, the majority of E18 labelled cells were found within the remnants of the proliferating zone and in the white matter, at postnatal ages. These nuclei were slightly smaller and less regular in shape than those found at the upper cortical plate. The number of cells in the intermediate zone/white matter did not increase over time, as one would expect if cells were invading this region from the proliferative zone. Therefore it seems that these intermediate zone/white matter cells were probably 'born' *in situ*. In other words they did not migrate to the white matter from the proliferative zone, at least during the period of study.

The distribution of E19 labelled cells

The distribution of E19 labeled cells did not reveal a straightforward trend. This was due in part to the large decrease in cell numbers that occurred between +5d and +9d post-injection that made it difficult to determine if cells were moving from one region to another. There was however a decrease in both the number and proportion of cells in the proliferative zone between two days and five days and a simultaneous increase in both numbers and proportions in the cortical plate. This suggests that cells were moving from the proliferative region into the cortical plate. The distribution in the intermediate zone/white matter was more complicated, but as for the E18 population there was no obvious invasion of cells from elsewhere. Thus, this population probably arises *in situ*.

The distribution of P0 labelled cells

The distribution of the P0 labelled cells was even more difficult to interpret because of the large loss of cells. Although there was evidence for an increase in the proportion of cells in the cortex, this reflected the loss of cells from the lower regions of the cortex, rather than a real increase in cell number within the cortex. There was

no obvious migration of cells from one region to another. Instead, it appeared that cells of a particular zone arose *in situ*.

Characterising the cells

I used markers of both neuronal and glial cells to identify the BrdU labelled cells. I conclusively identified a small proportion of cells as members of the astroglia class because they expressed GFAP. The morphological transformation of radial glia to astrocytic cell classes has been shown following the uptake of fluorescent dyes by embryonic radial glial cells *in vivo* (Voigt, 1989). This transformation is not directly preceded by mitosis (Gressens *et al.*, 1992), though radial glia are thought to be actively mitotic in the embryonic brain (Misson *et al.*, 1988). The cells that were double labelled could be (i) radial glial cells that were undergoing mitosis on the ages when BrdU was injected that subsequently transform into astrocytes or (ii) a newly generated population of astrocytes like the astrocytes of the supragranular cortical layers that are generated soon after neurogenesis from cells dividing in the proliferative zone (Gressens *et al.*, 1992). I favour the second explanation since the evidence used by Misson *et al.*, 1988 to show that radial glia are mitotically active in the embryonic brain (after the birth of radial glia in the early embryo) is disputable. Although they showed that cells labelled with [^{3}H] thymidine were RC2 positive, and had an apical and basal process, they did not demonstrate conclusively that these cells were radial glia. Indeed all cells (neuronal, astrocytic precursors and radial glial cells) in the proliferative zone have such processes. Further, it has been suggested that RC2 might not be a specific marker of radial glia, but instead a marker of neuroepithelial cells that give rise to all cell types (M. Gotz, personal communication).

Although I positively identified this small population of astrocytes, the majority of BrdU labelled cells could not be conclusively identified. However, based on their shape, size, location, proximity to B-tubulin III and birthdate, I am confident that the E18 labelled cells that were found under the marginal zone are neurons. The nuclei of these cells were similar in terms of size, location and appearance to that of

neuronal cells born during the peak period of neurogenesis (own observations). They were also significantly larger than the cells of the lower cortical regions.

Of the remaining BrdU labelled cells (of all ages of injection) those in the white matter were associated with a strong band of CNPase expression, suggesting that they are oligodendrocytes. Since Kamel *et al.*, (1998) showed that cells of the astroglia lineage divide almost exclusively in the proliferative zone during embryonic and early postnatal life, it seems possible that the cells dividing in the intermediate zone/white matter give rise exclusively to oligodendrocytes. The cells in the intermediate zone/white matter account for approximately 30% of 40% of all cells labelled at E18, E19 and P0. At +20d, all the cells in what remained of the proliferative zone were identified as ependymal cells, based on their location and shape. Their nuclei were long and narrow, running parallel to the ventricular edge. The remaining BrdU labelled cells are likely to be multipotent stem cells, that only becomes committed to a particular fate after further divisions, and/or microglia or non GFAP positive astrocytes. I took care to avoid counting cells that resembled endothelial cells, therefore it is unlikely that any of the cells derive from this lineage.

It is clear, that to categorically identify these cells, a more comprehensive labelling strategy is required. This would include the use of additional tissue processing methods, because some of the glial cell markers will only work on, for example, vibratome sections or cryostat. The reason that wax processing was used in this study was because a large number of brains required to be processed, and this was the most efficient method in terms of the time required to carry out the study. It was also a tried and tested method for processing tissues for BrdU labelling in this lab. Another problem associated with identifying the cells was that the primary antibody to BrdU and many of the markers of differentiated neuronal and glial cells are of the same immunoglobulin class. This meant that, in such cases, I had to employ a sequential labelling technique for the double label study. This was not nearly as efficient as simultaneous labelling because the first secondary antibody faded to some extent during the fixation step before the second primary antibody was

applied. This partly explains the inefficiency of the B-tubulin III labelling. This antibody also seems to be an inefficient marker of wax embedded mouse tissue (personal communication, technical support Sigma). A few polyclonal anti-BrdU antibodies are now available, although they have been found to be less efficient markers of BrdU +ve cells than the antibody used in this study (A. Chenn, personal communication).

This study leaves a number of questions unresolved. This is due partly to the loss of BrdU labelled cells (possibly through continuous division) and the inability to identify all the BrdU labelled cells using markers of differentiation. However, it was a necessary first step to guide the design of future experiments. Now that it is clear at what stage cells begin to disappear the nature of that loss can be tested using the double or triple labelling studies described above and markers of cell death. Retroviral studies to follow migration of glial cells in the postnatal brain are well established. Similar studies might be expected to reveal more of the nature of migration of glial cells in the embryonic brain. This study also makes clear that if markers of dividing cells are to be used to investigate these cells the tissues need to be fixed at very short time intervals, perhaps 12, 24 and 36 hours after labelling to follow the movement of cells before these labels become diluted (or the cells disappear by death or migration elsewhere).

In summary, this chapter provides quantitative information on the output of cells in the neocortical proliferative zone during the transition between neurogenesis and gliogenesis. It also reveals the spatial distribution of proliferating cells at the time during which they are dividing and subsequent to their division. Finally, it begins to identify these cells in terms of the phenotypic markers that they express.

CHAPTER 4: A TISSUE CULTURE APPROACH TO LOCALISING LAMINAR FATE CUES

INTRODUCTION

Laminar fate determinants in the developing neocortex

Following the study in Chapter 2 that confirmed the correlation between a neurone's birthdate and its ultimate laminar fate, I decided to investigate the factors that specify laminar fate.

As described in detail in the introduction to this thesis (Chapter 1), neuronal precursors becoming terminally mitotic on a particular day in the ventricular zone, attach to radial glia and migrate along their processes until they reach the marginal zone where the become detached from the radial glia and contribute to the formation of a particular cortical layer. They are displaced at the marginal zone by later born cells that migrate through them. This consistent and predetermined pattern of laminar development can be exploited to elucidate the factors that regulate laminar fate.

McConnell and Kaznowski (1991) showed that environmental factors specify the laminar fate of deep layer cortical neurons. These authors used transplant techniques to show that environmental factors act on precursors in the ventricular zone during the final stages of the cell cycle, between the end of S-phase and mitosis (Fig. 3, Chapter 1). Subsequently, Bohner *et al.* (1997) showed that short range cues within the telencephalon induce precursors to a deep layer fate. Conceivably, different factors could act on precursors of different cortical layers.

Finding the source of laminar fate cues

The purpose of this study, which began before Bohner *et al.* (1997) published their findings, was to localise cortical laminar fate cues. A tissue culture technique was employed to do this. The study was designed to show if cells that were born *in vitro*, that is, cells undergoing the final stages of the cell cycle in a defined environment,

would migrate and form normal cortical laminae. The tissue culture technique developed for this study was based on a protocol that was already established in the lab (Gillies and Price, 1993; Rennie and Price, 1994; Price and Lotto, 1996). It was found that the youngest age at which cortical slices could be successfully cultured using this technique, in which embryonic cortical slices were grown on a collagen filter, is E16. Since the study was to investigate the behaviour of cells born *in vitro*, this meant that the earliest born cells that could investigated were those undergoing their terminal division on E16. These cells give rise to layers IV and III of the mature neocortex (Caviness, 1982; Polleux, 1997).

Layer IV is the principal cortical recipient of afferents from the thalamus (Lund and Mustari, 1977), though layer III cells also receive thalamic afferents (Frost and Caviness, 1980). Thalamic axons reach the cortical subplate at around E15 in the mouse, and begin to project into the cortical plate between E17 and E18 (Table 1, Molnar and Blakemore, 1995). Their main cortical targets are layers III and IV which they reach at around P1 (Lund and Mustari, 1977). Thalamic axons have already been shown to play a role in the organisation of the cytoarchitecture of the cerebral cortex, for example in the formation of the barrel cortex in the rodent (Welker and Van der Loos, 1986; Jensen and Killackey, 1987; Erzurumla and Jhaveri, 1990; Schlaggar and O'Leary, 1991, 1994; Killackey *et al.*, 1994) and the visual cortex in the monkey (Rakic, 1988, Dehay *et al.*, 1991; Rakic *et al.*, 1991). The thalamus has also been shown to play a role in the expansion or maintenance of Otx2 expressing cells in the posterior prospective cortex (Nothias *et al.*, 1997). These studies illustrate a role for the thalamus in shaping and maintaining areal organisation in the cortex. It seems possible therefore that the thalamus might also play a role in specifying laminar fate, at least of the middle and superficial cortical layers, the precursors of which undergo the final stage of their terminal cell cycle after thalamic axons have entered the developing cortex.

Developing a tissue culture technique

I hypothesised that laminar fate cues for the middle cortical layers may come from either earlier born cells of the cortex as suggested by Gillies and Price (1993), or from the thalamus, as implied by its important role in areal organisation. To test this, I asked if neocortical neuronal precursors that undergo the final stages of the cell cycle *in vitro* would proliferate and laminate normally in isolated developing neocortical slices, cultured with and without thalamic tissue. By assaying the behaviour of cells undergoing their final division *in vitro,* I ensured that the only source of laminar fate cues for these cells was from within the defined culture environment. Roberts *et al.* (1992), Gotz and Bolz (1992), Price and Lotto, (1996) and Gillies and Price (1993) have shown that cells that are born *in vivo* will migrate relatively normally in cortical slices cultured in isolation. Roberts *et al.* (1992) and Gotz and Bolz (1992) also showed that radial glial cells are present in cortical slice cultures and retain their normal radial arrangement, therefore cortical slice cultures provide a suitable assay for these experiments.

Since the experiments were designed to address the possibility of the action of some factor in the cultured tissue, it was important to conduct these experiments in defined medium. This would eliminate the possibility of an effect from a factor in serum, which is commonly used to supplement culture medium. However, long term cell survival is compromised in the absence of serum. Magowan and Price (1996) showed that in thalamic explants cultured in defined serum free culture medium, 50% of E17 thalamic cells died within 3 days of culture. These authors demonstrated that raising the concentration of K^+ in the culture medium from 5mM to 9mM increased cell viability by up to 30%. Therefore I cultured neocortical slices in one of two conformations (i) alone or (ii) with thalamic tissue in culture medium supplemented with 5 mM KCl.

In order to study *in vitro* born cells, BrdU was added at the start of the experiment to label dividing cells. In preliminary experiments, BrdU was added for 24 hours to label cells dividing on a specific day. However, Takahashi *et al.* (1996)

have suggested that the sequential number of the cell cycle (and not specifically the birthdate) of cortical precursors correlates with their ultimate laminar fate. Therefore, given that the length of the cell cycle at E16 has been calculated to be 17.5 hours (Takahashi *et al.*, 1995), and since cells in the proliferative zone cycle asynchronously (Takahashi *et al.*, 1993), it was inevitable that a BrdU pulse of 24 hours duration would label cells belonging to at least two sequential cell cycles (Fig. 1). Thus, it was decided to reduce the BrdU pulse to one hour on the assumption that this would reduce the likelihood that cells undergoing successive cell cycles would be labelled. It was hoped therefore that this would lead to the formation of a tightly clustered layer of cells. I found that a one hour pulse was sufficient to label a reasonable sized population of cells.

The initial results of this set of experiments showed that the thalamus had a positive effect on the behaviour of the *in vitro* born cells. That is, it improved their ability to migrate normally. To test if this was likely to be a permissive (as opposed to instructive) effect, I assessed and compared the cortical slices cultured alone with those cultured with thalamus. The distribution of earlier born cells, nature of the radial glial fibres and cell viability were analysed. Finally, to test if the effect of the thalamus was specific, the experiments were repeated, replacing the thalamic tissue with other embryonic tissues.

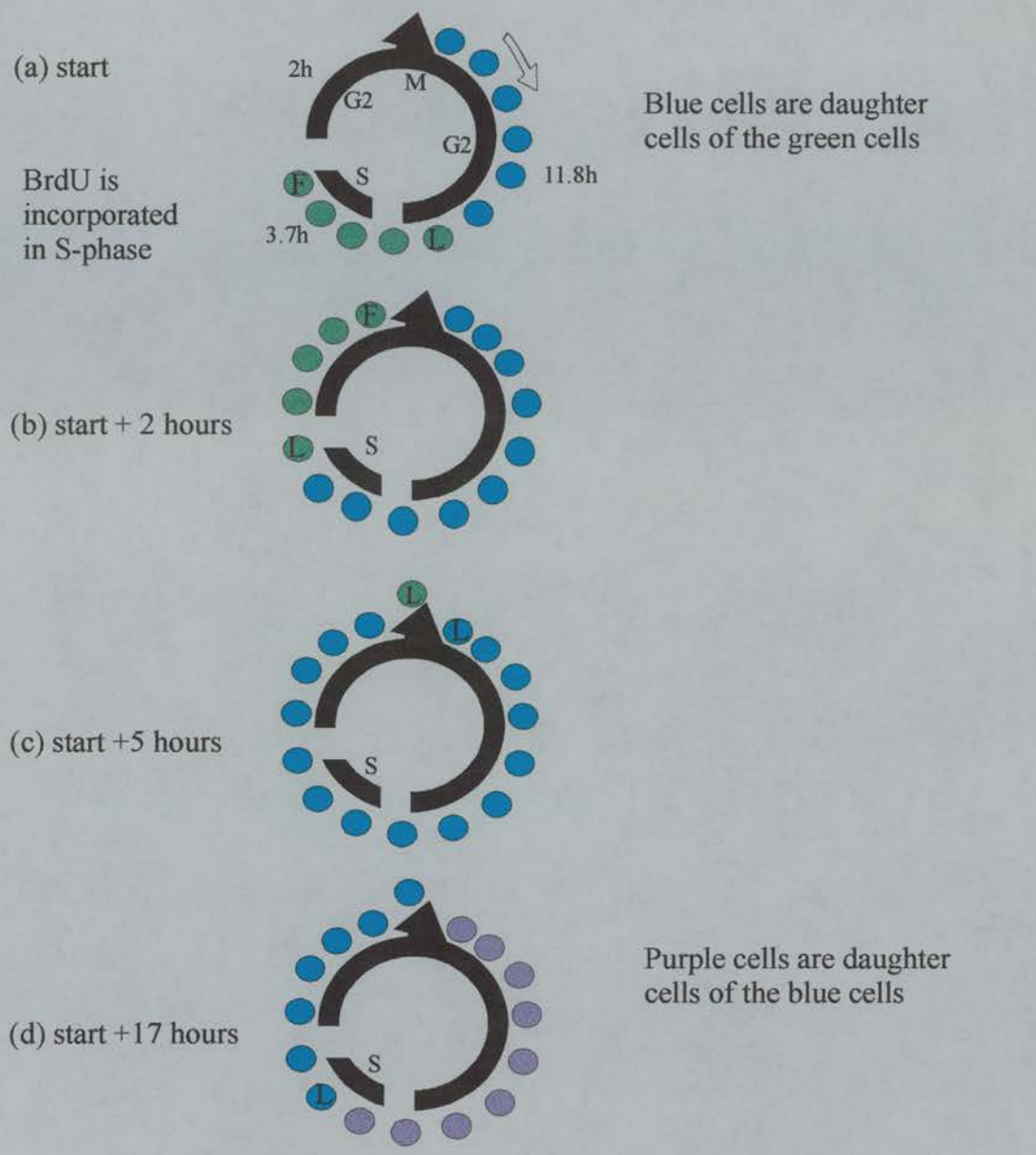


Figure 1. Diagramatic representation to show how cells belonging to three successive cell cycles could potentially become labelled with BrdU during a single BrdU pulse lasting 24 hours. The different coloured cells represent cells belonging to different cell cycles. The unfilled arrow represents the direction of progression of cells through the cell cycle. The approximate duration of each stage is indicated. F: the first cell to become labelled at the start of the BrdU pulse. L: the last cell of a particular cell cycle. (a) Cells in S-phase (S) at the start of the pulse (green cells) become BrdU labelled. (b) These cells progress to mitosis (M) within about two hours. They will redivide and their daughter cells will re-enter the cell cycle (light blue cells) or become terminally postmitotic and migrate out of the ventricular zone. (c) The blue cells will progress through the cell cycle and incorporate BrdU in S-phase. (d) Daughter cells of the blue cells (purple cells) will enter S-phase before the last blue cell leaves the cell cycle.

METHODS AND MATERIALS

The techniques for studying migration and lamination of E12, E14 and E16 born cortical precursors *in vivo* were described in Chapter 2.

Slice cultures

Balb/C mice from an isolated laboratory colony were mated overnight and checked for a vaginal plug at 9 a.m. the following day. The day of plug was considered embryonic day one. For the purposes of studying E12 and E14 born cells, in E16 slice cultures, mothers were injected (i.p.) with 5-bromo-2-deoxyuridine (BrdU) in saline solution (70μg g^{-1}) at midday on embryonic day 12 or 14.

Pregnant mice were anaesthetised on E16 with 0.3 ml of a 25% urethane solution (i.p.). Fetuses were removed by Caesarian section and the brains were removed and stored in ice cold Earl's Balanced Salts Solution (EBSS) for 45 minutes. The purpose of this was to dilute excitotoxic enzymes and possible diffusible factors that might supply laminar fate cues. Cortical slices were obtained by chopping brains parasagittally at 350 μm, using a MacIlwain tissue chopper. The presumptive neocortex (Fig. 2a) was then separated form the rest of the brain. Dorsal thalamic explants were dissected from littermates as described in Rennie *et al.* (1994; Fig. 2a) and sliced at 350 μm, using a MacIlwain tissue chopper. Cortical slices were cultured in pairs, alone or with thalamus, tectum or heart tissue (Fig. 2b), on collagen coated filters (Costar; see Fig. 2c) in culture wells containing 2.2 ml defined serum free culture medium (for recipe see Appendix). Tissues were incubated in warm (37° C) culture medium for one hour. For the purposes of studying E16 *in vitro* born cells, a one hour pulse of BrdU was given to specifically label cells undergoing S-phase in culture. The filters were removed and transferred to wells containing BrdU (40 μg ml^{-1}) in culture medium (see Appendix). After one hour, the filters were drained and returned to the wells containing culture medium alone. Some tissues were fixed at this stage to reveal the location of BrdU labelled cells at the start of the culture. Others were cultured at 35° C in 95% O_2/5% CO_2 for two or five days. Tissues from animals that had been treated with BrdU on E12 and E14 were not

labelled at this stage. They were either fixed to reveal the location of labelled cells at the start of the culture, or 'washed' as above and placed on collagen filters alone, or with thalamic tissue. The number of animals and slice cultures that were analysed in this study is shown in Table 1.

Fixation and processing of slices for BrdU immunohistochemistry.

Fixation was achieved using 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4; 1 hour). The tissues were then rinsed and stored overnight in PBS at 4° C. They were then dehydrated in a series of alcohols: 75% ethanol (2 x 15 minutes), 95% ethanol (2 x 15 minutes), 100% ethanol (2 x 15 minutes) and cleared in chloroform (90 minutes). The tissues were removed from the collagen sheets using a scalpel blade and transferred to paraffin wax (60°C; three hours). Tissues were sectioned at 10μm on a wax microtome, following the parasagittal plane of the tissue, mounted on poly-l-lysine coated slides and left overnight (or longer) at 37°C, to dry. Slides were dewaxed in xylene (12 minutes), rehydrated in a series of alcohols: 100% ethanol (3 minutes), 95% ethanol (3 minutes), 75% ethanol (3 minutes) and tap water (3 minutes), then reacted to reveal BrdU labelling. The BrdU reaction was described in Chapter 2.

Cell distribution analysis

In order to reveal the distribution of BrdU labelled cells within each cortical slice, camera Lucida drawings were made (at x25 magnification) of BrdU labelled cell profiles (subsequently referred to as cells, except if indicated otherwise) within a 0.5 mm wide bin, spanning the entire width of the cortical wall (from ventricular edge to pial edge; Fig. 2b). Four alternate sections were drawn from each slice. Sections at the cut edges of the slice were not analysed on the assumption that they would contain severed radial glial cells. Cortical slices provide an environment in which radial migration can be supported. However, tangential migration is prohibited to some extent by the finite width of the slice. Therefore, in the rostrocaudal direction, analysis was made in the center of each slice because this represents the region of the cortical wall in which there is least tangential dispersion of cells (Cepko *et al.*, 1990).

To determine the position of labelled cells within the cortical slices, camera Lucida drawings were divided into ten equal sized bins, from ventricular edge to pial edge. Cells were categorised as densely or lightly labelled as described in Chapter 2. Only cells that were fully labelled throughout their nucleus were considered to be densely labelled. The average number of cells in each bin was calculated. This was converted to a percentage and the average percentage of cells in each bin was plotted against bin number. Only densely labelled cells were used in this analysis, except at the start of the experiment when both densely and lightly labelled cells were counted as a single population, because, as described in detail in Chapter 3, they represent the product of a single cell cycle.

RC2 staining

Radial glial cells were studied using an antibody to RC2 (a gift from Dr P. Gressens. University of Louvain, Brussels, Belgium). Cortical cultures were removed from the collagen filters using a scalpel blade and placed in O.C.T. embedding medium (Miles Inc. Diagnostics Division) on a wooden chuck. They were immediately immersed in isopentane (BDH) on dry ice then stored at –70 ° C. Cryostat sections were cut at 10 μm, mounted on poly-l-lysine coated slides, dried and stored with desiccant at – 70° C, until required. Slides were thawed for 1 hour at room temperature and allowed to air dry. Sections were covered with 4% paraformaldehyde in PBS (10 mins at 4 ° C) then washed three times in PBS (5 mins/wash). The slides were immersed in acetone (10 mins at 4° C) to permeabilise the cell membranes. The acetone was removed and the slides were rinsed and washed (3 x 5 mins) in PBS. The PBS was replaced with TBS + 0.1% tween (3 x 5 min washes) then blocked with goat serum (1 in 5) in tris buffered saline (TBS)/tween (1 hour). Anti-RC2 in 10% fetal calf serum (1 in 3 in TBS/tween) was applied and left overnight at 4° C. Slides were washed in TBS/tween (3 x 5 mins) and Texas Red goat anti-mouse IgM (Vector TI 2020; 1 in 50 in TBS/tween) was applied for 1 hour at room temperature. The slides were washed in PBS and mounted in PBS/glycerol (1 in 3).

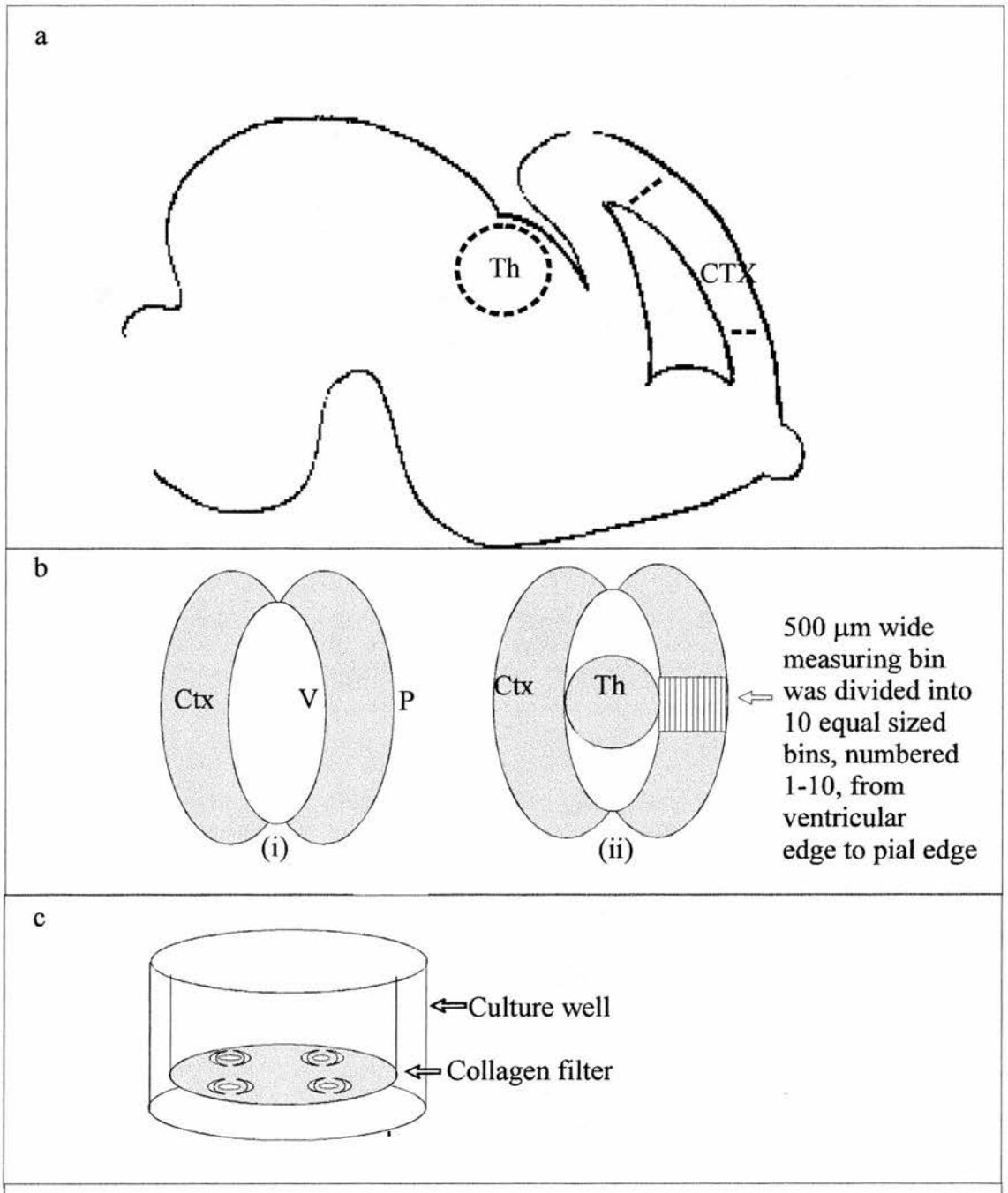


Figure 2 a: Diagramatic representation of a parasagittal section of an E16 mouse brain. Cortical slices were obtained from the presumptive neocortex (Ctx) and thalamic explants were obtained from the region of the dorsal thalamus (Th). b: Cortical slices were cultured in pairs (i) alone or (ii) with other embryonic tissue. Cameral lucida drawings were made of cells in a 500 μm wide bin stretching the width of the cortical wall c: Tissues were cultured on collagen coated filters in culture wells. CTX: cortex Th: thalamus V: ventricular edge P: pial edge

The same primary antibody was used on whole slices that had been fixed for one hour, washed in PBS and reacted free floating. Whole slices were removed from the collagen filters and fixed for 3 hours in 4% paraformaldehyde in PBS. They were rinsed thoroughly in PBS, treated with 3% H_2O_2 in methanol (1 in 4) to reduce endogenous peroxidase activity, washed with PBS containing 2% triton X (2 x 10 minutes), blocked with 5% rabbit serum in PBS/2% triton X (1 hour) and incubated in anti-RC2 (as above) in blocking solution (overnight at 4° C, on shaker). The slices were then washed in PBS/2% triton (3 x 20 minutes, on shaker) incubated in biotinylated rabbit anti-mouse antibody (Dako, 1 in 300) in blocking solution (2 hours at room temperature, on a shaker), washed as before, incubated in ABComplex (Dako) diluted in PBS/2% triton (1 hour at room temperature on shaker), washed in PBS then tris buffer (0.1M; pH 7.4), reacted with DAB in 750 µl tris buffer (0.1M, pH 7.4), with 20 µl 1% H_2O_2 and one flake of imidazole (8 minutes), rinsed in tris buffer and then PBS and mounted on slides in PBS/glycerol (1 in 3).

Calretinin staining

To reveal thalamocortical axons, an antibody to calretinin was used. Whole slices were removed from the collagen filters and fixed for 3 hours in 4% paraformaldehyde in PBS, then reacted free floating. They were rinsed thoroughly in PBS, treated with 3% H_2O_2 in methanol (1 in 3) to reduce endogenous peroxidase activity, washed with PBS containing 2% triton X (2 x 10 minutes), blocked with 5% pig serum in PBS/2% triton X (1 hour) and incubated in rabbit anti-calretinin (Chemicon; 1 in 1000) in blocking solution (overnight at 4° C, on shaker). The slices were then washed in PBS/2% triton (3 x 20 minutes, on shaker) incubated in biotinylated pig anti-rabbit (Dako, 1 in 300) in blocking solution (2 hours at room temperature, on a shaker), washed as before, incubated in ABComplex (Dako) diluted in PBS/2% triton (1 hour at room temperature on shaker), washed in PBS then tris buffer (0.1M; pH 7.4), reacted with DAB in 750µl tris buffer (0.1M, pH 7.4), with 20 µl 1% H_2O_2 and one flake of imidazole (8 minutes), rinsed in tris buffer and then PBS and mounted on slides in PBS/glycerol (1 in 3)

Di I labelling

Radial glia and thalamic axons were also visualised in whole slices using the lipophilic tracer dioctadecyl tetramethyilindocarbocyaninine pechlorate (DiI; Molecular Probes). Slices were fixed by immersion in 4% paraformaldehyde in phosphate buffer (Pb) after 2, 5 or 7 days in culture. DiI crystals dissolved in 70% alcohol was injected into the cortical marginal zone to reveal radial glial cells or into the thalamus to reveal thalamic axons, using a glass micropipette attached by a plastic conduit and rubber tube to a mouth pipette. The endfeet of radial glia terminate in the marginal zone therefore DiI can diffuse along the length of the cell membrane to label processes that span the cerebral wall. Slices were maintained in fixative at room temperature for about a week to allow for diffusion of DiI. Fluorescent cells were subsequently visualised using a rhodamine filter on a Leica epifluorescence microscope.

Cell viability

Cortical sections that had been reacted for BrdU were counterstained in cresyl-violet. This revealed both live and pyknotic cells. Pyknotic cells were identified by their small, dark nucleus. In contrast, healthy cells were pale coloured with a clear nucleus. For each cortical slice examined, counts were made from four adjacent sections. These were taken throughout its width (in the region in which cell counts had been made) in a 150 μm wide bin at x 40 magnification. The number of pyknotic cells was expressed as a proportion of all the cells counted.

Cortical width

The width of the cortical wall was measured from camera Lucida drawings of whole brains and slice cultures. For each slice/whole brains three lines were drawn, 250 μm apart, perpendicular to the ventricular and pial edges, through each drawing, and the average length of these lines was taken as the width of the cortical wall.

Cell size

At some points in this study it was considered necessary to compare cell numbers (see Results section). However, the cell profiles that are visible in tissue sections do not necessarily represent whole cells, because the profiles also reveal fragments of cells that belong to adjacent sections. Therefore profile counts do not accurately reflect the true cell number within a given mass of tissue (Abercrombie, 1946), that is the cell density. In addition, the number of cell profiles that are visible in a tissue section is influenced by the size of the cells being counted. Thus, when comparing profile numbers between different tissues, differences in the size of nuclei in the tissues being compared will lead to different counts, even if the cell density is exactly the same. Thus, where it was considered necessary to compare cell numbers, cell sizes were measured and compared. The length of the cells were measured, parallel to the ventricular and pial surfaces, at x100 magnification, using a micrometer eye piece. In all the cases where a comparison was made, there was no significant difference in the cell sizes between tissues, therefore it was considered that for the purposes of comparing cell numbers between different tissues, the profile counts provided a sufficiently accurate assessment of the relative number.

TABLE 1

	E16 (1h post BrdU administration	+2d	+5d
	Number of slices/brains (number of litters)		
E12 BrdU			
Whole brain	3 (2)	5 (2)	4 (2)
Cortical slice	3 (2)	3 (2)	3 (2)
Cortical slice + thalamus		5 (3)	4 (2)
E14 BrdU			
Whole brain	3 (2)	4 (2)	4 (2)
Cortical slice	3 (2)	5 (2)	3 (2)
Cortical slice + thalamus		3 (2)	3 (2)
E16 BrdU			
Whole brain	5 (3)	5 (3)	4 (2)
Cortical slice	4 (2)	5 (3)	4 (2)
Cortical slice + thalamus		7 (3)	5 (3)

Table 1 shows the number of whole brains and cortical slices that were analysed at each time point of fixation. For every time point, at least two different litters were used. In total 60 slices and 37 whole brains were analysed in this series of experiments. Four sections from each slice/whole brain were counted.

RESULTS

Cells proliferate and migrate in culture

The aim of this project was to test if cells that were born in a defined culture environment would migrate and form cortical layers. The migration of cells that are born *in vitro* in (i) isolated cortical slices or (ii) cortical slices cultured with thalamus was compared to the migration of cells born and allowed to develop *in vivo*. BrdU was added to culture medium to label cells dividing *in vitro* or (for the *in vivo* study) injected i.p. to label cells dividing *in vivo*. Cortical slices and whole brains were fixed one-hour post BrdU administration to reveal the distribution of cells dividing at the start of the study. Both darkly and lightly labelled cells were included in this analysis. Subsequently only densely labelled cells were analysed. This ensured that only cells that became terminally postmitotic at the start of the study were investigated.

Slices that were not fixed after one hour were allowed to develop in culture for two or five days, and the distribution of labelled cells was compared with those born *in vivo*. Cell distribution is described in terms of relative positions within the cortical wall. For ease of description the cortical wall is described in terms of the ventricular half (bins 1-5) and the pial half (bins 6-10).

Slice cultures

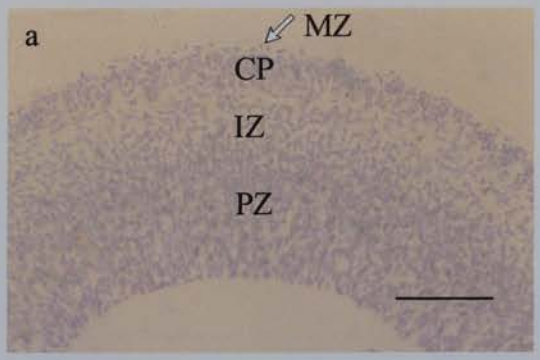
Cortical slice cultures remained healthy for up to 5 DIV (described in detail later). In slices (from at least three mothers) that had been cultured for seven days, a large proportion of the cells were pyknotic and it was decided not to include data from these cultures in the analysis. Slices were placed on the collagen filter so that their cut edges lay parallel to the filter and the ventricular and pial surfaces were perpendicular to it. Several slices folded over in the culture well or failed to sit flat in the wax pots at the point of wax embedding and were not suitable for analysis because they did not section in the correct plane. The incorporation of BrdU varied greatly, even within a single experiment, and many cultured slices contained no

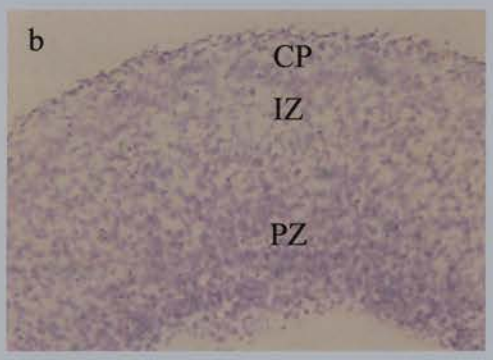
BrdU label. Therefore these could not be included in the study. Less than a quarter of all the slices cultured were used in the final analysis.

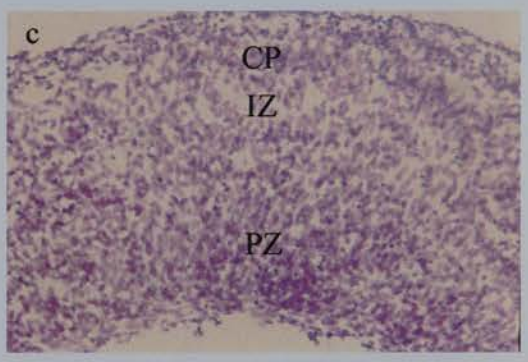
Cultured slices maintained their approximate organotypic organisation for at least two days. Figure 3 shows sections of counterstained cortical slices cultured alone or with thalamic tissue (a) after dissection (referred to as acute slices) and after (b and c) two and (d and e) five days *in vitro* (2 and 5 DIV). Three or four regions were visible in acute and 2 DIV slices. These were identified by their location and cell densities, as described in Chapter 2. They are the proliferative zone (PZ), intermediate zone (IZ), cortical plate (CP) and, in the acute slices, the marginal zone (MZ). At 5 DIV these regions were no longer discernible.

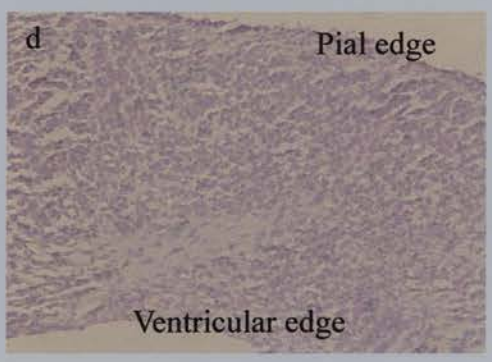
Cortical slices varied in width, both from slice to slice and from slice to whole brain. To normalise for this, and because the zones of the cortical wall were not discernible at 5 DIV, the relative position of cells within the cortical wall (as described in the Methods and Materials section) was described to reveal relative cell movement. The average width of the cortical wall is shown in Table 2.

There was a large difference between the average width of cultured cortical slices and whole brain cortices. Acute cortical slices were not significantly different from whole brain cortices at E16. However, cortical slice cultures were significantly narrower than whole brains at 2 DIV and 5 DIV (Student t-test $p < 0.01$) and cortical slices cultured alone were significantly narrower than cortical slices cultured with thalamus at 2 DIV (Student t-test $p < 0.05$).









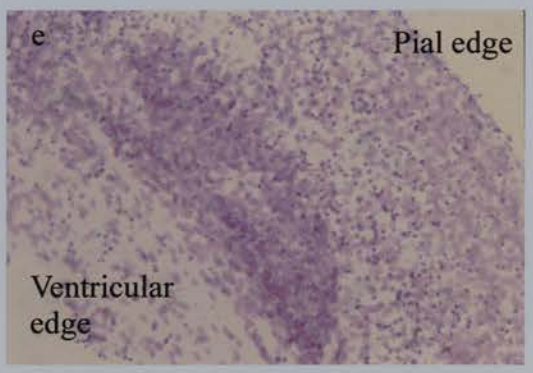


Figure 3 shows cresyl violet stained cortical slices (a) after two hours in culture (b) after culturing alone for two days (c) after culturing with thalamus for 2 days (d) after culturing alone for five days (e) and after culturing with thalamus for five days. Cortical slices maintained an approximate organotypic orgnaisation for 2 DIV. After 5 DIV the cortical zones could not be discerned. PZ: proliferative zone; IZ: intermediate zone; CP: cortical plate; MZ: marginal zone. Scale bar: 100 μm

TABLE 2

Tissue	Time after BrdU administration	Average width of the cortical wall (μm)
Cortex *in vivo*	1 hour	238.50 (s.e.m. = 16.79)
Cortex *in vivo*	2days	536.50 (s.e.m. = 11.82)
Cortex *in vivo*	5 days	966.50 (s.e.m. = 19.96)
Acute cortical slice	1 hour	217.00 (s.e.m. = 15.13)
Cortical slice	2 days	259.82 (s.e.m. = 19.96)
Cortical slice	5 days	341.00 (s.e.m. = 16.78)
Cortical slice + thalamus	2 days	387.18 (s.e.m. = 35.87)
Cortical slice + thalamus	5 days	390.50 (s.e.m. = 17.39)

Table 2 illustrates the average width of the cortical wall *in vivo* and *in vitro*, at all time points investigated.

Analysis of cell distribution

Within a single cortical slice, four 10 μm thick sections were drawn. Every second section was counted. Since the average length of the labelled nuclei was 4.34 μm, ranging from 7 μm to 2 μm (s.e.m. = 0.128), this means that no single cell nucleus will span more than two sections. Therefore taking counts in every second section avoids the problem of counting the same cell twice.

The distribution of labelled cells one hour post BrdU administration

At one hour post BrdU administration there was no obvious difference in either cell numbers or cell distribution between the two culture protocols (that is, between cortical slices cultured alone and cortical slices cultured with thalamus), therefore these counts were combined. Both *in vivo* and *in vitro,* most BrdU labelled cells were found in the ventricular half of the cortical wall at the start of the experiment. This is illustrated in Figures 4a and 5a which show representative camera Lucida drawings of a section taken from a whole brain and a cortical slice, at E16. These results are shown quantitatively in Figures 6a and 7a. Ninety eight and 94% of densely labelled cells were found in bins 1-5 in whole brains and cortical slices respectively. Therefore the proportion of cells in the pial half of the cortical wall at the start of the investigation was minimal. The total number of cells labelled *in vivo* was 168.90 (s.e.m. = 19.89) and *in vitro* was 168.63 (s.e.m. = 51)

Migration patterns *in vivo*

The migration of E16 born cells *in vivo* was described in detail in Chapter 2. By two days post-injection, the distribution of labelled cells had changed from that at the start. Cells were found throughout the width of the cortical wall (Fig. 4b and 6b). There was peak in their distribution in bins 5 and 6 which contained 40% of all cells . Bins 5 and 6 are between 268-321 μm from the ventricular edge. A small proportion of cells (12%) was found in bins 8, 9 and 10, a distance of 230-536 μm from the ventricular edge. By five days post injection there was a distinct peak in the distribution of cells in the upper cortical wall (Fig.4c and 6c). Sixty nine percent of

all densely labelled cells were found in bins 8, 9 and 10 (Fig. 6c). This is a distance of between 773-966 μm from the ventricular edge.

Migration in slice cultures

As described above, only 6% of all labelled cells were found in the pial half of the cortical slice at the start of the culture. Assuming the remaining 94% complete their interkinetic movement, as has been described previously in acutely dissected cortical slices (Chenn and McConnell, 1995; Bohnar *et al.,* 1997), they will begin their migration from the ventricular edge. Since this was not tested, bins 1-5 have been designated as the start point of migration. Examination of the positions of BrdU labelled cells in cultured slices at 2 DIV revealed a movement of cells into the pial half of the slices (Fig. 5b and c). In cortical slices cultured alone, bins 6-10 contained 28% of densely labelled cells (Fig. 7b). In cortical slices cultured with thalamus, bins 6-10 contained 69 % of densely labelled cells (Fig. 8b). This compares with 44% *in vivo* (Fig. 6b). After 5 DIV labelled cells were seen at various cortical depths (Fig.5d and e). The proportion of cells in bins 6-10 in cortical slices cultured alone had increased to 38% (Fig. 7c) whereas in slices cultured with thalamus it had decreased to 35% (Fig. 8c). This compares with 78% *in vivo* (Fig. 6c).

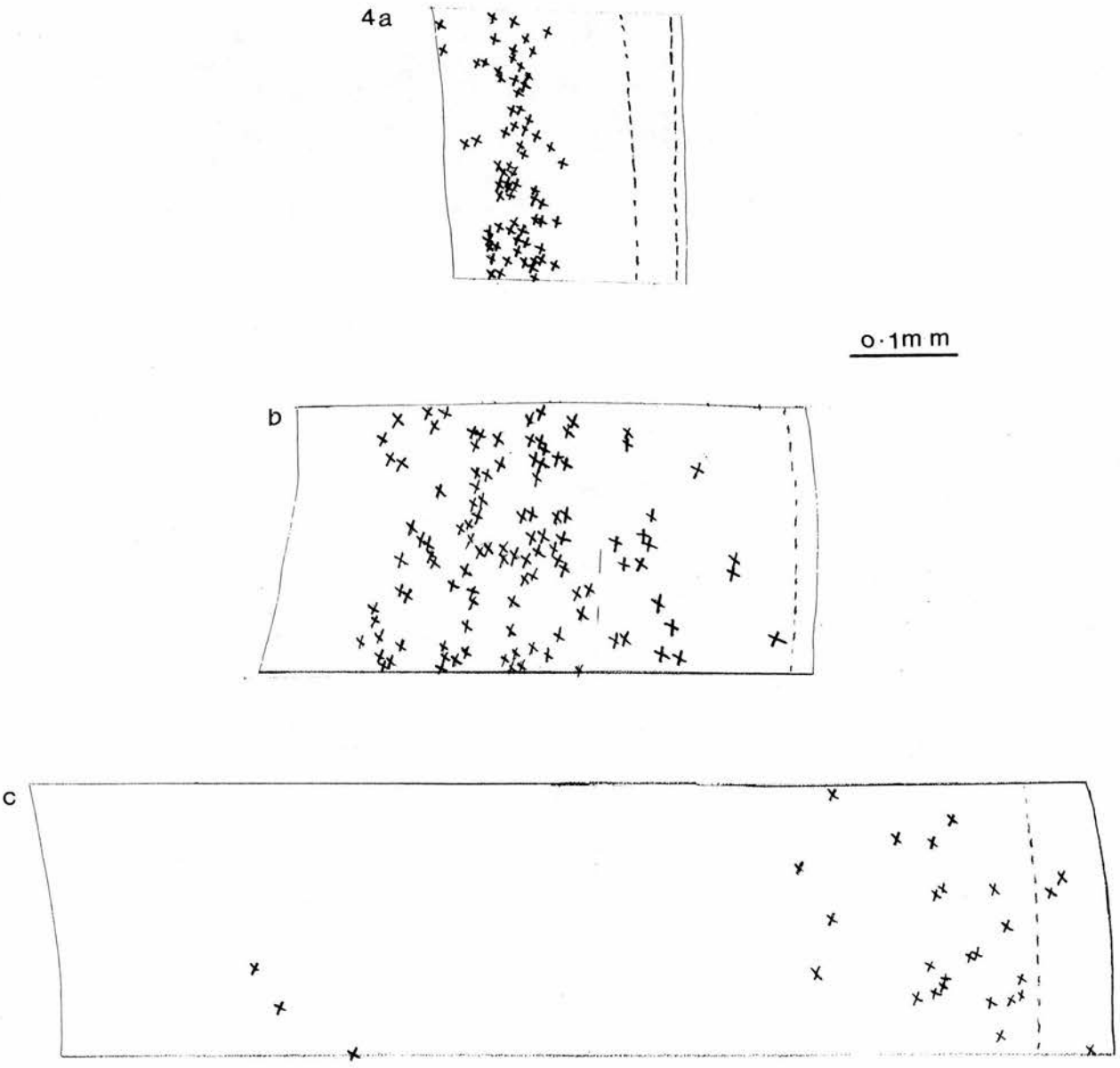
Comparison of the distributions (Figs. 6, 7 and 8) reveals little similarity between cell distribution in cortical slices cultured alone with those that were allowed to develop *in vivo,* except that the two day culture data resembles the five day *in vivo* data. This is discussed later. In the cortical slices cultured with thalamus, the two-day culture data resembles the five-day *in vivo* data. Both distributions reveal a peak in the distribution in bin 9 (compare Fig. 5c and 7b). Given the difference in the average width of the cortical wall in cultures with that *in vivo* (387 μm versus 536 μm) at E16+2 days it seemed feasible that the cells migrating in the co-cultures behaved like those migrating *in vivo*, but that they reached their ultimate laminar destination in a much shorter time because they had a much shorter distance to travel. The distribution of cells at five days was much

flatter than at two days. However there was a small peak in the distribution in bin 8. This might represent a downward movement of the two day peak which could be due to the E16 born cells being displaced by later born cells at the pial edge, as occurs *in vivo* (Caviness, 1981 and Polleux, 1997).

The results of this study are summarised in Table 3 which illustrates the proportion of cells that are located in the pial half of the slice (bins 6-10) at the beginning and end of the culture period and the proportion of cells that reach the top of the cortical slice (bins 8-10). In cortical slices cultured alone the proportion of cells in the pial half of the cortical slice (bins 6-10) after 2DIV was significantly different from that at the start (Student t-test $p < 0.01$). This is true too in cortical slices cultured with thalamus ($p < 0.01$). The proportion of cells at the top of the cortical slice (bins 8-10) was not significantly different from that at the start in cortical slices cultured alone, but it was significantly different in cortical slices cultured with thalamus ($p < 0.01$). After 5DIV , the distribution of cells was significantly different from that at the start in cortical slices cultured alone, bins 6-10 ($p < 0.01$) and in bins 8-10 ($p < 0.01$) and in cortical slices cultured with thalamus, in bins 6-10 ($p < 0.01$) and in bins 8-10 ($p < 0.05$). The difference in the proportional distributions between cortical slices cultured alone and cortical slices cultured with thalamus was also significantly different after 2DIV, in bins 6-10 ($p < 0.05$) and in bins 8-10 ($p<0.05$). After 5DIV there was no significant difference between the proportion of cells in the pial half of the slice or in the upper cortical wall between cortical slices cultured alone and cortical slices cultured with thalamus.

Figure 4 shows representative camera Lucida drawings of BrdU labelled cells *in vivo,* on (a) E16 (b) E18 and (c) P1. The ventricle is to the left and the pial edge is to the right in each figure. At E16, most of the cells are found in the proliferative zone. At E18 labelled cells are seen throughout the width of the cortex and at P1 the majority of labelled cells are found in the cortical plate, just below the marginal zone. Scale bar: 100 μm.

Figure 5 shows representative camera Lucida drawings of BrdU labelled cells *in vitro* at (a) E16, (b) after 2 DIV in cortical slices cultured alone (c) after 2 DIV in cortical slices cultured with thalamus (d) after 5 DIV in cortical slices cultured alone and (e) after 5 DIV in cortical slices cultured with thalamus. Scale bar: 100 μm.


4a
o·1m m
b
c

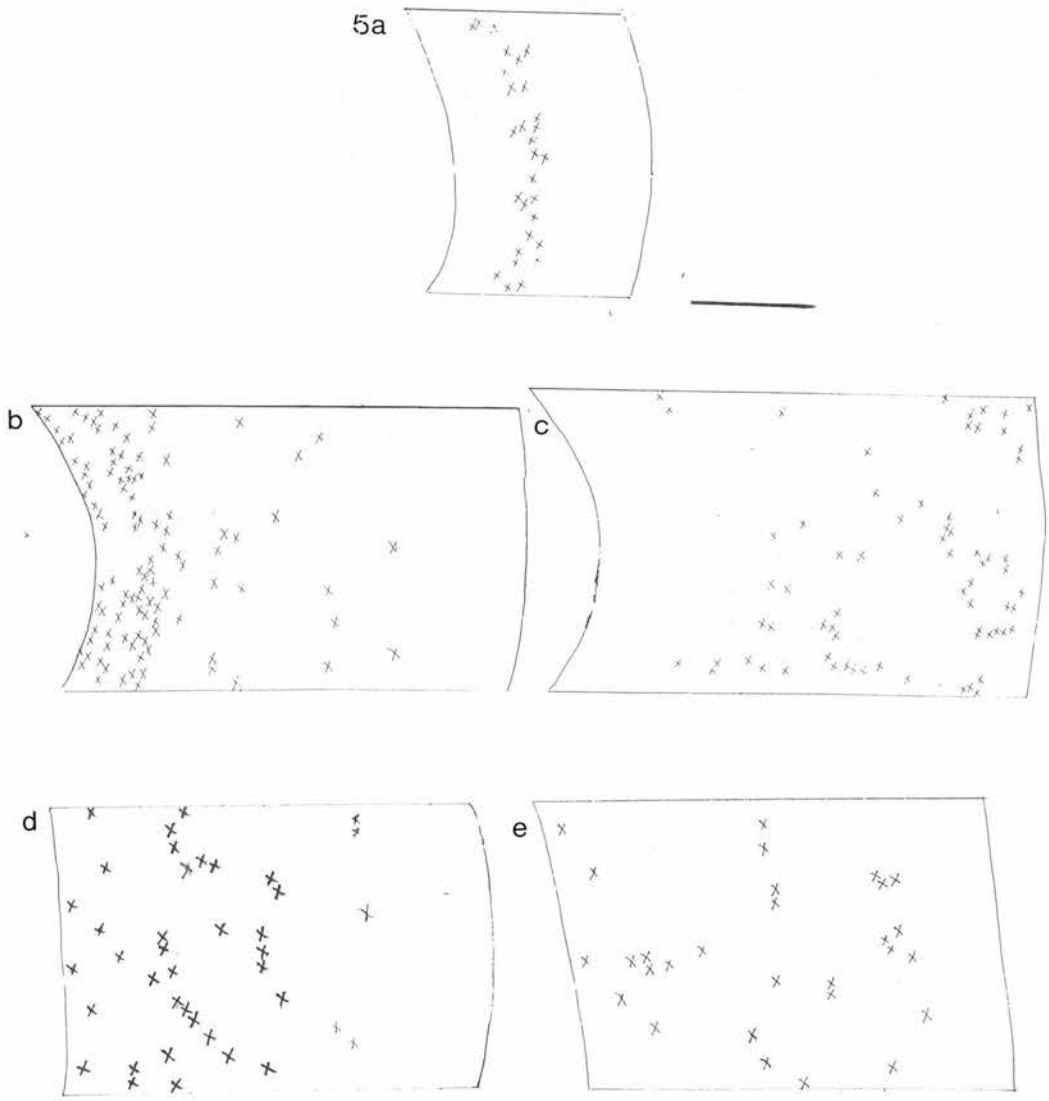
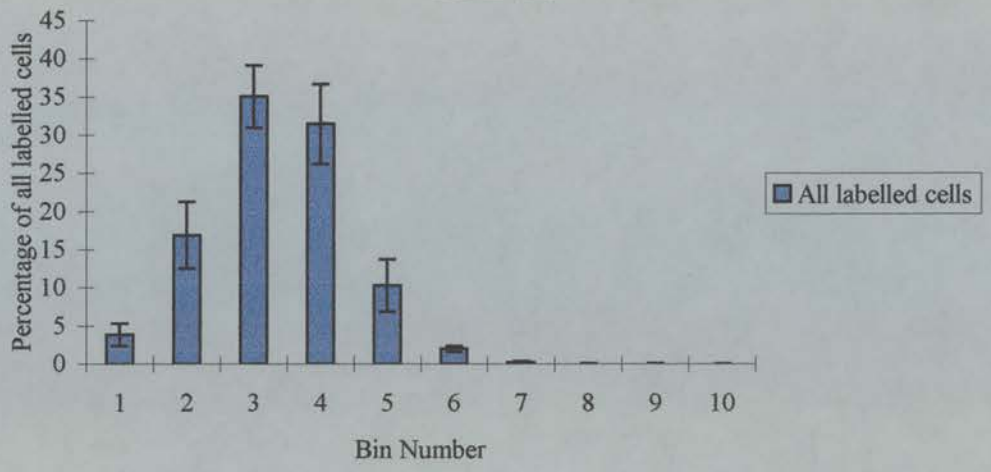

5a
b
c
d
e

Figure 6 shows the relative distribution of BrdU labelled cells *in vivo,* on (a) E16 (b) E18 and (c) P1. The proportion of labelled cells is plotted against bin number. The cortical wall was divided in to ten equal bins, numbered 1-10, from ventricular edge to pial edge. At E16, most of the cells are found in bins 1-5. At E18 labelled cells are seen throughout the width of the cortex, with a peak in their distribution in bins 5 and 6, and at P1 the majority of labelled cells are found in bins 8, 9 and 10, with a peak in their distribution in bin 9.

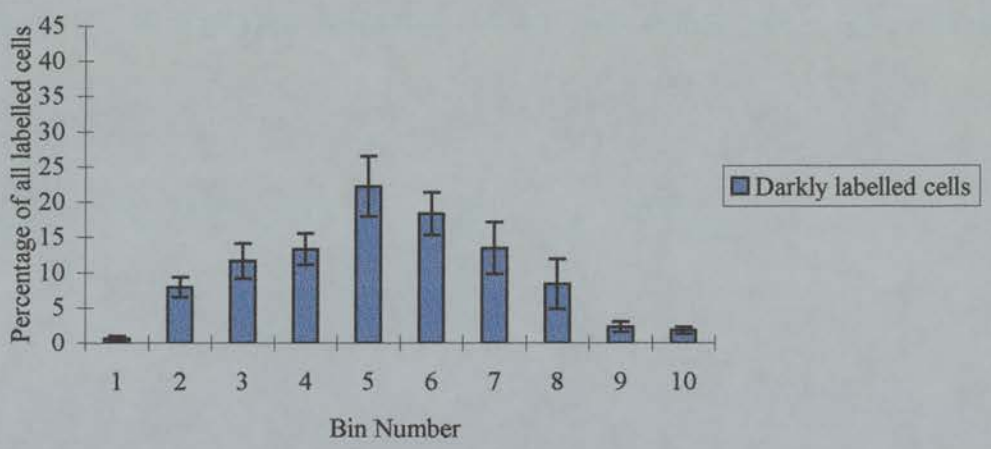
Figure 7 shows the relative distribution of BrdU labelled cells *in vitro,* on (a) E16 (b) after 2 DIV(c) after 5DIV, in cortical slices cultured alone. The proportion of labelled cells is plotted against bin number. The cortical wall was divided into ten equal bins, numbered 1-10, from ventricular edge to pial edge. At E16, most of the cells are found in bins 1-5. After 2 DIV labelled cells are seen throughout the width of the cortex, but the majority remain in the ventricular half. After 5 DIV labelled cells are seen in all cortical bins. There is a small peak in their distribution in bins 5 and 6.

Figure 8 shows the relative distribution of BrdU labelled cells *in vitro,* on (a) E16 (b) after 2 DIV(c) after 5DIV, in cortical slices cultured with thalamus. At E16, most of the cells are found in bins 1-5. After 2 DIV labelled cells are seen throughout the width of the cortex, but the majority are found in bins 8, 9 and 10. After 5 DIV labelled cells are seen in all cortical bins. There is a small peak in their distribution in bins 3 and 8.

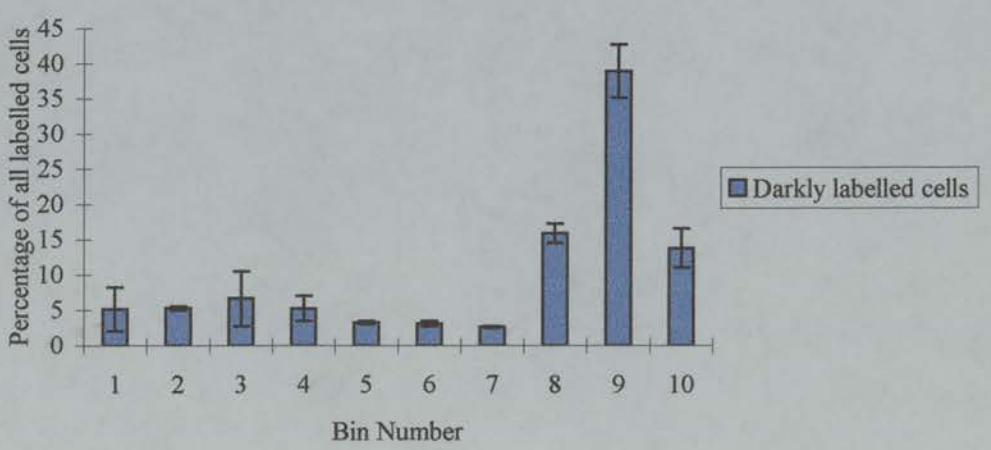
6a


Change in the distribution of all cells labelled with BrdU for one hour on E16 in vivo
Percentage of all labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
All labelled cells

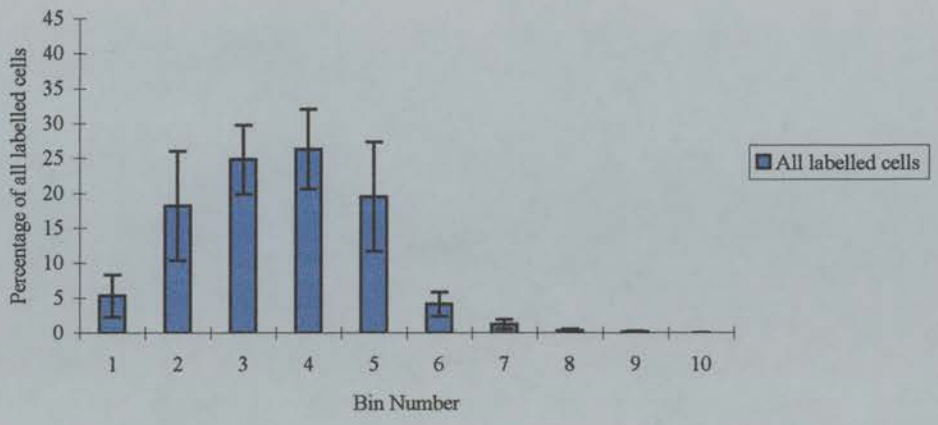
b


Change in the distribution of all cells labelled with BrdU on E16 after two days in vivo
Percentage of all labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
Darkly labelled cells

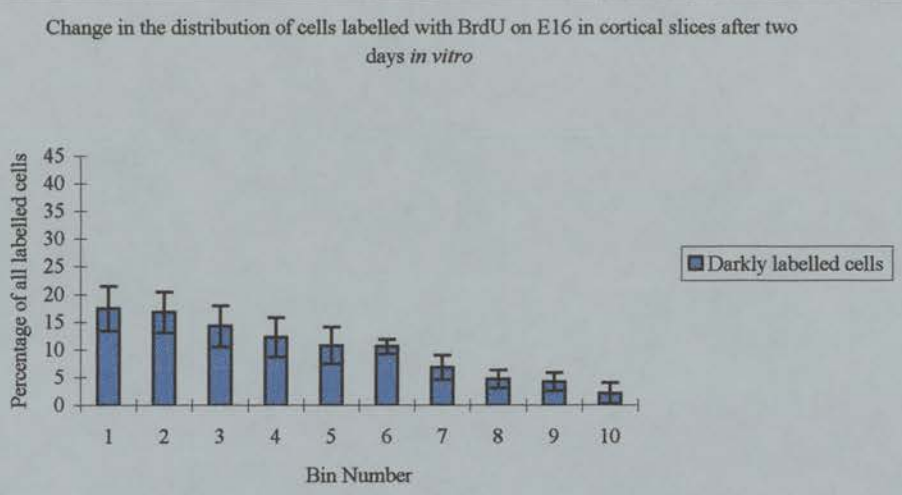
c


Change in the distribution of all cells labelled with BrdU on E16 after five days in vivo
Percentage of all labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
Darkly labelled cells

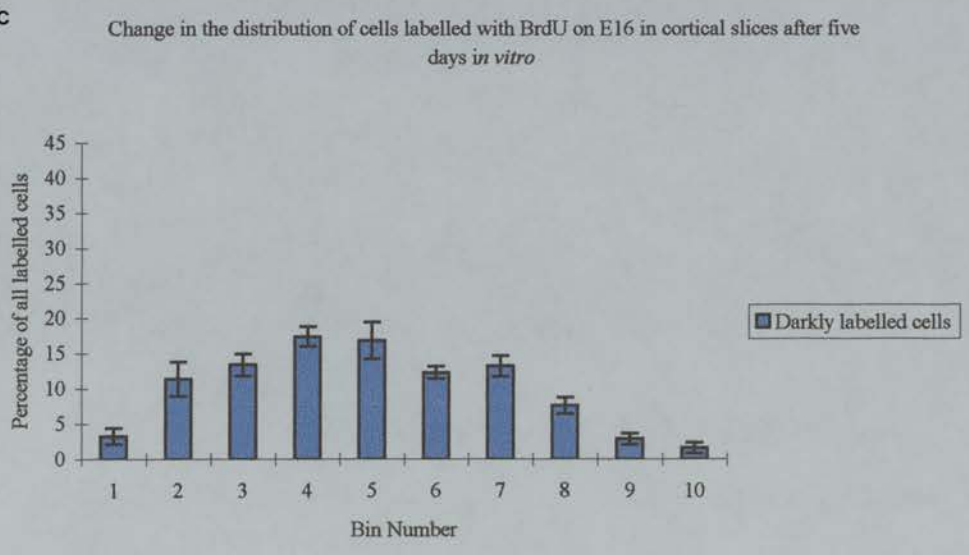
7 a


Relative distribution of cells labelled with BrdU for one hour in vitro
Percentage of all labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
All labelled cells

b

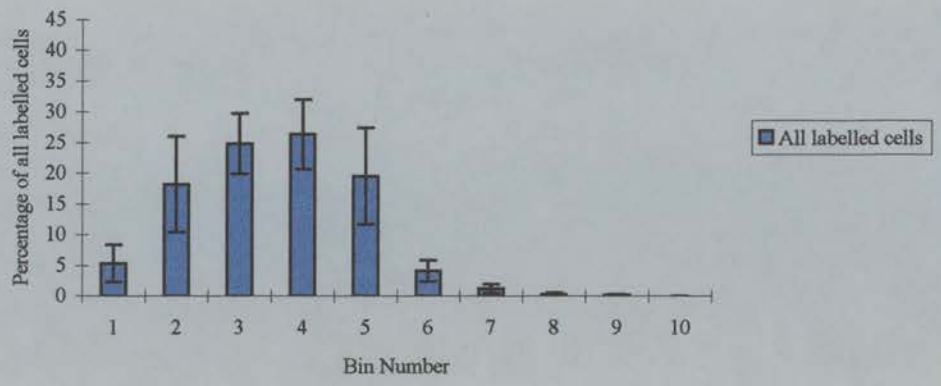

Change in the distribution of cells labelled with BrdU on E16 in cortical slices after two days *in vitro*
Percentage of all labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
Darkly labelled cells

c


Change in the distribution of cells labelled with BrdU on E16 in cortical slices after five days *in vitro*
Percentage of all labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
Darkly labelled cells

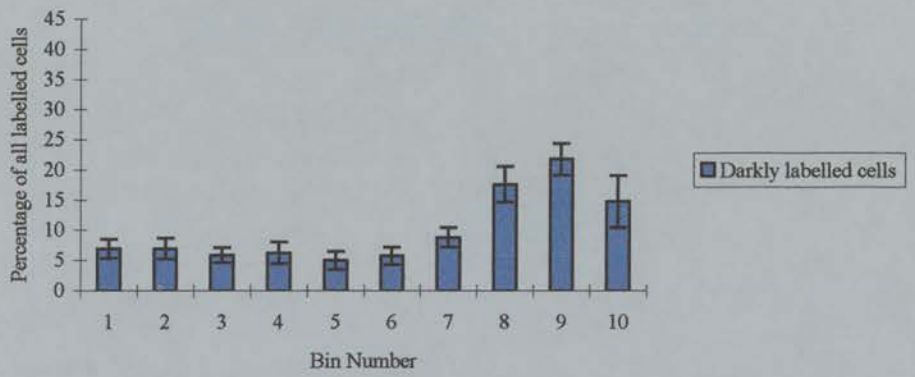
8 a

Relative distribution of cells labelled with BrdU for one hour in vitro



b

Change in the distribution of cells labelled with BrdU on E16 in corticothalamic co-culture after two days *in vitro*



c

Change in the distribution of cells labelled with BrdU on E16 in corticothalamic co-culture after five days *in vitro*

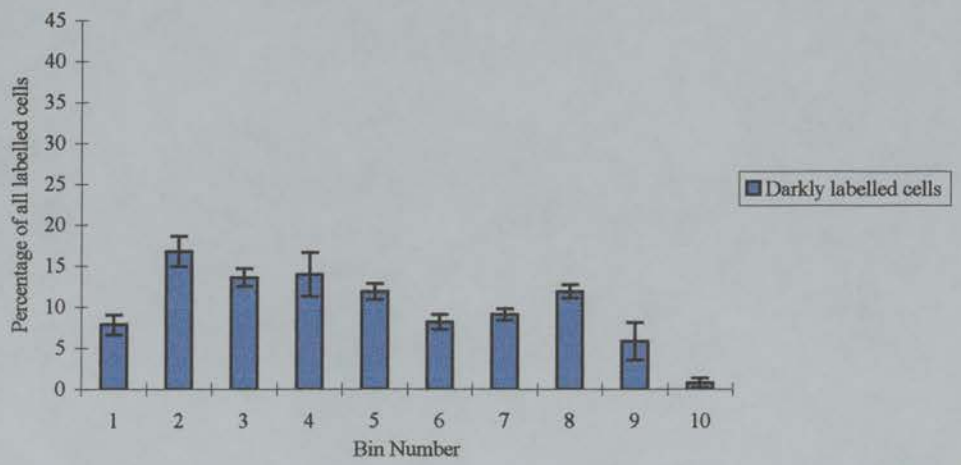


TABLE 3

Tissue	Percentage of cells in bins 6-10	Percentage of cells in bins 8-10	Number of slices (number of experiments)
Acute cortical slice	5.74 (s.e.m. = 0.36)	0.41 (s.e.m. = 0.24)	4 (3)
Cortical slice alone 2DIV	28.46 (s.e.m =4.40)	11.05 (s.e.m. =4.00)	5(3)
Cortical slice alone 5DIV	37.65 (s.e.m. = 3.28)	12.08 (s.e.m. = 1.10)	5(3)
Cortical slice + thalamus 2DIV	68.83 (s.e.m. = 8.22)	54.21 (s.e.m. = 19.76)	7(3)
Cortical slice + thalamus 5DIV	35.86 (s.e.m. = 3.99)	18.54 (s.e.m. = 3.26)	5(3)

Table 3 shows the mean percentages of cells in bins 6-10 and bins 8-10 at the start of the culture period and after 2 and 5 DIV, in cortical slices cultured alone and with thalamic tissue

The results described above reveal a difference in the behaviour of cells born in isolated cortical slices from those born in cortical slices cultured with thalamus. In both cases a proportion of cells born *in vitro* migrated out of the ventricular half of the slice. However, in isolated cortical slices a much smaller proportion of cells did so and an even smaller proportion made it to their normal destination at the top of the cortical slice during the period of the study. To determine if was also the case after 1DIV and 3DIV in cortical slices cultured alone a few cultures were examined and it was found that the distribution of labelled cells was like that at 2DIV (data not shown). In contrast, cells born in cortical slices cultured with thalamus did form a tight band at the pial edge, but they did so sooner than *in vivo*, possibly because they had a shorter distance to travel. They were then apparently displaced downwards, possibly by later born cells coming to lie above them.

In order to test if the difference in behaviour might be due to the thalamus making the environment more permissive to migration, the distribution of earlier born in the two culture systems were examined and compared. Radial glia were also examined and cell survival was assessed.

The distribution of E12 and E14 born cells *in vivo*

At E16, E12 born cells were found in the marginal zone and subplate (Fig. 9a). After two (Fig. 9b) and five (Fig. 9c) days the marginal zone and subplate populations had become increasingly separated. At E16, many E14 born cells were in position in the cortical plate (Fig.10a). They become increasingly displaced downwards after two and five days (Figs.10b and 10c). This is due to later born cells coming to lie above the earlier born cells. The relative positions of E12, E14 and E16 born cells and their relationship to each other was described in detail in Chapter 1 and is summarised in Figure 11.

The distribution E12 and E14 born cells *in vitro*

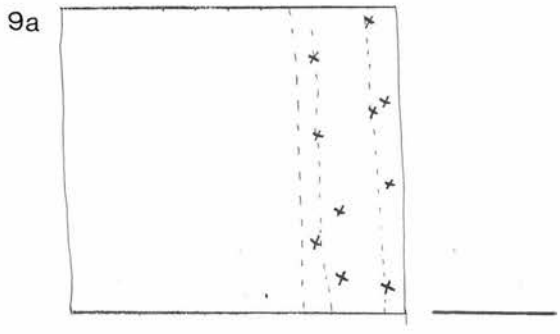
In acute cortical slices, E12 labelled cells were distributed in a similar pattern to those *in vivo* (compare Figs 9a and 12a and yellow bars Fig. 11a and 14a).

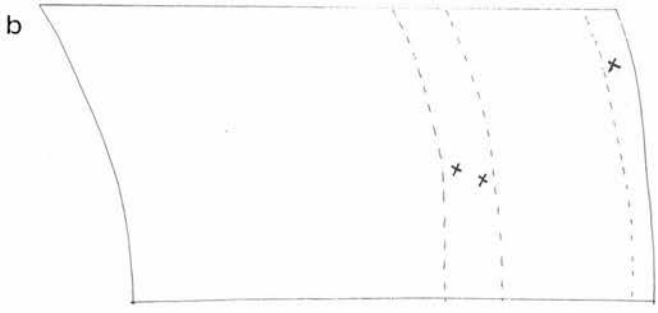
However, as the cameras Lucida drawings suggest, the proportions in each region were different. Seventy one percent of densely labelled cells were found in bins 7 and 8, corresponding to the subplate, and 16% were in bin 10, corresponding to the marginal zone (Fig. 14a). These percentages represent 5.09 and 1.33 BrdU +ve cells. This compares with 52% and 35% *in vivo* (Fig. 11a), which represents 8.25 and 7.75 BrdU +ve cells. E14 born cells were also found in a similar pattern to those in the whole brain at the start of the culture (compare Figs 10a and 13a and brown bars Figs 11a and 14a). The great majority of cells (77%) were found in bins 8, 9 and 10, corresponding to the cortical plate (Fig.14a). This compares to 69% *in vivo* (Fig. 11A).

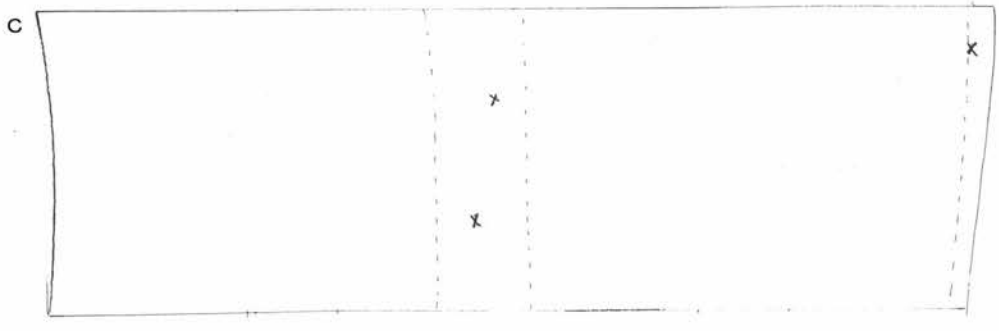
After 2 and 5DIV, in cortical slices cultured alone, the majority of E12 born cells were found at the top of the cortical plate (Figs 12A and d) , corresponding to bins 9 and 10 (Figs. 14B and c). To show if this was possibly due to the preferential loss of subplate cells, which begin their lives in bins at a short distance from the top of the cortical plate, corresponding to bins 7 and 8, as described previously, the number of BrdU positive cells was examined and compared at the start of the culture and after 2 and 5 DIV. At the start of the culture, the total average number of E12 labelled cells located in bins 7-10 was 7.00 (s.e.m. = 0.87), after 2DIV the total number was 9.75 (s.e.m. = 0.25) and after 5DIV the total number was 7.14 (s.e.m = 2.94). In cortical slices cultured with thalamus (Figs 11C and e), the distribution of E12 born cells was slightly broader (Figs 11c and e), corresponding to bins 8,9 and 10 (Fig. 15b), after 2DIV and bins 7,8,9 and 10 (Fig. 15c) after 5 DIV. The total average number of cells at 2 and 5 DIV was also examined. At 2 DIV the total average number of cells was 8.15 (s.e.m. = 2.25) and at 5 DIV the total number was 7.56 (s.e.m. = 2.40). The average length of these cells was calculated in acute slice, two and five days cultures. The range of average cell lengths was between 4.55 μm and 5.0 μm. There was no significant difference in the length of these cells (Student's t-test) therefore the use of cell profile numbers is an adequate measure of cell density for the purposes of this comparison.

Figure 9 shows representative camera Lucida drawings of cells labelled with BrdU on E12 *in vivo,* on (a) E16 (b) E18 and (c) P1. The ventricle is to the left and the pial edge is to the right in each figure. At all ages, cells are found in the marginal zone and subplate. A few cells are found in the cortical plate at E16. Scale bar: 100 μm.

Figure 10 shows representative camera Lucida drawings of cells labelled with BrdU on E14 *in vivo,* on (a) E16 (b) E18 and (c) P1. The ventricle is to the left and the pial edge is to the right in each figure. At E16 cells are seen mainly in the cortical plate. At E18, most cells are seen in the lower part of the cortex and the subplate and intermediate zone. At P1, most cells are found in the region representing the deep cortical layers. Scale bar: 100 μm.


9a


b


c

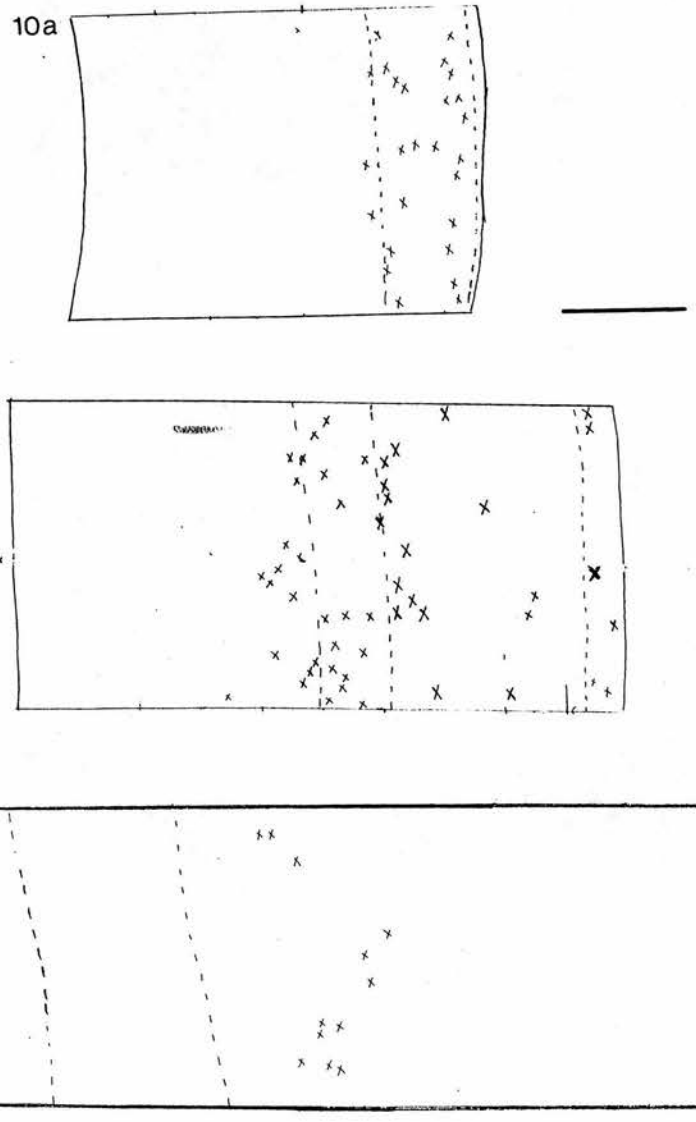
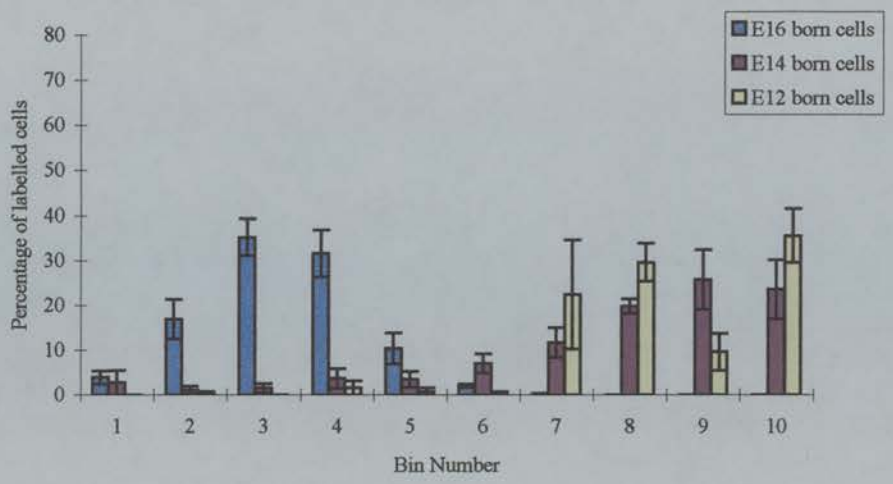
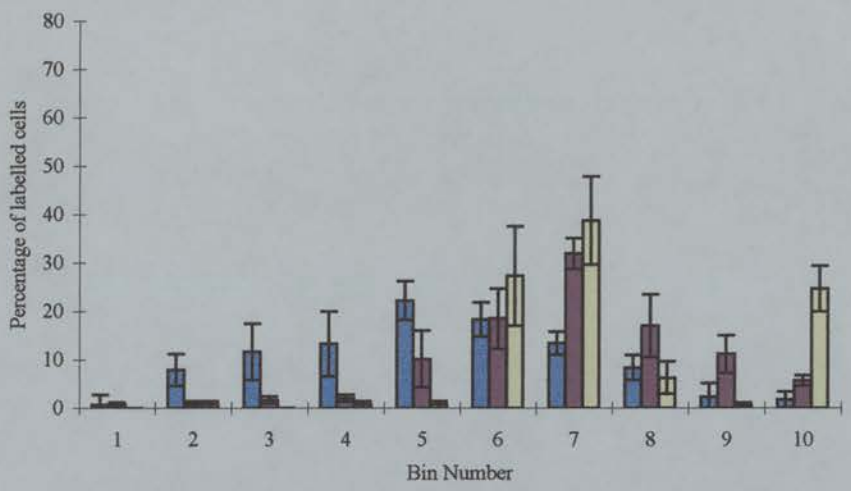

10a
b
c

Figure 11 shows the relative distribution of cells labelled with BrdU on E12 (yellow bars), E14 (brown bars) and E16 (blue bars) and their relationship to each other *in vivo,* on (a) E16 (b) E18 and (c) P1. At E16, most E12 born cells are found in bins 7 and 8 and 10; E14 born cells are approximately sandwiched between the E12 labelled cells and are found predominantly in bins 8, 9 and 10; E16 born cells are found in bins 1-5. On E18, most E12 labelled cells are found in bins 6 and 7 and 10; E14 labelled cells have moved downwards to bins 6, 7 and 8; E16 labelled cells are found in all bins with a peak in their distribution in bins 5 and 6. On P1, E12 labelled cells have almost completely disappeared from bin 10 and the remainder are found in bins 5 and 6; E14 labelled cells have moved downward to bins 4, 5 and 6; E16 labelled cells have reached bins 8, 9 and 10 at the top of the cortical wall.

a The relative distribution of cells labelled with BrdU on E12, E14 and E 16 on E16



b The relative distribution of cells labelled with BrdU on E12, E14 and E 16 on E18



c The relative distribution of cells labelled with BrdU on E12, E14 and E 16 on P1

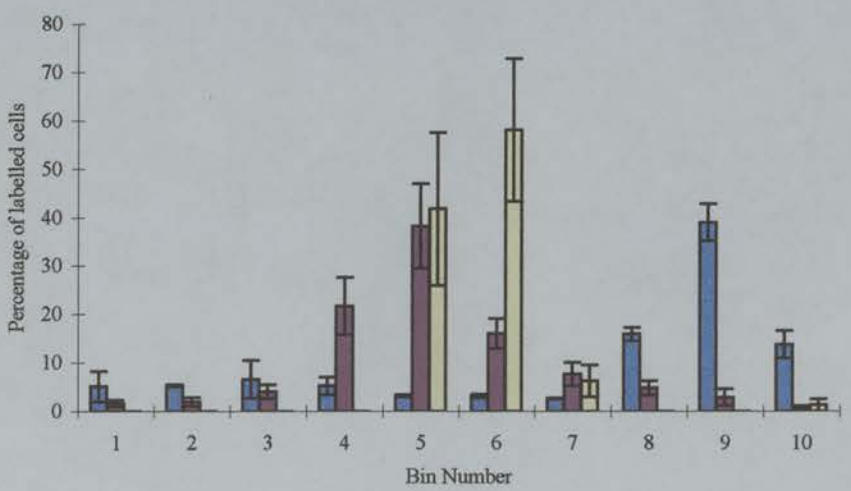
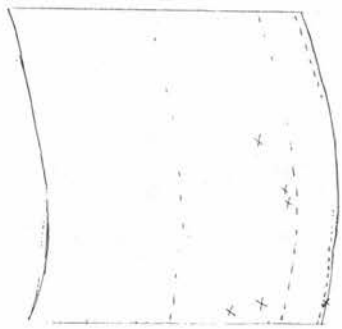


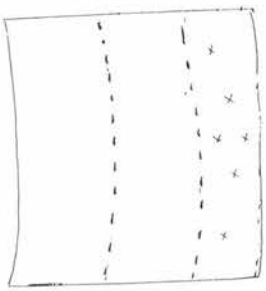
Figure 12 shows camera Lucida drawings of cells labelled with BrdU on E12 in cortical slices prepared on E16 at (a) in acute slices (b) after 2 DIV(c) after 5DIV, in cortical slices cultured alone (b and d) and with thalamus (c and d) . At all stages and in both slices cultured alone and with thalamus the majority of cells are seen at the top of the cortical wall. Only in the acute slice (a) was it possible to discern a marginal zone and subplate contingent.

Figure 13 shows camera Lucida drawings of cells labelled with BrdU on E14 in cortical slices prepared on E16 (a) in acute slices (b) after 2 DIV(c) after 5DIV, in cortical slices cultured alone (b and d) and with thalamus (c and d) . At all stages and in both slices cultured alone and with thalamus the majority of cells are seen at the top of the cortical wall. At 5 DIV, some cells were also found in lower regions of the cortical wall in both slices cultured alone and slices cultured with thalamus.

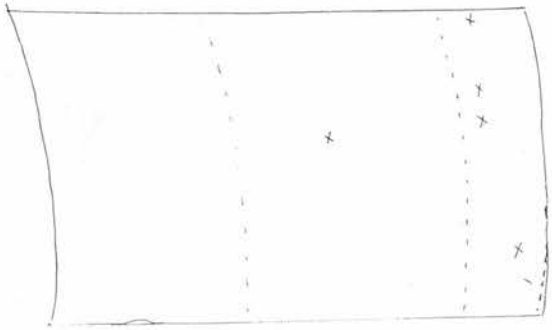
12a



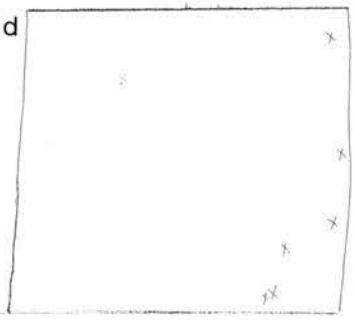
b



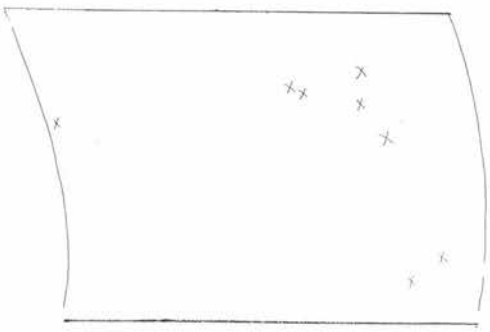
c



d



e



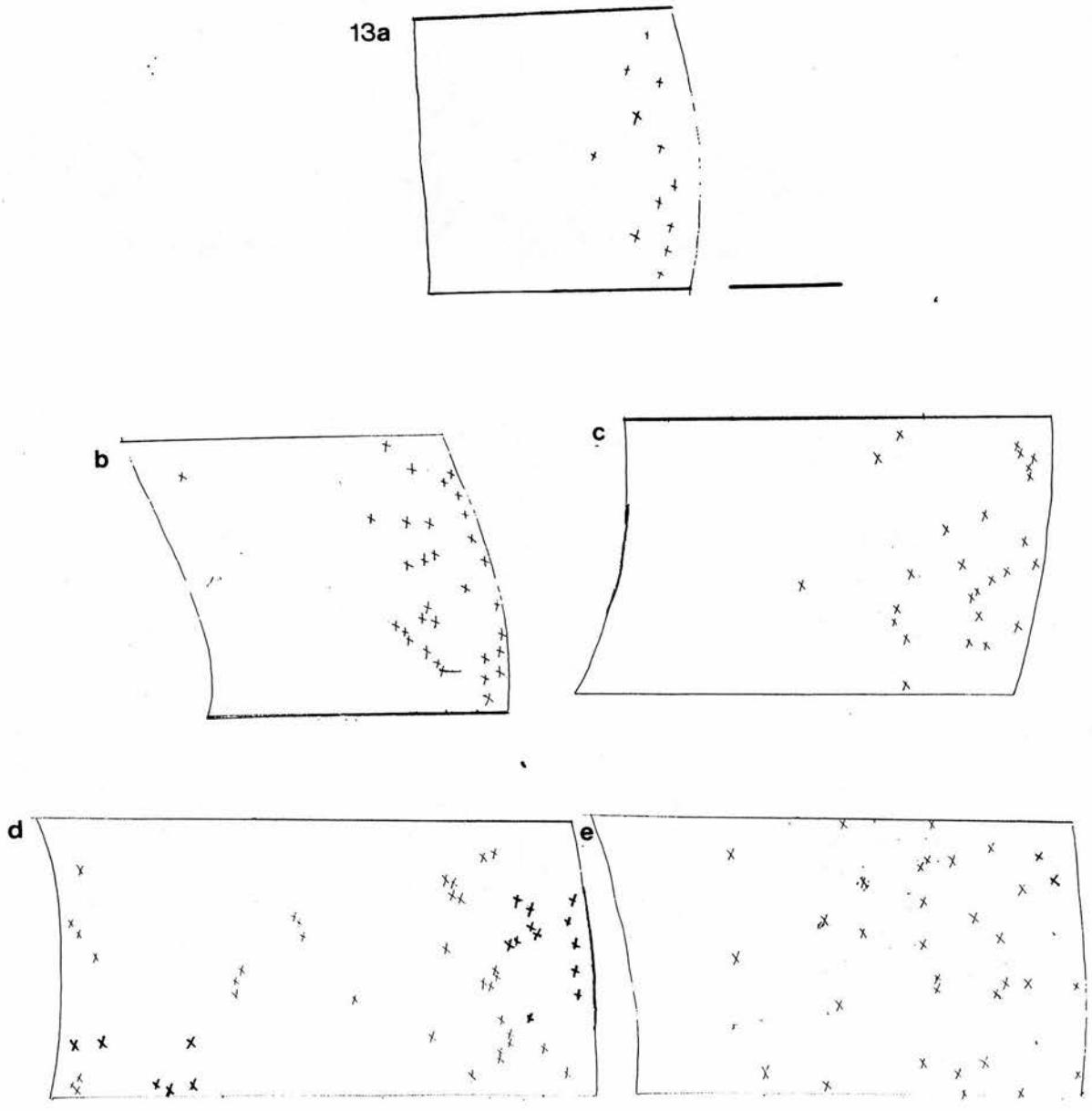
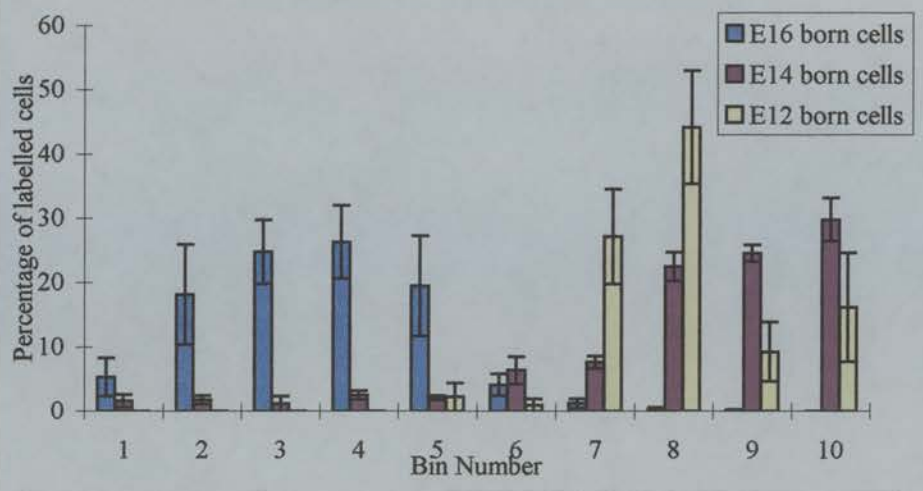
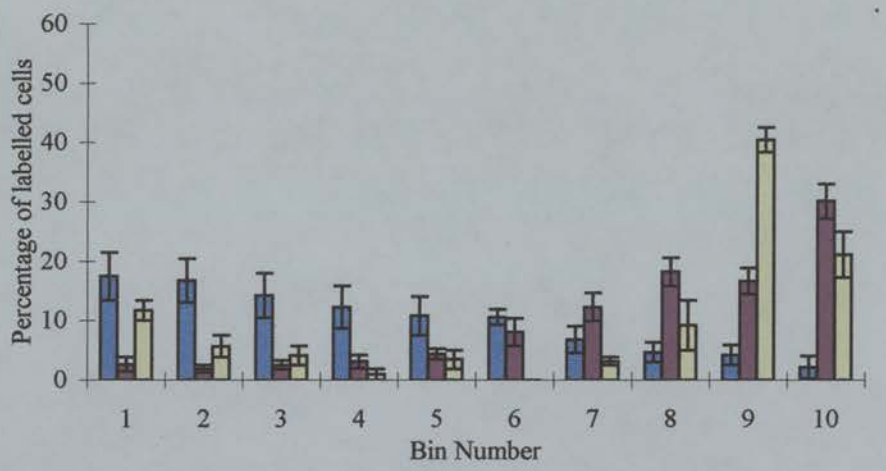

13a
b
c
d
e

Figure 14 shows the relative distribution of cells labelled with BrdU on E12 (yellow bars), E14 (brown bars) and E16 (blue bars) and their relationship to each other in cortical slices cultured alone (a) in acute slices (b) after 2 DIV(c) after 5DIV. In acute slices most E12 born cells are found in bins 7 and 8 and 10; E14 born cells are approximately sandwiched between the E12 labelled cells and are found predominantly in bins 8, 9 and 10; E16 born cells are found in bins 1-5. After 2 DIV, the E12 labelled cells have moved to the top of the cortical slice; the E14 labelled cells retain the distribution they had at the start; a small proportion of the E16 labelled cells have moved into bins 6-10, but the majority remain in bins 1-5. After 5 DIV, the distribution is similar to that at 2 DIV, but the E1 distribution has flattened slightly and cells are found in all bins and the E16 labelled cells have formed a peak in bins 4 and 5.

a


The relative distribution of cells labelled with BrdU on E12, E14 and E 16 in cortical slice cultures after two hours in vitro
E16 born cells
E14 born cells
E12 born cells
Percentage of labelled cells
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9
10
Bin Number

b


The relative distribution of cells labelled with BrdU on E12, E14 and E 16 in cortical slice cultures after two days in vitro
Percentage of labelled cells
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9
10
Bin Number

c

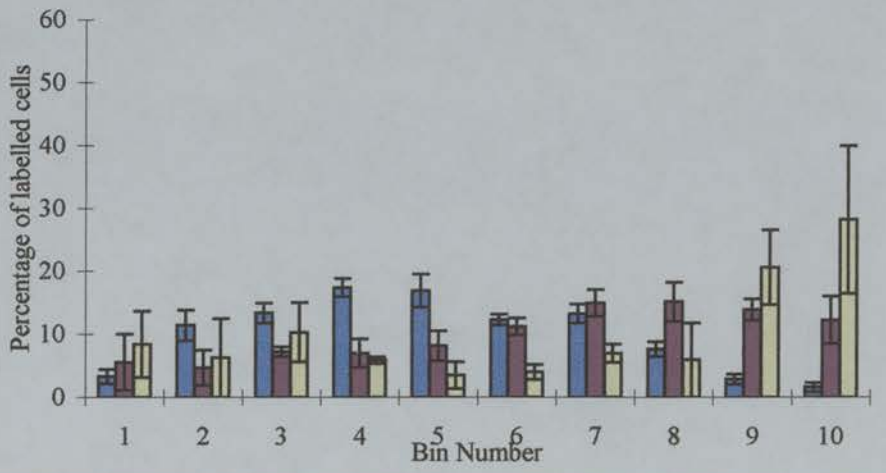

The relative distribution of cells labelled with BrdU on E12, E14 and E 16 in cortical slice cultures after five days in vitro
Percentage of labelled cells
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9
10
Bin Number

Figure 15 shows the relative distribution of cells labelled with BrdU on E12 (yellow bars), E14 (brown bars) and E16 (blue bars) and their relationship to each other in cortical slices cultured with thalamus (a) in acute slices (b) after 2 DIV(c) after 5DIV. In acute slices most E12 born cells are found in bins 7 and 8 and 10; E14 born cells are approximately sandwiched between the E12 labelled cells and are found predominantly in bins 8, 9 and 10; E16 born cells are found in bins 1-5. After 2 DIV, the E12 labelled cells have moved to the top of the cortical slice; the E14 labelled cells retain the distribution they had at the start; a large proportion of the E16 labelled cells have moved into bins 6-10, with a peak in their distribution in bins 8-10. After 5 DIV, the distribution of E12 labelled cells has flattened out slightly and most cells are found in bins 7,8,9 and 10; E14 labelled cells have retained their position at the top of the cortical wall and there is a peak in their distribution in bin 10; E16 labelled cells are found in all bins with peaks in their distribution in bins 2 and 8.

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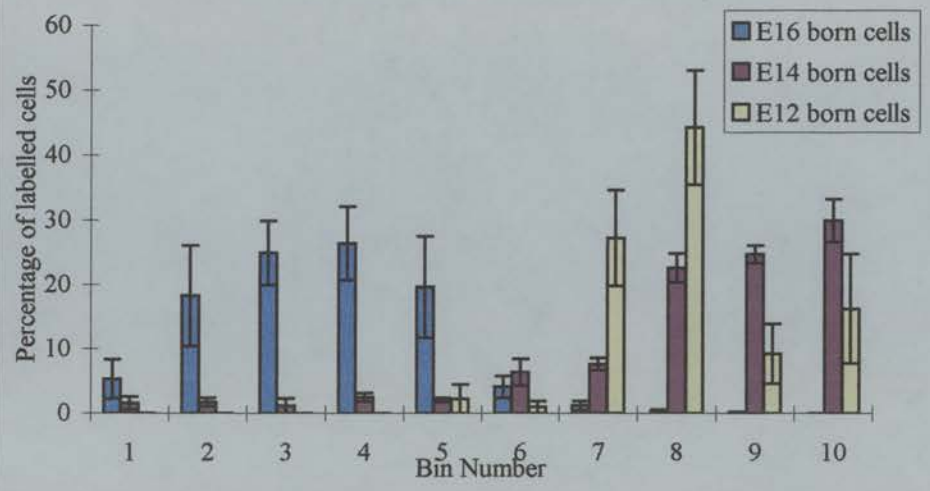
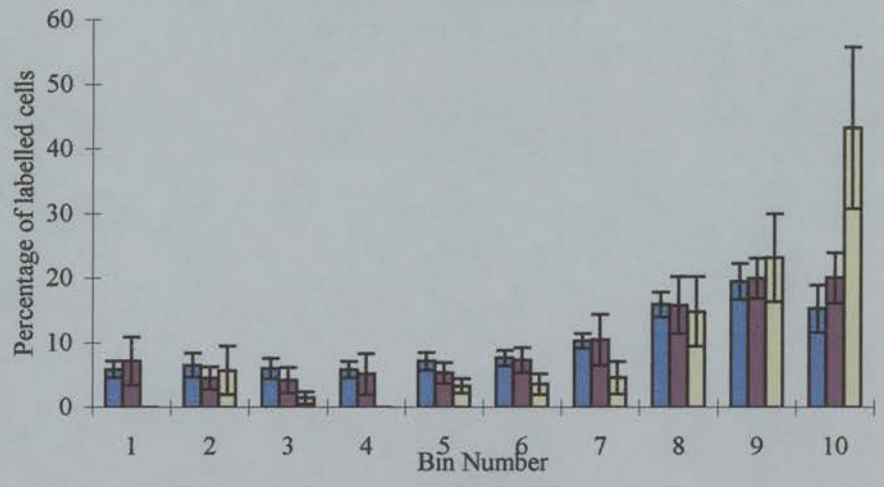
a

The relative distribution of cells labelled with BrdU on E12, E14 and E 16 in cortical slice cultures after two hours in vitro



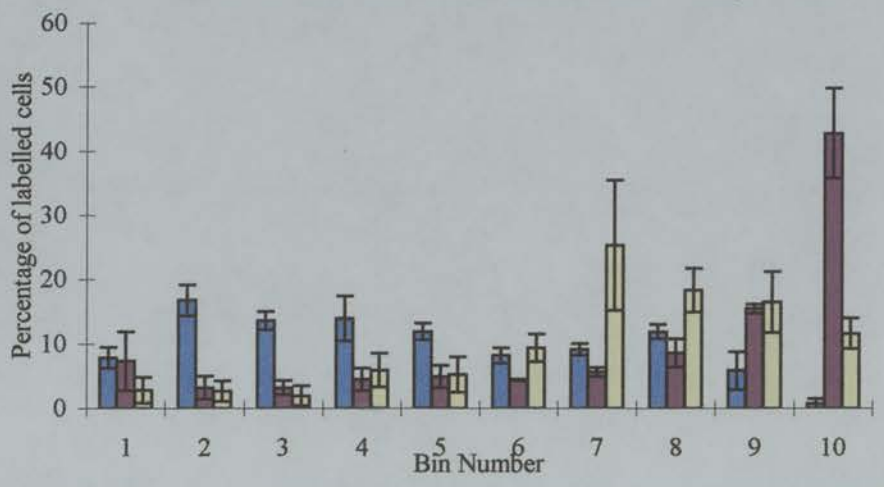
b

The relative distribution of cells labelled with BrdU on E12, E14 and E 16 in cortical slice cultured with thalamus after two days in vitro



c

The relative distribution of cells labelled with BrdU on E12, E14 and E 16 in cortical slice cultured with thalamus after five days in vitro



In cortical slices cultured alone, after two days *in vitro*, the E14 born cell distribution was similar to that at the start of the experiment (compare Figs 13 and b and 14 a and b). However, after five days, the distribution flattened out (Fig.13c and 14c). In corticothalamic co-cultures the majority of E14 born cells maintained their position at the upper cortical plate (Figs 13c and e and 15b and c) for the duration of the experiment.

These results showed that E12 born cells did not retain their normal bimodal distribution in either cortical slices cultured alone or with thalamus. They also suggest that the thalamus helps E14 born cells maintain their normal position in the cortical slices compared to cortical slices cultured alone. However, the presence of the E14 born cells at the top of the cortical slice, indicates that no new laminae were formed *in vitro*. Thus the downward movement of the E16 born cells in corticothalamic co-cultures at plus five days was not due to their being displaced by later born cells.

Since neurons are thought to migrate along the processes of radial glial cells, radial glial were examined in the slice cultures to determine if there was any difference between radial glia cells in cortical slices cultured alone and in cortical slices cultured with thalamus. Three different methods were used to look at the radial glial cells. An antibody to RC2 was used to look at radial glia in 10 μm cryostat sections of cultured slices. Radial glia were seen in slices cultured for up to seven days, both with and without thalamus. This method of visualising radial glial cells was, however, unsatisfactory because whole radial glial fibres were not present within a single 10 μm section. To counter this an attempt was made to label radial glial cells in whole cortical slices. However this also proved unhelpful because the antibody only penetrated the superficial depths of the tissue and therefore revealed only the radial glia on the cut edge of the slice. So although, this also showed that radial glia were present and that they ran radially within the cortical slices, it did not show if individual radial glial fibres spanned the entire width of the cortex. To test this the lipophilic tracer DiI was used. A tiny crystal of DiI was placed on the pial

edge of the whole cortical slice and a fluorescent microscope was used to visualise the DiI labelled processes. Many radial processes were seen running from the pial edge to the ventricular edge. Some of these ended in what appeared to be neuronal cell bodies. But others ended in small cell bodies in a region close to the ventricular edge of the slice where the cell bodies of radial glial cells are found *in vivo*. There was no obvious difference between slices cultured alone and slices cultured with thalamus (Fig. 16). After five days *in vitro* the radial glia became obscured by large, flat, multi-nucleate cells, though many radial fibres were seen in both isolated cultures and co-cultures.

To test if the thalamus had any effect on the survival of cortical cells the proportion of pyknotic cells was counted throughout the width of the cortical slices. These results are illustrated in Table 4. There was no significant difference between the proportion of pyknotic cells in the 2DIVor 5DIV cortices cultured alone compared to those cultured with thalamus (Student's t-test). Pyknotic cells are indicated by the white arrows, and healthy cells by the black arrows in Fig.17, which shows the pial portion of a cortical slice cultured alone for 5DIV.

TABLE 4

Tissue	2DIV	5DIV
	Percent pyknotic cells	Percent pyknotic cells
Cortex	2.20% (s.e.m. 0.24)	24.66 (s.e.m. 9.6)
Cortex + thalamus	4.11% (s.e.m.0.80)	21.16 (s.e.m. 1.99)

Table 4 illustrates the proportion of pyknotic cells in cortical slices cultured alone compared to cortical slices cultured with thalamus, at 2 and 5DIV.

These results suggest that, other than assisting E14 born cells to maintain their laminar position compared to isolated cortical slices, the thalamus does not have any obvious effect on the environment in which the cells are attempting to migrate. However, to test if the thalamus extended axons into the cortical slices, the cortical slices were viewed under the microscope prior to fixation using phase contrast and/or

the fixed tissue was stained with DiI or an antibody to calretinin. Inspection of the cultures prior to fixation showed that the thalamic tissue extended fibres toward the cortex after only a few hours in culture. DiI and calretinin staining revealed that thalamic axons grew into the co-cultured slice and coursed around the top of it. Figure 18 shows an example of a five day co-culture stained with calretinin. Axons are seen entering the cortex (asterisk, Fig.18a) at a particular point along its ventricular surface. These axons had growth cones on their ends (arrow Fig.18b) and they tended all to run in the one direction along the top of the cortical wall.

The effect of other embryonic tissues

So far the results show that *in vitro* born precursors will migrate towards their normal position and form a relatively tight band in cortical slices cultured with thalamus. In slices cultured alone, only a small proportion of cells leave the ventricular half of the slice and they do not form a tight band at the top of the cortical wall within the period of the study. To test if the effect is specific to the thalamus, the two-day culture protocol was repeated with other embryonic tissue in place of the thalamus. The thalamic explant was replaced with either another piece of cortex, a piece of tectum or embryonic heart tissue from the same animals from which the cortical slices were derived. The results of these experiments show that the other tissues did not mimic the effects of the thalamus Figure 19 shows the relative position of cells labelled with BrdU on E16 *in vitro*, in cortical slices cultured (a) with thalamus, (b) alone (c) with cortical tissue (d) as (a) for comparison with (e) with tectal tissue and (f) heart tissue. These results are summarised in Table 5. Table 5 shows the proportion of cells in the pial half of the slice (bins 6-10) and in the upper part of the cortical slice (bins 8-10). These results show that the only condition in which a substantial proportion of cells reach the upper part of the cortex, is in cortical slices cultured with thalamus The proportion of cells in bins 6-10 and 8-10 in cortical slices cultured with thalamus was significantly greater than that in cortical slices cultured alone (Student's t-test $p < 0.05$), with cortical tissue ($p < 0.05$) with heart tissue ($p < 0.05$), with tectal tissue ($p < 0.05$).

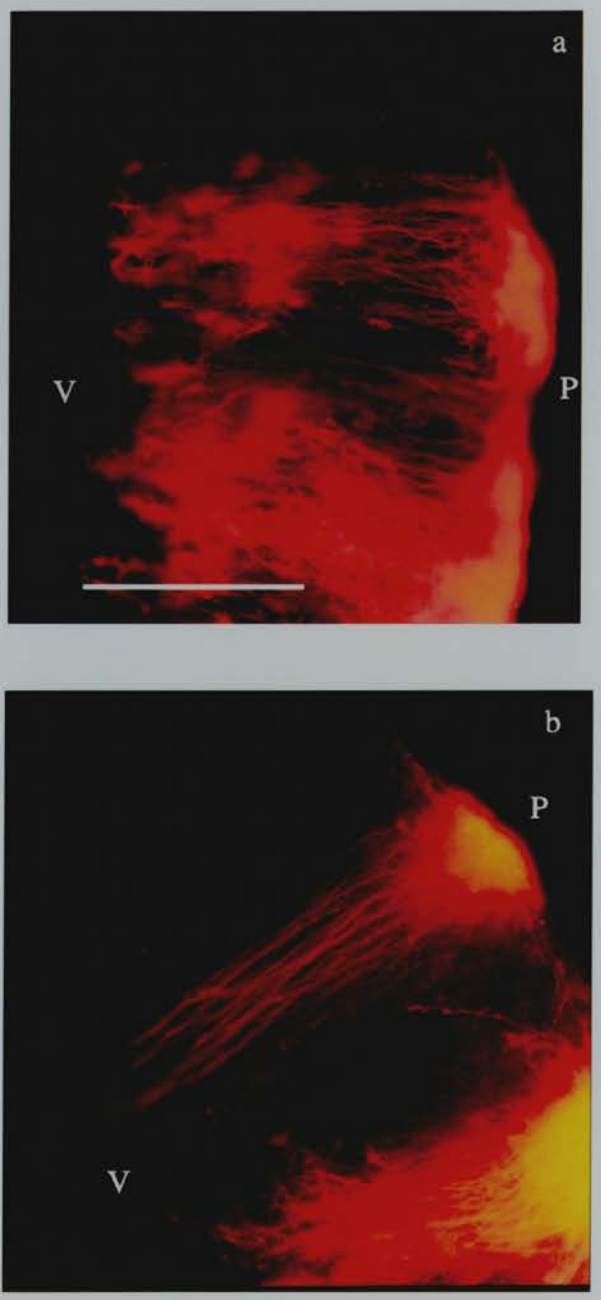


Figure 16 shows DiI labelled processes in (a) a cortical slice cultured alone for two days and (b) in a cortical slice cultured with thalamus for two days. Many of these processes end in cell bodies in the area corresponding to the proliferative zone. P: pial edge; V: ventricular edge. Scale bar 100 μm.

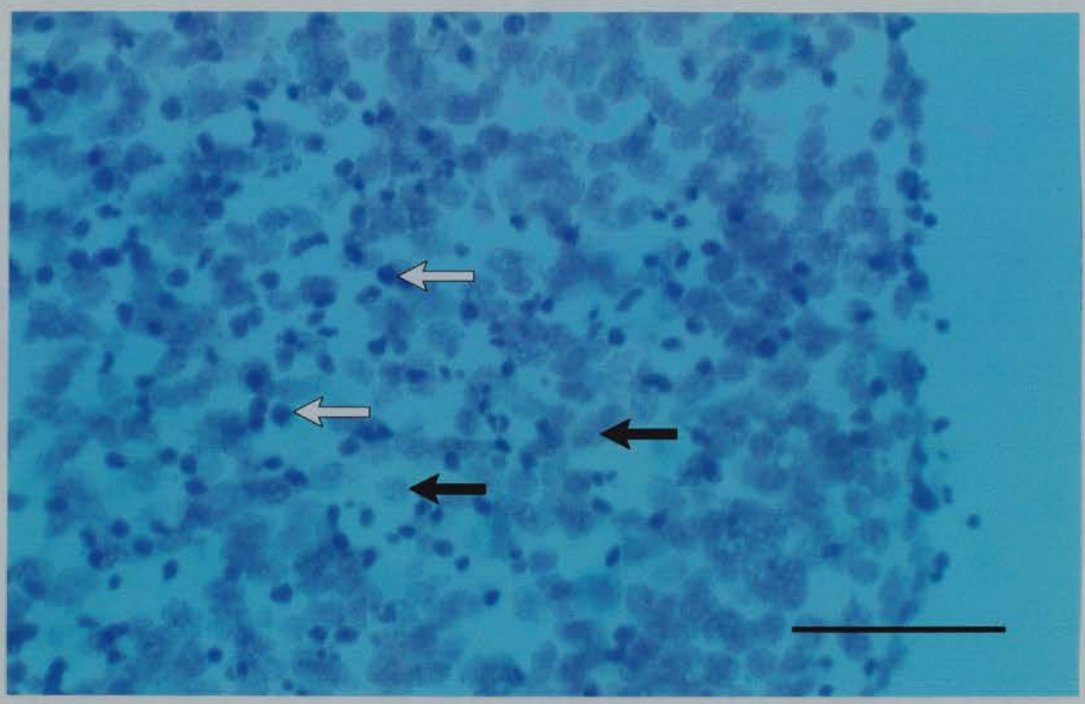


Figure 17 shows the pial portion of a cortical slice cultured alone for five days. Pyknotic cells are indicated by the white arrows and healthy cells are indicated by the black arrows. Scale bar 50 μm.

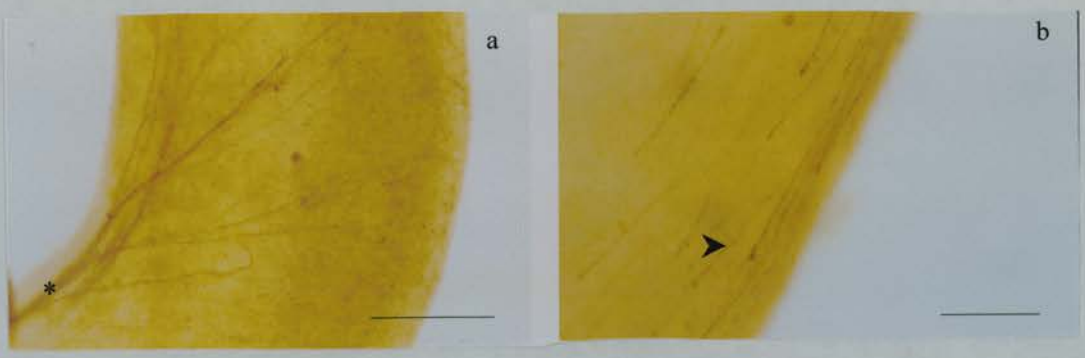
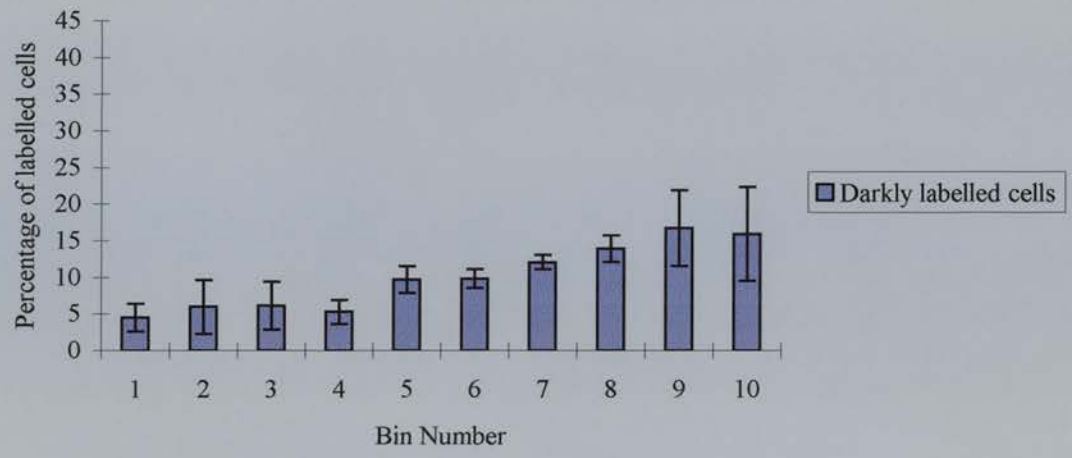


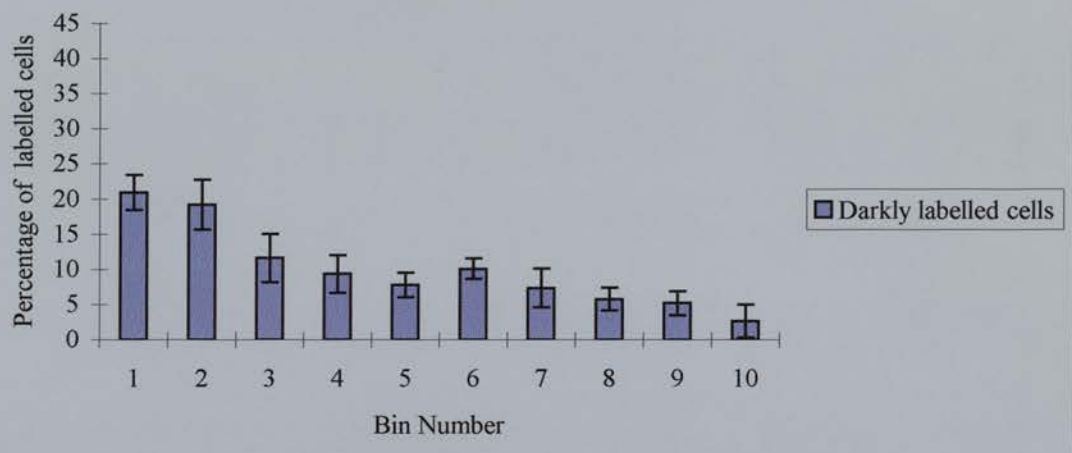
Figure 18 shows a five day corticothalamic co-culture stained with an antibody to calretinin. (a) Axons from the thalamus are seen entering the cortex (asterisk). (b) The axons, which course around the top of the cortical slice, have growth cones on their ends (arrowhead). Scale bars a: 500 μm; b: 100 μm

Figure 19 shows the relative distribution of cells in cortical slices cultured (a) with thalamus (b) alone (c) with cortical tissue (d) with thalamus (for comparison with e and f) (f) with tectum and (g) with heart, for 2DIV.

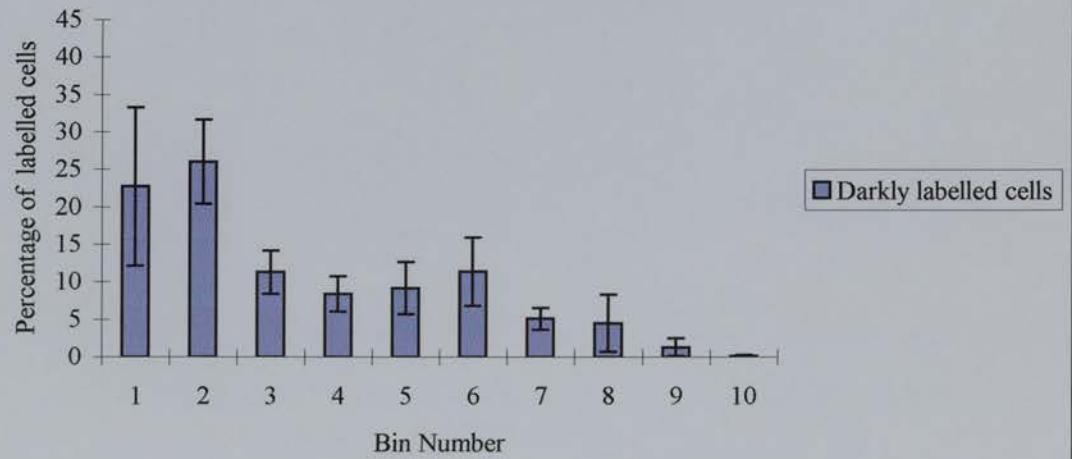
a


Relative distribution of cells labelled with BrdU on E16 in cortical slices cultured with thalamus after 2DIV
Percentage of labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
Darkly labelled cells

b

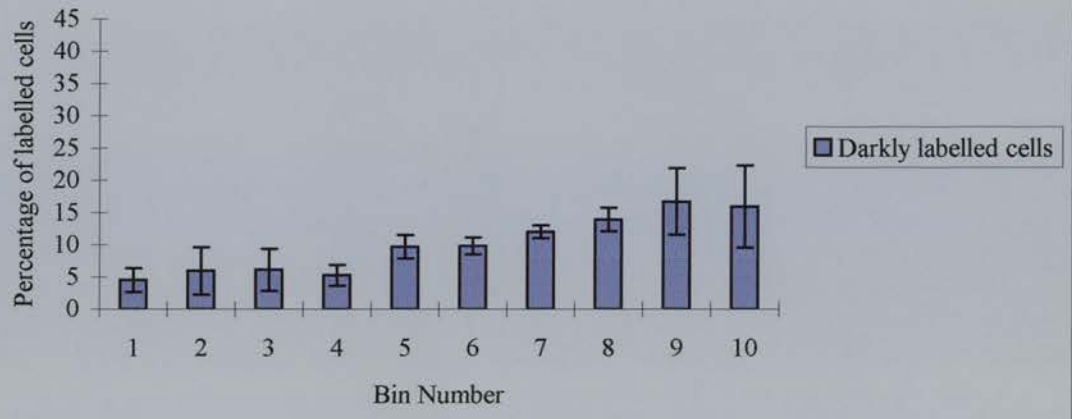

Relative distribution of cells labelled with BrdU on E16 in cortical slices cultured alone after 2 DIV
Percentage of labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
Darkly labelled cells

c


Relative distribution of all cells labelled with BrdU on E16 in cortical slices cultured with cortical tissue after 2DIV
Percentage of labelled cells
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Bin Number
Darkly labelled cells

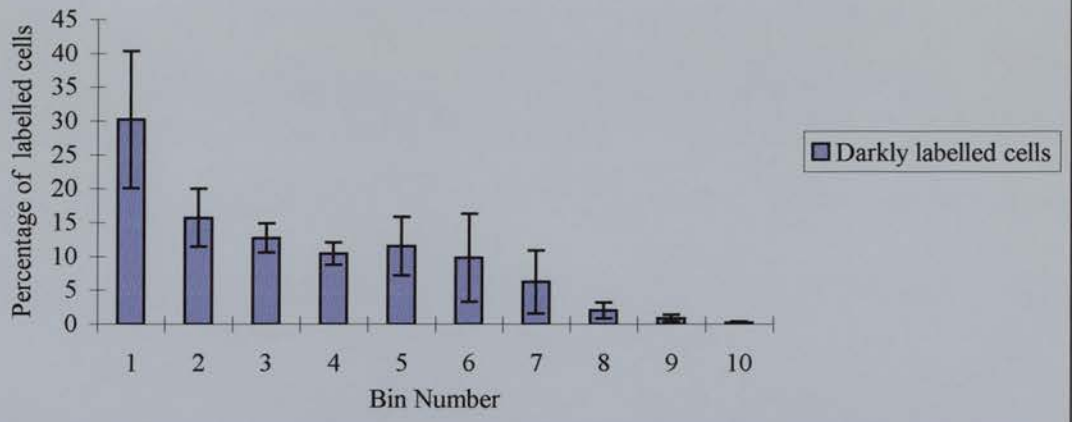
d

Relative distribution of cells labelled with BrdU on E16 in cortical slices cultured with thalamus after 2DIV



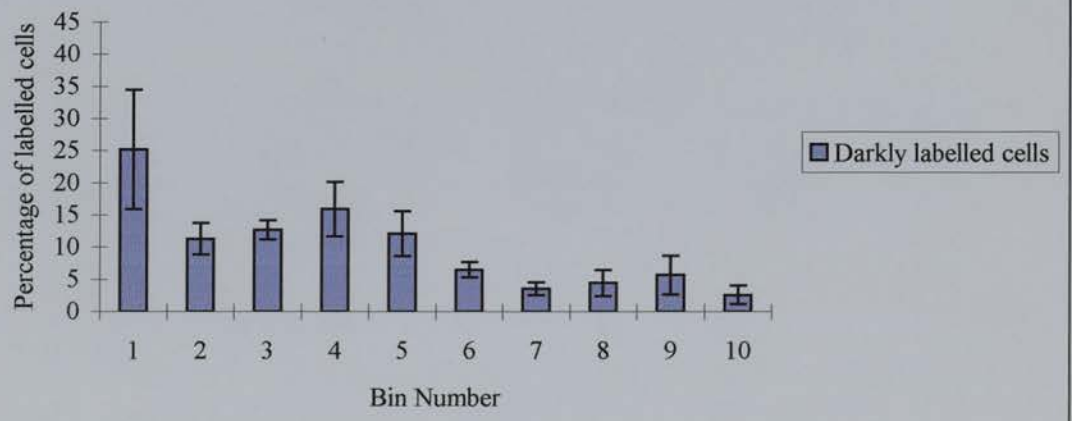
e

Relative distribution of all cells labelled with BrdU on E16 in cortical slices cultured with tectal tissue after 2DIV



f

Relative distribution of all cells labelled with BrdU on E16 in colrtical slices cultred with heart tissue after 2DIV



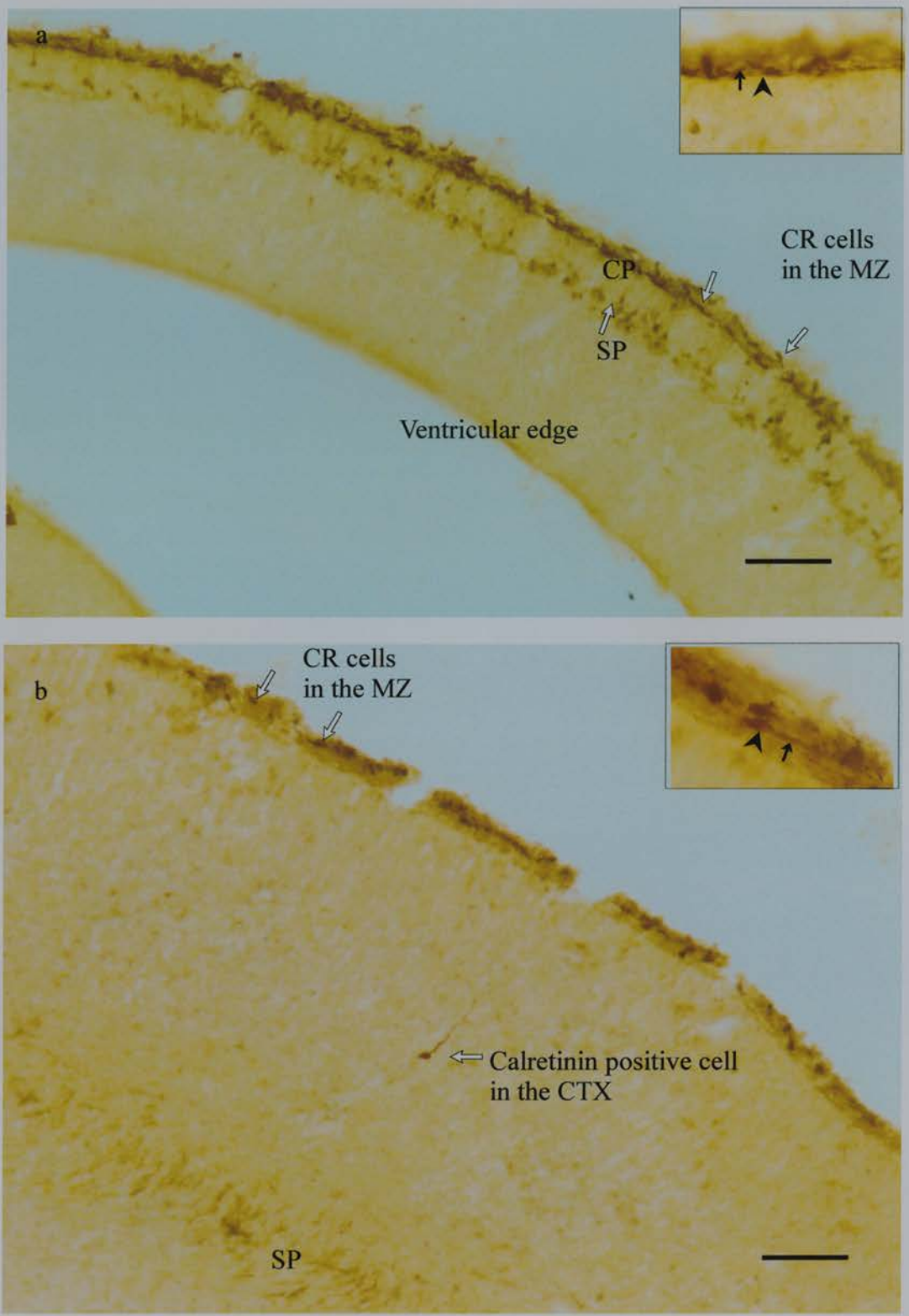


Figure 1 shows calretinin positive cells and fibres in the neocortex (a) at E16 and (b) at P1, *in vivo*. The insets show higher magnification views of CR cells in the marginal zone. The arrow heads indicate CR cell bodies and the arrows indicate the horizontal dendritic processes. CR: Cajal-Retzius cell; CP: cortical plate; SP: subplate; MZ: marginal zone; CTX: cortex. Scale bars: 100 μm

TABLE 5

	Proportion of cells in bins 6-10	Proportion of cells in bins 8-10	No.cultures (animals)
Cortex alone	27.24% (s.e.m 4.60)	13.63% (s.e.m 3.96)	4(3)
Cortex + cortex	22.46% (s.e.m. 9.35)	5.99%(s.e.m 4.95)	3(2)
Cortex + tectum	19.25% (s.e.m. 12.95)	3.19%(s.e.m 1.83)	3(2)
Cortex + heart	22.73% (s.e.m. 7.35)	12.72%(s.e.m 6.38)	4(2)
Cortex + thalamus	68.46% (s.em. 11.16)	46.60% (s.e.m 6.38)	5(3)

Table 5 shows the proportion of cells in the pial half of the cortical slice (bins 6-10) and in the upper cortical wall (bins 8-10) after 2DIV in cortical slices cultured alone and with other embryonic tissues.

DISCUSSION

This analysis was designed to investigate the source of cortical laminar fate cues. However, before the implications of the results with respect to this question are addressed, the appropriateness of the *in vitro* system as a model for studying migration and lamination is discussed. If the system was to provide a suitable assay then some requirements had to be met. Firstly cortical precursors must divide *in vitro*. Secondly, the cortical slices must provide an environment in which cell migration can occur. This discussion deals firstly with these issues then describes any conclusion that can be drawn about laminar fate cues.

Cell division *in vitro*

Cell division during embryogenesis is a necessary requirement for the formation of the central nervous system. Ross (1996) has suggested that the process of cell division is intrinsically driven but responsive to the local environment. Previous studies have shown that mitotic activity continues for at least fourteen days in serum enriched cortical slice cultures (e.g. Gotz and Bolz, 1992), for up to five days in clusters of dissociated cells and not at all, or for only one division, in single dissociated cells grown in defined serum free culture medium (Ghosh and Greenberg, 1995). In this study I showed, using BrdU labelling, that cell cycle activity continues *in vitro*, at least for a short period at the start of the culture. In preliminary experiments during the development of the culture protocol used here, it was found that cells still incorporated BrdU after 24 hours in culture. The ability of cells to divide *in vitro* was essential to this study because the assay was based on cells receiving their laminar fate cues, which they do during the final stages of the cell cycle (McConell and Kaznowski, 1991), from within the defined culture environment.

The numbers and pattern of distribution of BrdU labelled cells in the cultured slices were similar to that *in vivo*. The majority of labelled cells were found at a short distance from the ventricular edge which probably coincides with the ventricular S-phase zone where cells replicate their DNA (and therefore incorporate

BrdU). Therefore the results of this study suggest that cell proliferation occurs normally, at least at the start of the culture period.

This is perhaps not too surprising given that cells were labelled only one hour after the slices were put into culture and the requirements for cell to cell interactions (Ghosh and Greenberg, 1995) were met. Labelling cells so soon after the start of the culture meant that these cells had undergone most of the early part of the cell cycle (G1-phase; during which factors that regulate cell cycle dynamics are thought to act) in a normal environment. It was decided to label cells with BrdU just one hour after isolation cortical slices to allow the possible effects of events occurring early in the cell cycle to be ruled out. Others have shown the S-phase of the cell cycle is prolonged in cortical slice cultures (Bohnar *et al.*, 1997), but, as these authors showed, it did not affect the ability of cells to respond to laminar fate cues.

Labelling cells so soon after putting them into culture could mean that laminar fate cues that arise at a distance from the cortex, but that were present in the ventricular zone *in vivo*, might still be present in the slice at the start of the culture and be capable of acting on these labelled cells. For this reason the slices were thoroughly washed in a large volume of EBSS after dissection. Not only does this 'wash out' cytotoxic enzymes but it will also wash away possible diffusible laminar fate cues. If the fate cues are cell attached molecules this is unlikely to be effective. However, it is unlikely that fate cues that were present *in vivo* are still present *in vitro* because, since precursor cells divide asynchronously, there must be a relatively tight temporal window in which fate cues act otherwise cells belonging to consecutive cycles would become specified to the same fate. However, we cannot rule out this possibility that laminar fate cues that were present in the intact animal are still present in the ventricular zone at the start of the culture. Had cells behaved identically in cortical slices cultured with and without thalamus, this would have had to be investigated. However, they did not, suggesting that there is a difference between the two culture systems.

Cortical slices provide an environment in which migration can occur

Cortical slice cultures provide an environment in which cell migration can occur. This has been shown in experiments in which BrdU was injected *in vivo* in the mouse (Gillies and Price 1993; McLauchlin, 1995) and in the ferret (Roberts *et al.*, 1993).

Cortical slices provide a suitable environment in which to study laminar fate cues

The fact that cortical precursors undergo cell division *in vitro* and cortical slices support cell migration shows that this is a useful system in which to study laminar fate cues. The ability of cells to respond to laminar fate cues should not be detrimentally affected by culturing cortical slices Bohner *et al.* (1997) showed that putting cortical slices into culture did not prevent the cortical precursors from migrating to a particular laminar position after they were subsequently transplanted into an *in vivo* environment.

The effect of adding K+ to the culture medium

It has been shown that late embryonic thalamic tissue does not survive well in defined serum free culture medium (Magowan and Price 1996). Therefore, to optimise survival of the E16 thalamic explants used in this study, 5mM K^+ was added to the culture medium, making its final concentration 9mM. This concentration of K^+ was shown to be optimal for enhancing cell survival in cultured thalamic explants (Magowan and Price 1996).

The membrane potential is a property of all living cells. It is determined by the relative magnitudes of the membrane permeability to K^+ and, to a much smaller extent, Na^+ and Cl^-. The resting potential is the stable state when there is no electrical stimulation, that is, when there are no changes in membrane permeability. The slightest change in the permeability can alter the membrane potential, causing electrical events such as action potentials. A cell's normal resting potential falls within the range of –60 to –90 mV. In the developing CNS it tends to be more

depolarised (McLeod *et al.,* 1996). LoTurco *et al.* (1991) showed that in embryonic rat brain, cells resembling migrating neurones had a resting membrane potential of between –60 and –45 mV and cells in the cortical plate had a resting potential of between –60 and –35mV. The membrane potential is due largely to the high diffusion rate of K^+ compared to other ions. K^+ tends diffuses out of the cell because of its high concentration gradient, leaving an excess of negative ions inside. This results in a driving force of negative ions out of the cell, and positive ions into the cell. However the cell's membrane is rather impermeable to ions other than K^+. Thus, at rest, K^+ has a chemical force pushing it out and an electrical force pulling it back. The amount of the electrical potential needed to counterbalance the concentration gradient at rest is determined by the Nernst equation: $E_{K+} = 58 \log_{10} [K^+]o/[K^+]i$, where E_{K+} is the equilibrium potential for K^+, $[K^+]o$ is the concentration of potassium outside the cell and $[K^+]i$ is the concentration of K^+ inside the cell. The idea that the resting membrane potential is the result of an unequal distribution of potassium ions between the extracellular and intracellular fluids was first proposed at the beginning of the 20th century (Bernstein, 1902, cited in Nicholls *et al.*, 1992). However, at that time it was not possible to test this directly. It was not until the use of the squid giant nerve fibre was introduced (Young, 1936, cited in Nicholls *et al.*, 1992) that direct measurements of membrane potentials could be made. Modern electrophysiological methods have shown that K^+ is not the only ion to contribute to the resting potential (Hodgkin and Keynes, 1966; Goldman, 1943; Hodgkin and Katz, 1949), however the effect of the other ions (Na^+ anc Cl^-) is small.

The Nernst equation was used to calculate the approximate effect of increasing $[K^+]$ from 4mM (in the normal culture medium) to 9mM. This shows that: if $E[K^+] = 58 \log_{10} [K^+]o/[K^+]$, then if $[K^+]o$ is increased from 4mM to 9mM and if $[K^+]i$ is constant then:

$E[K^+2] - E[K^+1] = 58 (\log_{10} 9mM/4mM + [K^+]o/c) - 58 (\log_{10} [K^+]o/c)$

$= 58 \log_{10} 2.25 = 20mV.$

If the permeability to other ions is considered, the effect of changing $[K^+]o$ is less, especially at $[K^+]o$ concentrations of less that 40mM (Nicholls *et al.*, 1992). Therefore the calculated value of 20 mV is the maximum amount by which the membrane will depolarise. However it is important to realise that these calculations only hold true if the developing cell behaves exactly like the 'model' adult cell. Assuming that in the developing mouse brain the resting potential of its neurones is similar to those in the rat, this level of depolarisation will bring the membrane potential to a maximum of -25 mV for migrating neurones and –15 mV for cortical plate neurones. It is difficult to determine what effect this will have on a developing cell. It is highly likely that it will affect ion channels to some extent, perhaps inactivating some Na+ channels and activating some types of Ca^{2+} and K^+ channels.

Migration of *in vitro* born cortical precursors

The results of this study showed that there was a difference in the migratory behaviour of cells born in isolated cortical slices compared to those in cortical slices cultured with thalamus. In isolated cortical slices, BrdU +ve cells were rarely seen at the outer part of the cortical wall, whereas in cortical slices cultured with thalamus, the majority of BrdU +ve cells had attained this position after 2 DIV. Neither result reflects the *in vivo* situation in which E16 born cells reach the upper cortical wall sometime between two and five days post injection. In the cortical slices cultured with thalamus, the location of cells at the pial edge after two days suggests that these cells reach their final laminar position sooner than *in vivo*. It has been suggested that the apparent increased rate of migration in slice cultures may be accounted for by the reduced expansion of the cerebral intermediate zone *in vitro* (Roberts *et al.*, 1993). Although thalamic tissue sends axons into the cortex in the co-culture system used in this study, these coursed around the outside of the cortical slice. Therefore it is unlikely that they contribute much to its radial expansion. Indeed, comparison of the width of the co-cultured slice and the cortical wall *in vivo* show that the cortical slice is less than 75% of the width of the cortical wall. Assuming cells begin migration from the ventricular edge *in vitro* as they do *in vivo*, as has been demonstrated previously (Chenn and McConnell, 1995; Bohner *et al.*, 1997) then the majority of

cultured cells, which were found in bins 8, 9 and 10 at plus two days, have attained a position of between 309 and 387 μm from the ventricular edge Thus, assuming these cells take forty eight hours to reach this position, the rate of their migration is, on average, 7.25 μm/hour. This compares to a rate of 11 μm/hour in the ferret brain *in vivo* (Roberts *et al.*, 1993). Therefore it is not unreasonable that these cells reach their normal position at the cortical plate in only two days. Cells migrating *in vivo*, in which the majority had reached bins 5-8 had attained a position of between 268 and 428 μm. from the ventricular edge. The average rate of migration, based on this distance is also 7.25 μm/hour. However, this is only an approximate measurement of the rate of migration *in vivo* because this distance reflects the thickening of the wall due to afferent ingrowth and it is not known to what extent these fibres contribute to the distance that the cells must migrate because many of them will grow in after the cells have passed the point of their ingrowth.

The fact that BrdU +ve cells were not seen at the pial edge in slices cultured alone for two days, could be due to the fact that these cells migrate more slowly than their counterparts in co-cultures. However, isolated cortical slices were on average about 2/3 the width of the co-cultured slices after 2DIV, so even if migration was slower, the reduced cortical width would compensate to some extent. The fact that the five day distribution resembled the two day *in vivo* distribution could be due to the fact that these cells get 'stuck' in the late stages of the cell cycle and only begin migration after several days. This is highly unlikely however, because previous studies show that cells continue cycling *in vitro* as long as cell-cell contact is maintained (Ghosh and Greenberg, 1995), and the cell cycle length is not increased massively. If cells migrated faster and reached the pial edge sooner in isolated slices, then the two day data could be due to subsequent slippage. However, a few cortical slices were examined after 1 and 3 DIV and the results were the same as at two days. Therefore it seems most likely that these cells simply failed to migrate substantially. This has been shown previously (Gotz and Bolz, 1992) for cells that were born after several days *in vitro.*

After 5 DIV many of the BrdU +ve cells in co-cultured slices became displaced downwards towards the ventricular half of the slice. This will be discussed later in this discussion and more fully in Chapter 5. In isolated slices there was little change from the two day result.

Is the role of the thalamus permissive or instructive

The results described above suggest that the thalamus positively affects the ability of cells to migrate to their normal position at the upper part of the cortical slice. In order to draw conclusion about the role of the thalamus in laminar fate mechanisms, it was important to know if the effect of the thalamus was simply to make the environment more permissive to migration. Since earlier born cells might provide directional cues for *in vitro* born cells, the distribution of cells born on E12 and E14 *in vivo* were studied in cultures and *in vivo*. The results showed little effect of the thalamus on these cells. E12 born cells failed to maintain a normal bimodal distribution in either culture system. Interestingly, there was some evidence for the loss of marginal zone cells at the start of the culture, as evidenced by the smaller number of cell in the marginal zone in acute slices compared to *in vivo* controls. This will be discussed at more length in the next chapter. Gotz and Bolz (1992) claimed that cortical layers that have already been established *in vivo* in the rat brain, remain preserved for several weeks in isolated cortical slice cultures. However, close inspection of their results show that although this is generally the case, E13 born cells (which form the subplate and marginal zone in the rat cortex) also failed to maintain a bimodal distribution. In this study, the only obvious effect of the thalamus on earlier born cells was to help E14 born cells to retain their laminar position for longer than they did in the isolated cortical slice.

Inspection of the radial glia showed that here was no obvious difference in the radial glia in isolated slices from those in co-cultured slices. Neither was there any significant difference in cell survival between the two systems, though it has been shown previously that serotonin (which is secreted by thalamocortical axons)

promotes the survival of glutamatergic neurons *in vitro* (Dooley *et al*, 1997). The effect of the thalamus on cortical cell survival might well be masked by the elevated potassium used in this study, or the level of serotonin in the thalamic explant might be too low to elicit a cell survival effect, especially in these short term cultures. Taken together these results suggest that the thalamus does not dramatically affect the structure of the cortical slice. Therefore this raises the possibility that the role of the thalamus is an instructive one. However the possibility that its role is permissive cannot be discounted.

Thalamic axons grew into the cortical slice

The results of the calretinin staining and DiI labelling revealed that thalamic axons entered the cortex and coursed around the top of the cortical slice. These axons extended growth cones therefore it is unlikely that they formed synapses with cortical cells. This raises the possibility that the thalamus releases some factor from the axonal growth cones that instructs cortical precursors to migrate, or is permissive to their migration. Non-synaptic neurotransmitter release has been reported previously (Ugrumov *et al.*, 1989; Hume *et al.*, 1983; Lockerbie *et al.*, 1985) and a role for neurotransmitters in brain development has been postulated previously based on evidence, for example, from studies on the formation of barrel fields in MAOA knockout mice (Vitalis *et al.*, 1998).

New laminae are not formed in slice cultures

After 5DIV, in cortical slices cultured with thalamus, BrdU +ve cells that had attained a position at the upper edge of the cortex were displaced downwards. This could have been due to their displacement by later born cells. However, inspection of E12 and E14 born cells showed that this was unlikely. After 5 DIV, the majority of E12 and E14 born cells remained at the outer edge of the cortex. If migration and lamination had continued normally, the E16 *in vitro* born cells would have displaced the E14 born cells and separated the E12 born cells. Instead, it appears that the E12, E14 and E16 populations became intermixed after 2DIV and the E16 born cells subsequently 'slipped' below the E12 and E14 born cells. Thus, no new laminae

were formed *in vitro*. This has been reported before by Roberts *et al.* (1993), who were investigating *in vivo* born cells, but their evidence came from inspection only of the migrating cells and not from their relationship to other cells in the cortical slice. This 'slippage' is dealt with in more detail in the next chapter.

In summary, the results of this study show that cells dividing in cortical slices cultured with thalamus can migrate to the pial edge of the slice culture, but fail to form new laminae. Cells dividing in isolated cultures failed to migrate substantially and did not reach their normal position at the upper cortical edge during the 5 DIV. These experiments were repeated by replacing the thalamic tissue with other embryonic tissues. These experiments suggest that the effect of the thalamus was specific, because a much larger proportion of cells moved into the upper part of the cortical wall, in cortical slices cultured with thalamus than in slices cultured alone or with other embryonic tissue.

What are the implications for the role of the thalamus in laminar fate mechanisms?

The results of this study suggest that the thalamus is important for the migration of *in vitro* born cells to the upper cortical wall. Since cells that are born *in vivo* and allowed to develop for some time between 24 hours and a few days *in vivo* will migrate normally in the absence of the thalamus (Gotz and Bolz, 1992, Gillies and Price, 1993; Roberts *et al.*, 1993) it seems most likely that the thalamus exerts its effect sometime early in the process. This could mean that the thalamus provides laminar fate cues, which act on precursors while they are still in the ventricular zone. Ghosh and Shatz, (1993) showed that preventing thalamocortical axons from invading the cortex leads to a loss of layer 4 stellate neurones in the cat. Windrem and Finlay (1991) showed that ablating the lateral geniculate nucleus leads to loss of layer IV neurones. Although these studies only show that the thalamocortical input is important for stabilisation of neocortical architecture and cell number, the possibility exists that layer IV cells were depleted because the some factor from the thalamus specifies layer IV precursors and is also required for their maintenance.

Bohner *et al.* (1997) showed that laminar fate cues for deep layer precursors were present in acutely dissected intact cortical slices, so that when cells that underwent the final stages of the cell cycle in these slices were transplanted into a novel environment they retained their ability to form deep layer neurons. The result obtained in this study suggests the possibility that, for middle layer neurones, laminar fate cues are not present in the intact cortical slice. Of course, the possibility that the cells are unable to respond to the cues cannot be ruled out. However, McConnell and Kaznowki (1991) showed that cortical precursors that had been dissociated and transplanted retained their ability to respond to laminar fate cues, therefore this seems unlikely. Another feasible explanation is that something else is absent from the isolated cultures that is required for precursor cells to migrate. A recent study has shown that in the p35 knockout mouse, cells of the middle and superficial layers fail to migrate through the earlier born cells. It was suggested that they needed activation of the cdk5 receptor to be able to attach to radial glial cells or navigate their way through the earlier born cells. It may even be the case that in the co-cultures, fate cues are absent, but cells migrate by default to the top of the cortical slice.

Since the thalamus sends axons into the cortical slice it is possible that migration is assisted by the presence of ingrowing thalamic axons. However these axons were sparse and tended to enter the slice at a particular point, therefore this seems unlikely. To test if the thalamus releases a soluble factor that promotes migration cortical slices could be cultured in thalamic conditioned culture medium. This was attempted but preliminary experiments were unsuccessful, therefore they remain to be completed. The thalamus produces a large number of neurotransmitters and trophic factors which might potentially play a role in migration. Komuro and Rakic (1993) showed that activity of the N-methyl-D-aspartate (NMDA) receptors regulated migration of granule cells in slice preparation of the developing mouse cerebellum, though in NMDA knockout mice, cortical migration is unaffectd (C. Shatz, personal communication). Behar *et al.* 1998 have shown that GABA can induce motility in dissociated ventricular zone and cortical plate cells from the

embryonic rate brain. It is possible therefore that the developing thalamus acts as a source of glutamate, GABA or some other neurotransmitter that allows migration to occur in the cortical slices cultured in the presence of the thalamus and is absent or present at low concentrations in isolated cortical slices.

In summary, this study suggests that the thalamus plays an important role in the migration (out of the ventricular zone) of cells ultimately destined for the middle and upper cortical layers; layers that receive direct thalamic innervation *in vivo*. The study does not reveal the role of the thalamus in this process, but suggests that it is not mediated through the maintenance (mechanical or trophic) of earlier born neurones and glia. Some suggestions for further experiments to elucidate the role of the thalamus in this process are made in the final discussion to this thesis.

CHAPTER 5: THE ROLE OF CAJAL-RETZIUS CELLS IN NORMAL MIGRATION AND LAMINATION

INTRODUCTION

The results of the study in Chapter 4 showed that cells that were born *in vitro* in co-cultures migrated to their normal position at the top of the cortical wall. However, these cells were unable to maintain their position there, so no new cortical layers were formed. Since Cajal-Retzius cells are known to play an important role in the formation of cortical layers *in vivo* (reviewed in Marin-Padilla, 1998) I decided to investigate the nature of these cells in the *in vitro* system described in Chapter 4.

The 'slippage' effect is not specific to *in vitro* born cells. The results from Chapter 4 of this thesis also provide indirect evidence for slippage of *in vivo* born cells that complete their migration in culture. This evidence comes from the fact that E14 born cells (that had completed their migration *in vivo*) retained their position at the top of the cortical slice throughout the period of the study. If cells that had been born *in vivo* and completed their migration *in vitro* (cells born after E14 and before the slices were prepared on E16), had formed new cortical laminae they would have displaced the E14 born cells at the top of the cortical slice. Thus, the inability of cells to retain their position at the top of the slice and form new laminae is unlikely to be specific to *in vitro* born cells. This suggests that whatever prevents the formation of new laminae, it is probably not related to events occurring early, that is, during cell proliferation or in the early stages of migration. The most feasible explanation is that something happens after the cells have been specified to a laminar fate and migrated to the top of the cortical plate, to prevent them from staying there.

Marin-Padilla (1984) proposed that the early formation and location of the preplate suggests a role for the preplate cells in the organised formation of the cortical layers. Indeed, the temporal expression of CR cells in the mouse coincides with the period of neuronal migration and lamination. Recently, evidence has come to light to support Marin-Padilla's suggestion. D'Arcangelo *et al* (1995) and Ogawa

et al (1995) showed the CR cells secrete reelin – a glycoprotein with characteristics of extracellular matrix molecules - that is absent in *reeler* mice in which there is an approximate inversion of the cortical layers. Further, Soriano *et al*, (1997) showed that CR cells play a key role in the regulation of the radial glial phenotype and suggested that they may exert a chemoattractvie influence on migrating neurones.

The fate of CR cells after neuronal migration is complete is controversial. Parnavelas and Edmunds (1983) provide evidence for the transformation of CR cells into nonpyramidal neurones, whereas Del-Rio *et al*, (1995) propose that the presence of degenerating calretinin and pyknotic BrdU immunoreactive cells in the mouse cortex suggests that CR cells disappear by cell death. Whatever the nature of the loss, which is mimicked *in vitro*, it can be prevented by the addition of TTX to early postnatal cortical slices (Del Rio *et al*, 1996). These authors take this result to suggest that CR cell death, after corticogenesis is complete, is triggered by neuronal activity.

However, although Del Rio *et al* (1996) suggested that the loss of calretinin positive CR cells from the marginal zone *in vitro* mimics in the *in vivo* loss, they did not show if the loss of cells *in vitro* occurred only in cortical slices obtained at early postnatal stages. Thus they did not rule out the possibility that it was an artefacutal effect due to placing the cells into an environment in which their normal afferent connections are absent. In addition, since they only counted the numbers of cells in the marginal zone, they did not specifically show if the effect of the TTX was simply to enable the CR cells to retain their normal laminar position. The results of the culture experiments reported in this thesis suggest that cells belonging to the preplate become disorganized, even after short times in culture, and become dispersed throughout the slice (Chapter 3). It seemed possible therefore that this might be induced by some sort of desynchronised activity resulting for the fact the extrinsic afferents were severed. The addition of TTX may have simply prevented such disorganisation.

Although it was shown in Chapter 3 that there was no obvious loss of E12 generated cells (subplate and marginal zone cells) between the point of culture and 5 DIV, the inability to differentiate between the subplate and marginal zone population made it difficult to assess the fates of the marginal zone cells. Thus although there was no obvious marginal zone present in the cultured slices (Figs. 3b, c, d, and e, Chapter 3), the presence of cells labelled with BrdU on E12, at the top of the cortical wall, which could have belonged to either subplate or marginal zone populations, made it difficult to assess the fate of the marginal zone cells.

To test directly if the loss of Cajal-Retzius cells occurs in cortical slices derived from E16 brains, cortical slices were cultured for five days with thalamic tissue, and an antibody to the calcium binding protein calretinin was used to stain CR cells. To test if CR cells could be kept alive and lamination improved by blocking Na+ dependent activity with TTX, the number of CR cells was counted and the position of cells born *in vitro* on E16 was analysed (as in Chapter 4). To test if TTX helped CR cells maintain their normal position in the marginal zone, the distribution of CR cells was investigated.

MATERIALS AND METHODS

Corticothalamic co-cultures

Cortical slices from E16 BALB/c mice were co-cultured with thalamus and labelled with BrdU as in Chapter 4. The culture medium was replaced after 2DIV (following the protocol of Del Rio *et al.*, 1996) with culture medium alone (controls) or culture medium supplemented with 10^{-8} or 10^{-7} M TTX (Sigma).

Calretinin staining

After 5 DIV, the co-cultured slices were fixed in 4% paraformaldehyde in PBS (3 hours). After rinsing thoroughly in PBS the sections were reacted to reveal calretinin staining as described in Chapter 4 and mounted on poly-l-lysine coated slides in PBS/glycerol (1 in 3). Whole brains (3 at each age) were obtained as described in Chapter 2 at E16 and P1 and fixed in 4% paraformaldehyde in PBS (3 hours or overnight at 4° C). They were then transferred to 10% sucrose in 4% paraformaldehyde (24 hours) and then stored in 30% sucrose in PBS until sectioned on the freezing microtome. They were rinsed thoroughly in PBS and reacted to reveal calretinin staining as described in Chapter 5, then mounted on poly-l-lysine coated slides in PBS/glycerol (1 in 3).

Cell counts

Cameral Lucida drawings were made through the middle of whole cortical slices at x10 magnification. The number of calretinin positive cells resembling CR neurones (following the criteria of Del Rio *et al.*, 1996) was counted in a horizontal bin (1 mm in length) through the outer 200 μm of the the cortical wall (following approximately the protocol of Del Rio *et al*, 1996). Cells situated at various depths in the slice could be visualised by focussing through the plane of the tissue.

CR cell distribution

Cameral Lucida drawings were made through the middle of whole cortical slices at x10 magnification. The number of calretinin positive cells resembling CR neurones

in a 500 μm bin through the entire width of the cortical wall was counted in ten equal sized bins as described in Chapter 3.

Cell migration

The migration of cells born *in vitro* on E16, following TTX treatment, was analysed as described in Chapter 3.

Table 1 shows the number of slices from which the calretinin and BrdU analyses were obtained.

TABLE 1

	Number of slices (cultures) in which calretinin staining was analyse	Number of slices (cultures) in which position of BrdU labelled cells was analysed
Control	12 (3)	7 (3)
10^{-8} M TTX	11(3)	7 (3)
10^{-7} M TTX	10(3)	4 (3)

Table 1 shows the number of cortical slices in which calretinin positive and BrdU positive cells were anlaysed. The numbers in brackets indicates the number of experiments that were carried out.

RESULTS

Cajal-Retzius cells *in vivo*

At E16 *in vivo*, calretinin positive cells were present in the cortical marginal zone. They were densely packed and formed a tight band along the marginal zone (Fig. 1a and 1a inset). Calretinin positive cells were also seen in the cortical plate and in the subplate (Fig. 1a). At P1 the marginal zone was still heavily populated by calretinin positive cells (Fig. 1b and 1b inset). They had a large ovoid cell body and extended a long dendrite parallel to the pial surface (Fig.1a inset). However there were no calretinin positive cells in the subplate and only an occasional calretinin positive cell in the cortical plate (Fib1 b). There were however a few calretinin positive fibres in the cortical subplate (Fig.1b)

Cajal-Retzius cells in five day corticothalamic co-cultures

Cortical slices from E16 brains were cultured in defined serum free medium for five days. After 5 DIV the cortical slices contained calretinin positive cells that were identified as CR cells (based on the criteria used by Del Rio *et al.*,1995; 1996). Only cells that lay parallel to the pial surface and had large ovoid perikarya from which a single, thick horizontal dendrite extended (Fig. 2a and b and 2a inset i) were judged to be CR cells. The slices also contained calretinin positive cells that did not resemble CR cells, either because the cell body was not ovoid in shape and/or because more than one dendrite extended from the cell body (Fig. 2a inset ii), or because they did not run parallel to the pial surface. Fig. 2b is taken at the same magnification as Fig.1 to enable a comparison between the slice culture and the cortical wall *in vivo*. The calretinin positive cells seen in this photograph were located mainly in outer cortical wall, but it is clear that they did not retain the tight band of expression that they do *in vivo* (compare Figs. 1b and 2b). The location and number of calretinin positive cells seen after 5DIV varied enormously from slice to slice, both within an experiment and across experiments. This is illustrated in Figures 3-5 which show camera Lucida drawings of calretinin positive cells with the

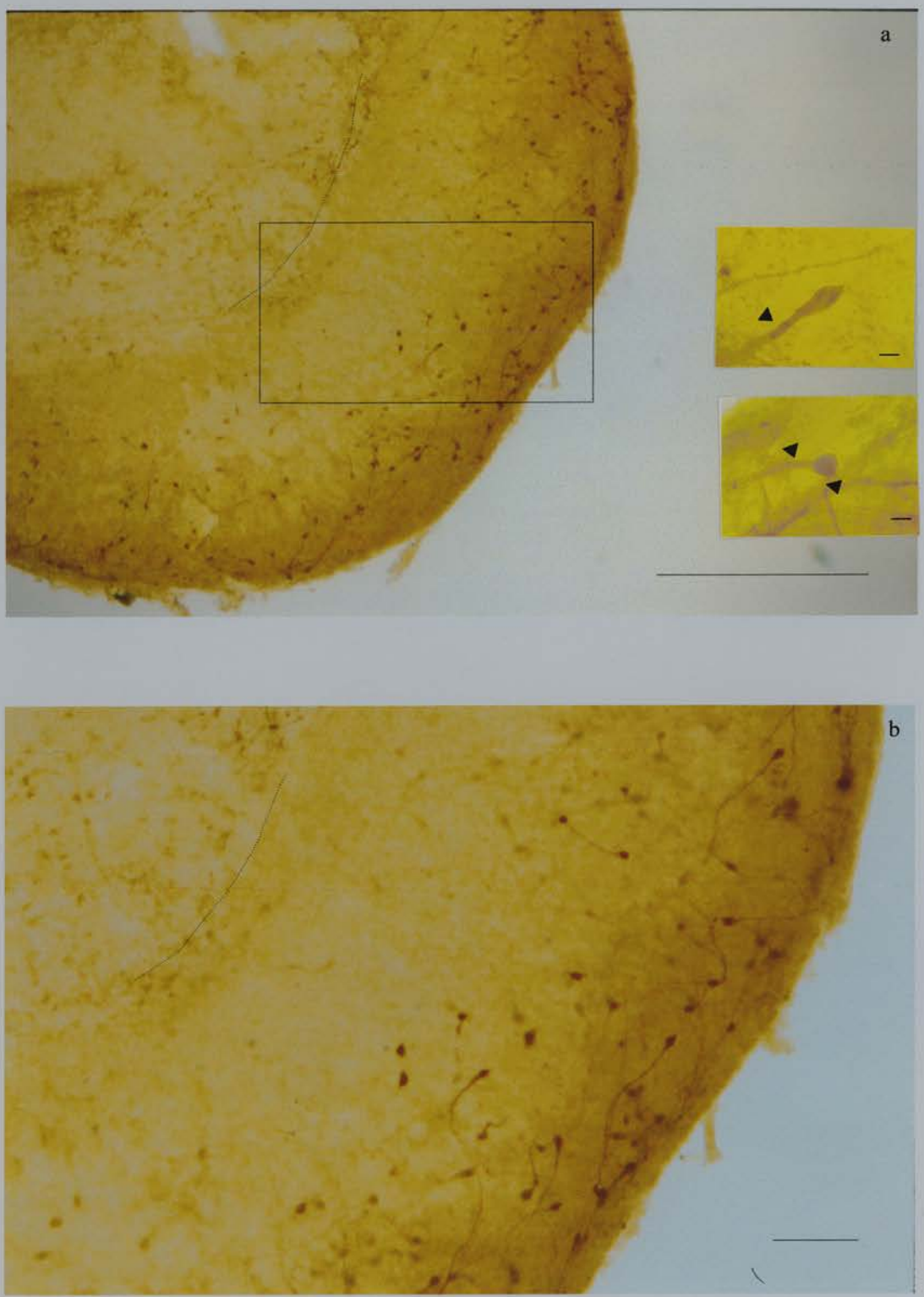


Figure 2 shows calretinin positive cells in a corticothalamic co-culture after 5 DIV. Scale bar a:500 µm; b:100 µm

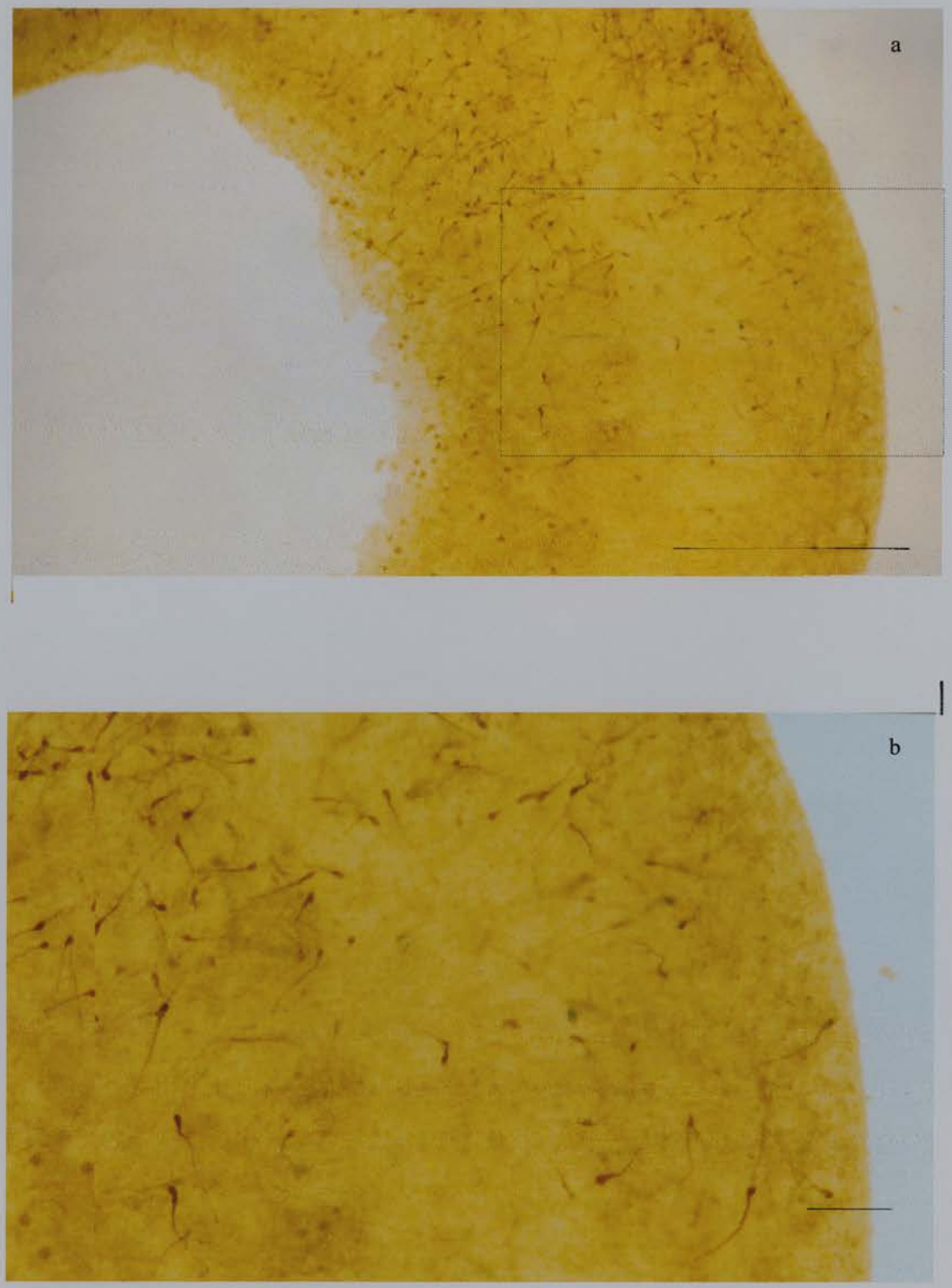


Figure 6 shows calretinin positive cells in a corticothalamic co-culture after 5 DIV and after treatment with 10^{-8} M TTX. Scale bar a: 500 µm; b: 100 µm

morphology of CR cells in three different cortical slices from the same culture well. Within a single culture, the number of CR cells located in a 1mm wide bin in the outer 200 μm of the slice varied from between 6 and 113.

To test the effect of TTX on CR cell survival and distribution, the experiment was repeated and TTX was added to the culture medium after 2 DIV, following the protocol of Del Rio *et al* (1996). Figures 6 and 7 are photographs of randomly chosen examples of cortical slices treated with 10^{-8} and 10^{-7} M TTX. Figure 6 shows a co-cultured slice (the region in which the thalamus was positioned is indicated by the dotted line marked Th) which was treated with 10^{-8} M TTX, in which calretinin positive cells are seen throughout its width. Many of these cells were not identified as CR cells, based on the criteria used in this study. Figure 7 shows a co-cultured slice which was treated with 10^{-7} M TTX, in which only a few calretinin positive cells are present. Only some of these cells were identified as CR cells. A few calretinin positive puncta are also seen in the this slice. This has been reported previously (Del Rio *et al.* 1996 and Fonseca *et al.* 1995)

The number of calretinin positive cells resembling CR neurons in TTX treated and non-treated cortical slices is illustrated in Figure. 8. The numbers of cells varied greatly in all conditions (as illustrated by the error bars which represent the standard error of the mean), from between 1 and 89, in a single experiment in which 10^{-8} M TTX was added and between and 2 and 74 in a single experiment in which 10^{-7} M TTX was added. However, the average number of cells was similar for all three conditions. To assess if TTX had an effect on cell distribution, the relative position of CR cells was investigated. Figure 9 shows the relative distribution of CR cells in TTX treated and non- treated slices. Only those slices that contained more than half the mean number of cells was included in this analysis. This was to eliminate the bias produced by converting very small numbers of cells that were found in only one area of the slice into percentages. In conclusion, the addition of TTX to the culture medium had no significant effect either on cell number or the distribution of cells within the slice.

In conjunction with these experiments the effect of TTX on lamination of *in vitro* born cells was tested. The addition of TTX at both 10^{-8} and 10^{-7} M TTX had a small effect on the proportion of cells in the upper cortical wall. However this effect was not significant. Figure 10 shows the effect of TTX on cell distribution from a single experiment, in which three slices from each condition were analysed.

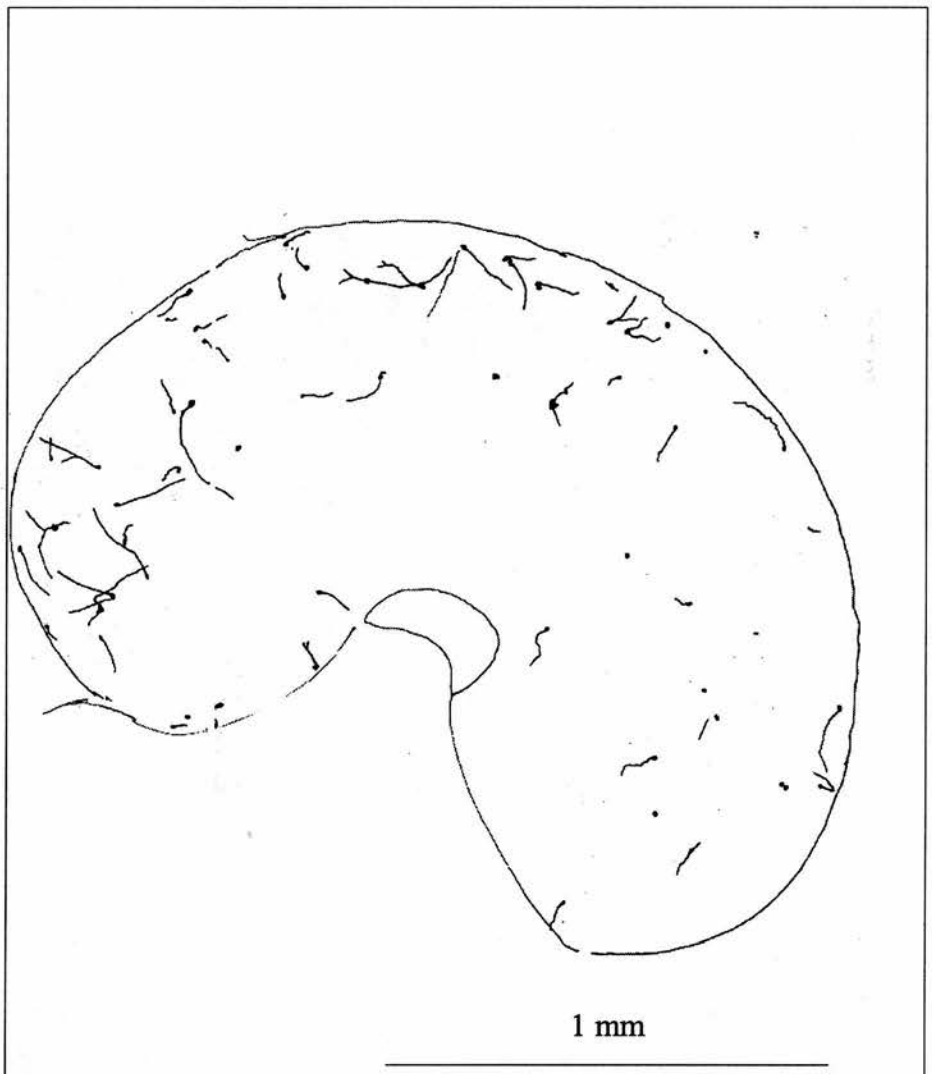


Figure 3 shows a camera lucida drawing of calretinin positive cells resembling CR cells in a cortical slice cultured with thalamus for 5 days. The thalamic explant became detached from this cortical slice

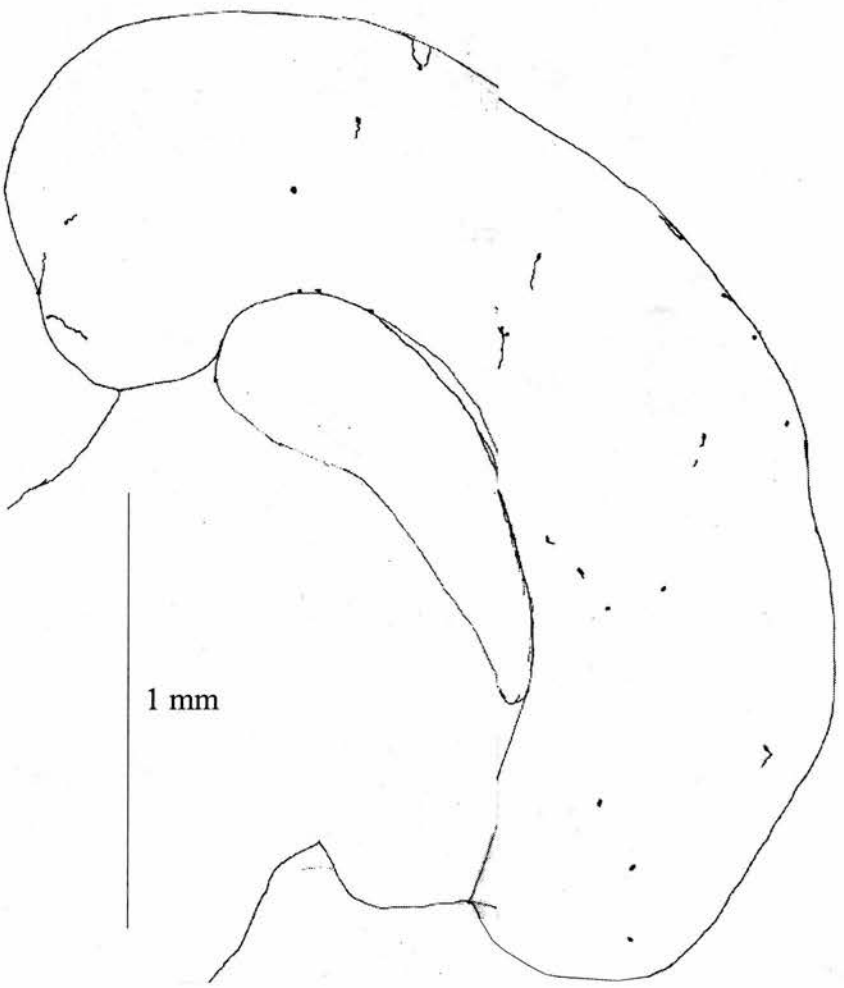


Figure 4 shows a camera lucida drawing of calretinin positive cells resembling CR cells in a cortical slice cultured with thalamus for 5 days.

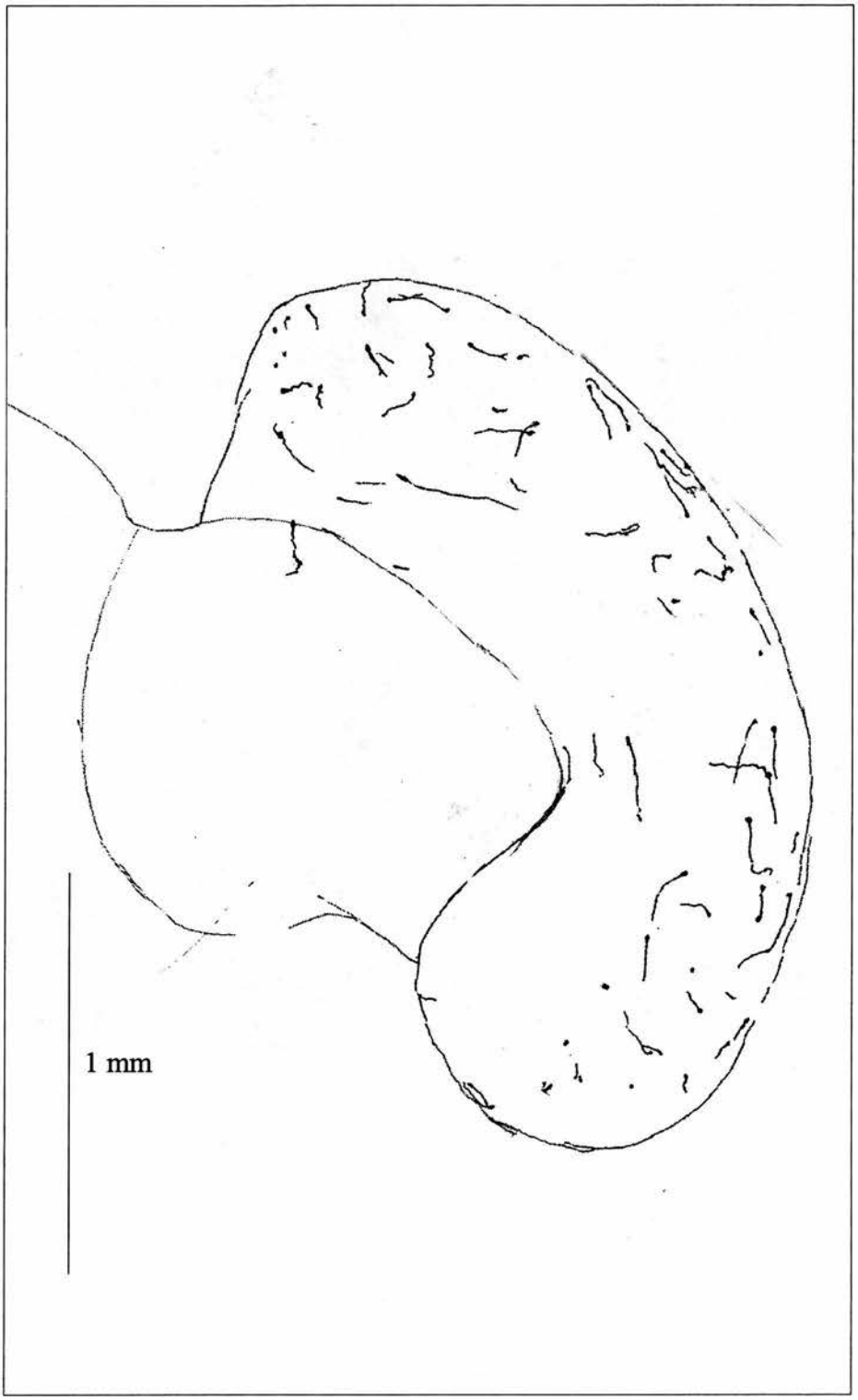


Figure 5 shows a camera lucida drawing of calretinin positive cells resembling CR cells in a cortical slice cultured with thalamus for 5 days.

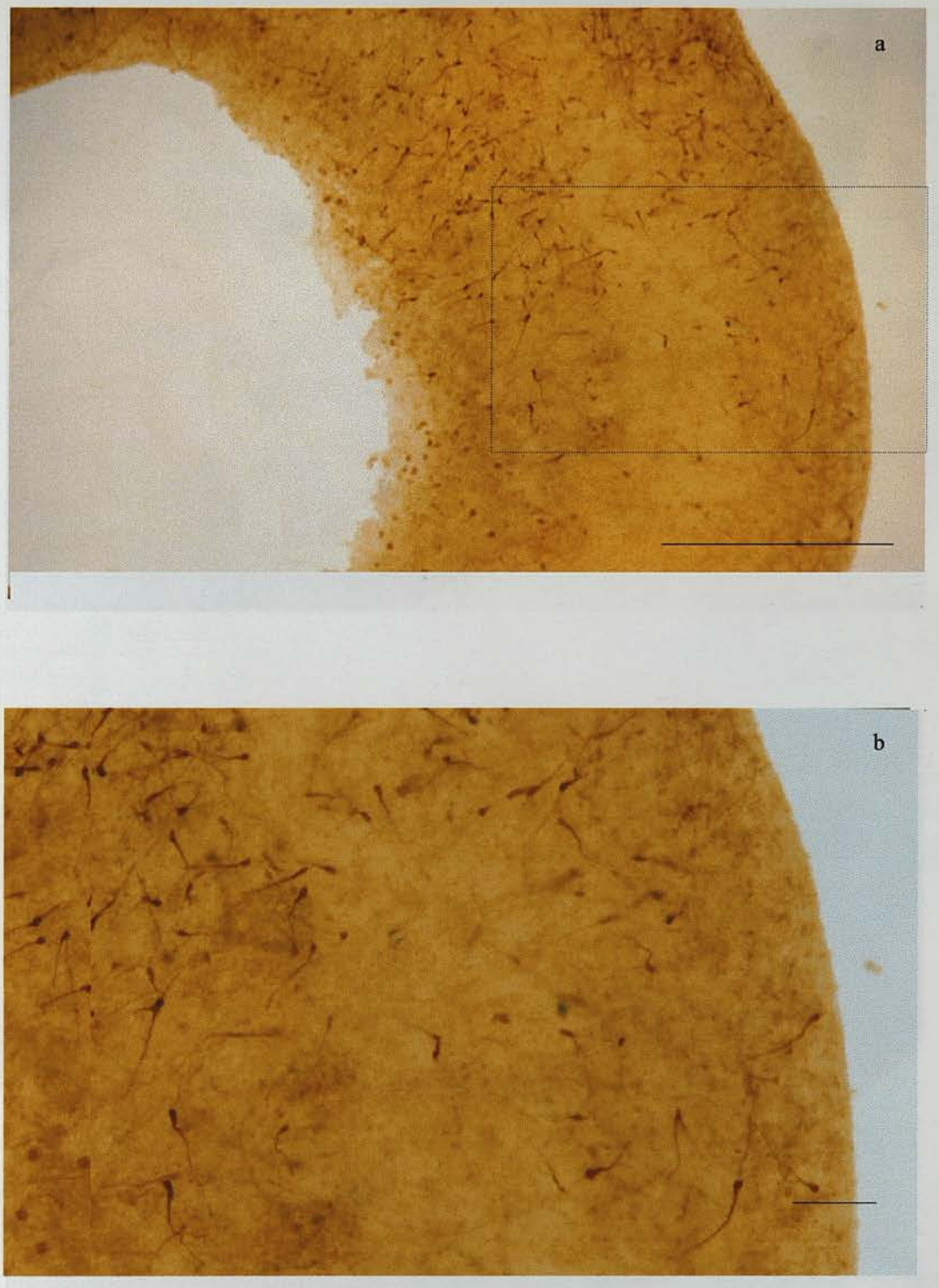


Figure 6 shows calretinin positive cells in a corticothalamic co-culture after 5 DIV and after treatment with 10^{-8} M TTX. Scale bar a: 500 µm; b: 100 µm

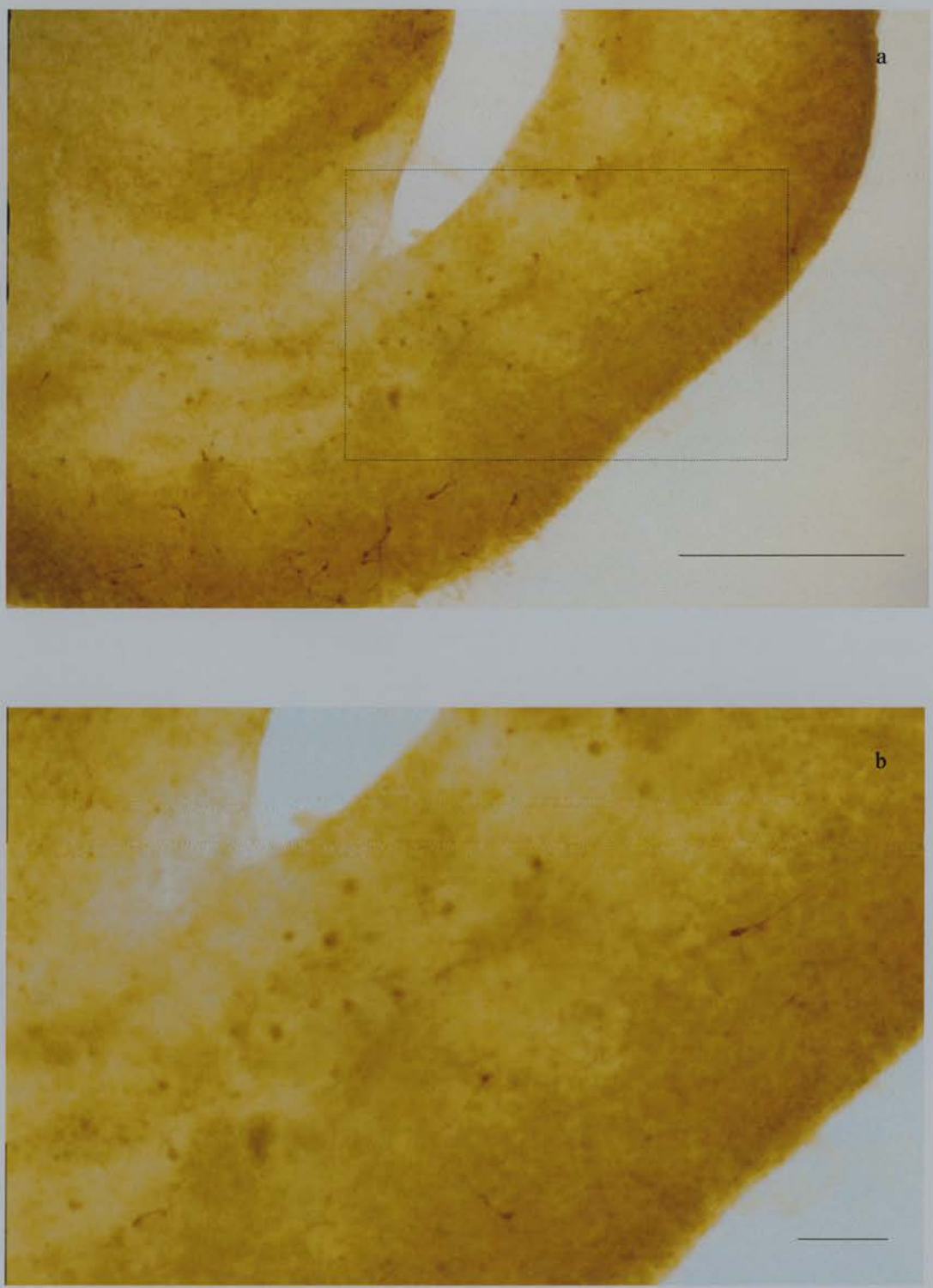


Figure 7 shows calretinin positive cells in a corticothalamic co-culture after 5 DIV and after treatment with 10-7 M TTX. Scale bar a: 500 μm b: 100 μm

Figure 8 shows the number of calretinin +ve cells resembling CR neurons in TTX treated and non-treated cortical slices after culturing with thalamus for 5 days

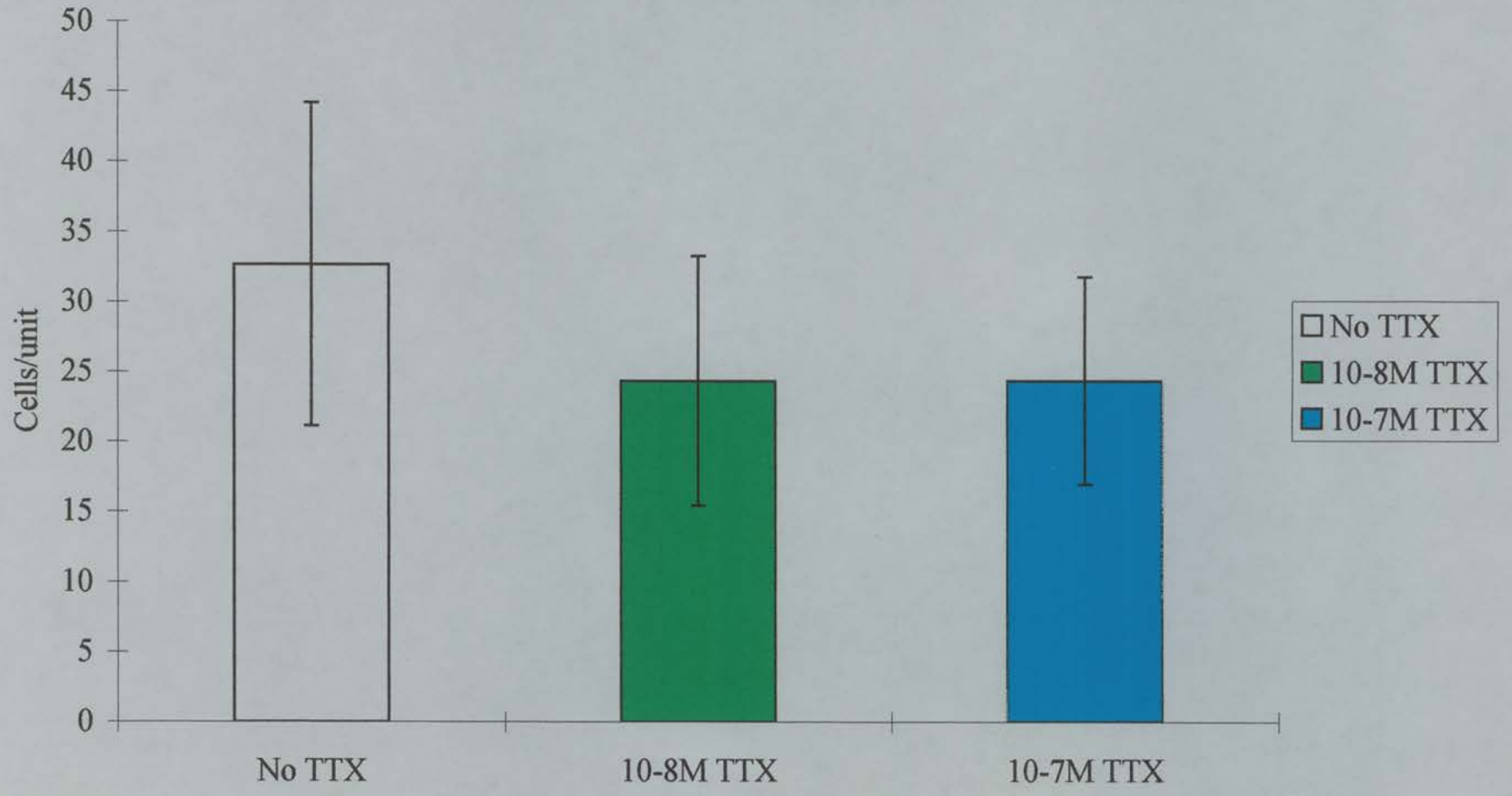


Figure 9 shows the relative distribution of calretinin in +ve cells resembling CR cells in TTX treated and non-treated cortical slices after culturing with thalamus for 5 days.

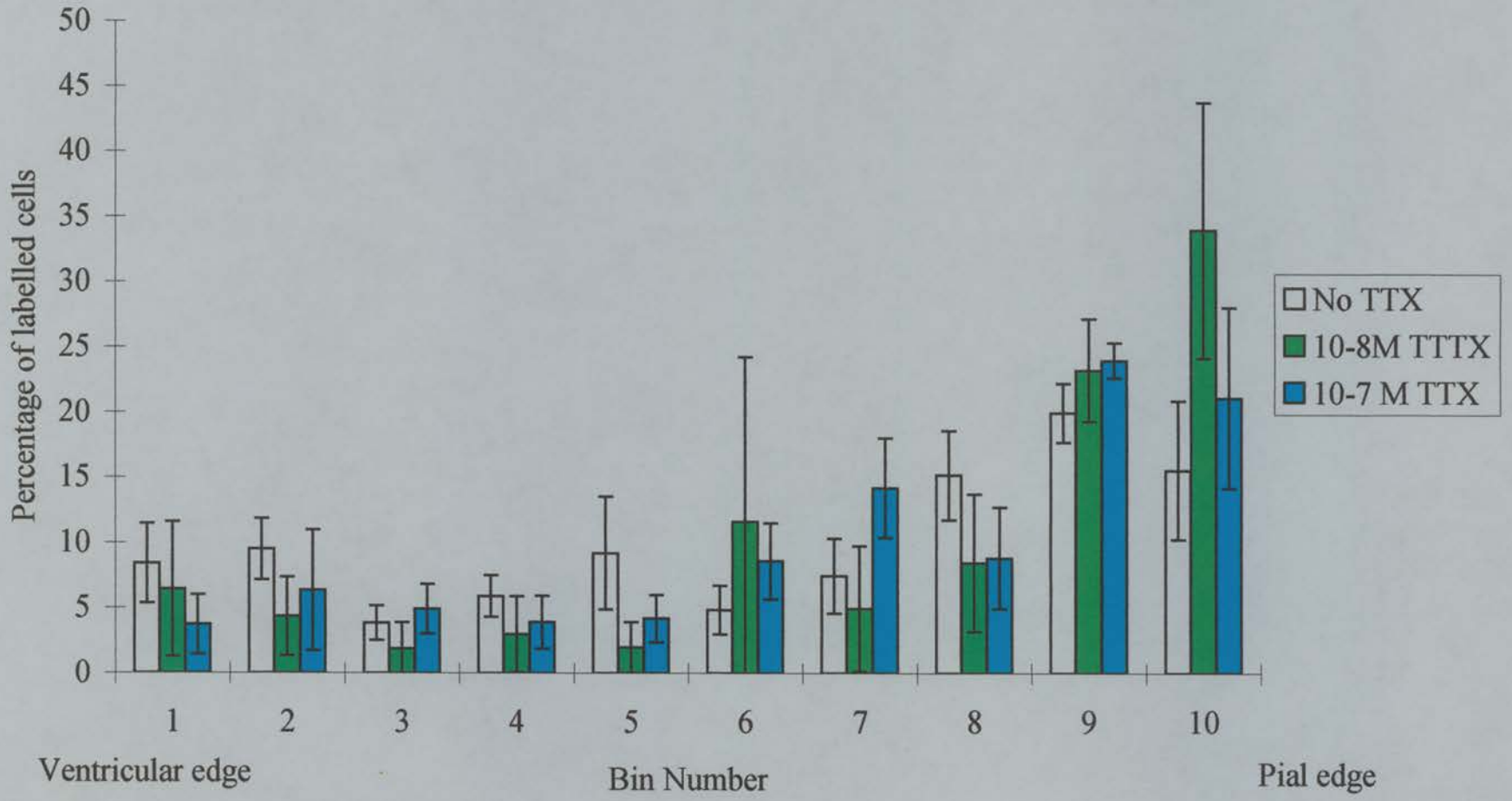
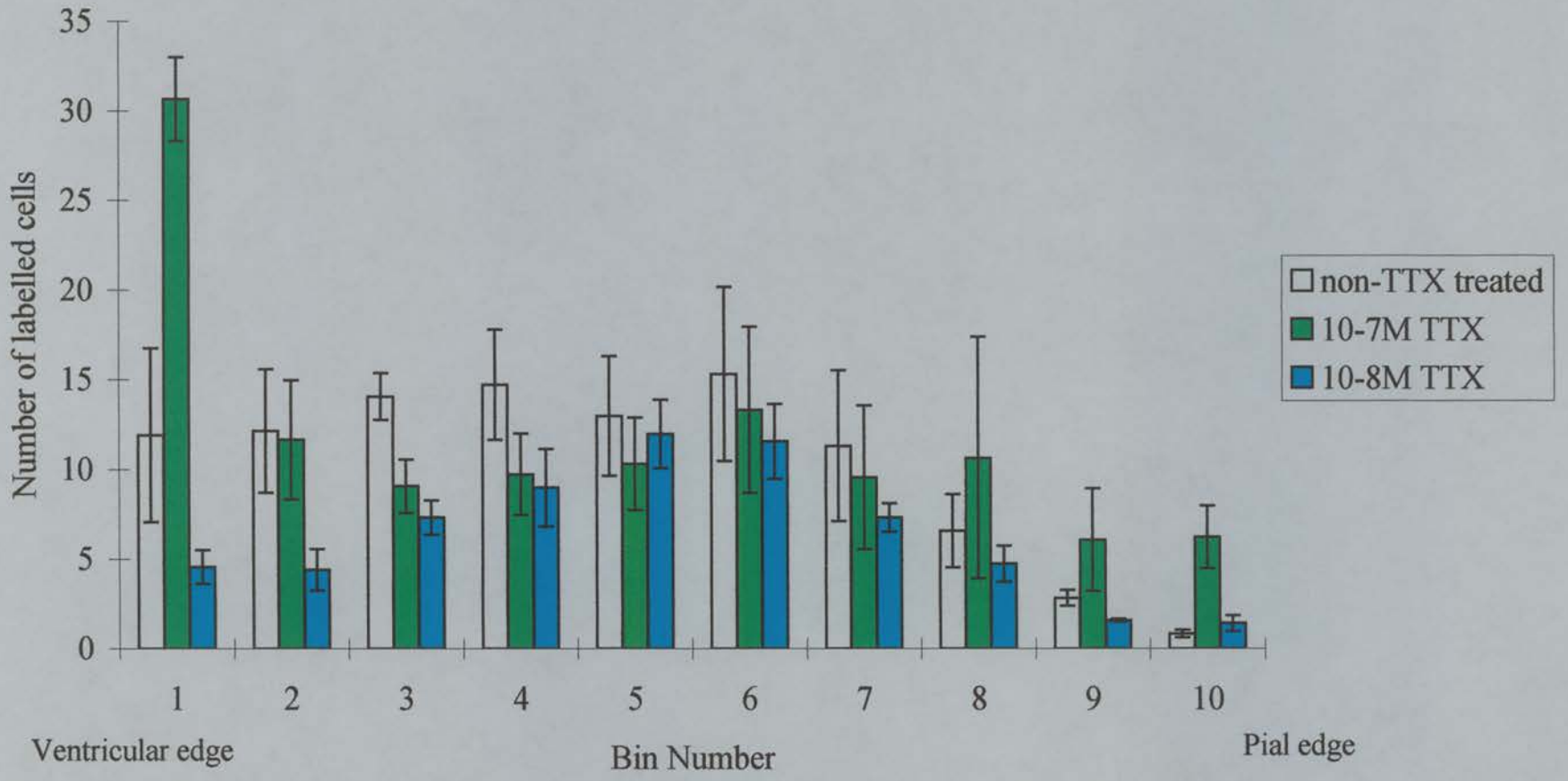


Figure 10 shows the relative distribution of BrdU labelled cells in the neocortex five days after culturing with thalamus in TTX treated and non-TTX treated cultures



DISCUSSION

This series of experiments was designed to investigate the inability of cells to from new cortical layers in culture. It was hypothesised that CR cells, which are important for normal laminar organisation, may die prematurely and/or become disorganised in cortical slices deprived of their normal afferent input. It was found that the numbers and distribution of cells identifed as CR cells varied greatly between cortical slices, and in many cases, only a few cells were seen after 5DIV. TTX had no significant effect on cell number, distribution or on the ability of *in vitro* born cells to retain their normal laminar position.

Cortical slice cultures were obtained from E16 mouse embryos. Calretinin expressing cells were seen on E16 *in vivo* in the cortical marginal zone, subplate and cortical plate. Cells in the marginal zone were identified as CR cells based on their shape, location and antigenicity for calretinin, which according to Del Rio *et al.* (1995) is specifically expressed by the CR population in the marginal zone of the mouse. At P1, heavily labelled were still present in the marginal zone. These were more easily recognised as CR cells because by this age they have acquired their characteristic size and morphology (as described by Meyer *et al*, 1998, in the rat brain). Although Foseca *et al.* (1995) reported seeing a large number of calretinin immunopositive cell in the early postnatal rat cortex, only the occasional cell was seen in P1 cortex in this study. I found no calretinin positive cells in the subplate at this age either. The absence of calretinin positive cells in the subplate at P1 might be due to loss of subplate cells through cell death. However subplate cells are still present in the mouse cortex at this time (Chapter 1 this study; Price *et al.*, 1997), therefore it is most likely that these cells have simply downregulated their expression of calretinin.

As described in the results section, calretinin positive cells were identified as CR cells based on the criteria of Del Rio *et al.* (1996). However, subsequent to this study a recent paper by Meyer *et al* (1998) suggests that calretinin expression in the

cortical marginal zone is not restricted to CR cells. They suggest that there are a lot of cells in the marginal zone that are immunopositive for calretinin and resemble CR cells but which are born slighly earlier than CR cells. Therefore, I cannot be certain that the cells I identified were CR cells. However, for the purposes of this discussion, they will be referred to as such.

After 5 DIV, many cortical slices contained very few calretinin positive CR cells. Those calretinin positive cells that were seen were much more diffusely spread than *in vivo*. It was not clear if the apparent loss of CR cells was due to cell death or downregulation of calretinin. This is discussed later. On the assumption that it might be due to cell death, as in P0 cortical slices (Del Rio *et al*, 1996), the effect of adding TTX to the culture medium was tested (based on the results of the study by Del Rio *et al.* (1996) which showed that TTX could improve the survival of CR cells in cultured slices of P0 cortex).

TTX was added to the culture medium after 2DIV. This followed the protocol of Del Rio *et al.* (1996). However there was another reason for postponing the addition of TTX until 2DIV. Since I investigated the effect of TTX on the ability of E16 born cells to retain their position at the outer edge of the cortical plate, it was important not to change the conditions under which they achieved that position in the first place. That is, it was important to follow the protocol used in Chapter 4, in which cells were seen to migrate to the upper part of the cortical wall during the first 2 DIV.

TTX was added to the culture medium at 10^{-8} M, as used by Del Rio *et al* 1996, and at 10^{-7} M. Preliminary experiments suggested that TTX had a profound effect on survival of CR cells, therefore, the effect of the addition of TTX was tested on cell migration (discussed below). However, after repeating the experiment, it became clear that the variability in cell number and distribution seen in the absence of TTX was not altered by the addition of TTX.

The very small number of CR cells in some slice cultures could be due to cell loss or down regulation of calretinin expression. For two reasons I suggest that it is due to cell loss. The first is that since calretinin expression is maintained by some cells, its down regulation is not a direct consequence of putting cortical slices into culture. Lavdas *et al*, 1997 have also shown that calretinin positive cells are present in cortical slices that were cultured for 7 days in defined medium. Therefore, it is not immediately obvious why some cells of the same population would down regulate calretinin and others would not. Secondly, Super *et al.* (1997) suggest that CR cells are dependent upon trophic support from meningeal cells for their survival. Since meningeal cells are wholly or largely absent in the cortical slice cultures used in this study (the pial membrane and dura mater becomes detached from the surface of the brain when the brain is sliced on the tissue chopper), this may account for the loss of some cells. However, this is slightly difficult to reconcile with that fact that (as shown in Chapter 3) there was no evidence for loss of cells labelled with BrdU on E12 (subplate and marginal zone cells), from the period between the start of the culture and 5 DIV. Nonetheless, since most of these cells were probably sublplate cells, this is not conclusive. Thirdly, the presence of pyknotic cells at the top of the cortical slices (data not shown) does suggest that there is some cell death, though it was not confined to marginal zone cells.

However, the variability in CR number as indicated by the calretinin labelling might be due, not only to cell death in culture, but to the fact that the marginal zone is damaged when the pial membrane is stripped off. This would explain (1) the fact that in acute slices, there were fewer E12 labelled marginal zone cells than *in vivo* (in which the pial membrane is retained during processing; Chapter 4) and (2) the variability in the number of CR cells seen in this study, because presumably the damage is not uniform. Thus, one could imagine that, as when paper is torn, a ragged edge, would allow some CR cells to survive and others to be detached with the pial membrane. I tried to test this directly by visualising CR cells in acute cortical slices. However, although it was clear that there was some calretinin positive cells and fibres in the marginal zone, the slices were too thick to be able to discern

individual cells. An attempt was made to section these slices on the freezing microtome, but they were much too small to make this possible. Although cryostat and wax sections can be obtained from acute slices the antibody to calretinin does not work well on tissue prepared in this way.

I conclude therefore that the variability in the number of CR cells was due to the loss of CR cells, either through removal of the pial membrane, or as a result of cell death *in vitro,* or by both mechanisms. Whatever the nature of the loss, the fact that many cortical slices contained very few CR cells, probably contributes to the inability of cells that complete their migration *in vitro* to maintain their position at the top of the cortical wall. If this is true, then it also suggest that the mechanism by which cortical plate cells acquire a laminar position and maintain that position are different, because the E14 born cells that had already reached the top of the cortical wall *in vivo*, were able to maintain that position. Therefore the CR cells seem not to be important for the maintenance of a laminar position (Chapter 4). These results are consistent with the suggestion by D'Arcangelo *et al.* (1995) that reelin might act to mediate cell adhesion. Based on my results, I propose that cortical plate cells migrate to the top of the cortical plate and 'stick' there as a result of an interaction with reelin. At this stage they would detach from the radial glia and start to express cell surface molecules that would then allow them to stick to each other, and therefore maintain their laminar position (as the E14 born cells did in Chapter 4). In the absence or reduction in reelin (due to loss of CR cells or their diffuse distribution) cells that reached the top of the cortical wall would not 'stick' there, therefore would not detach from the radial glia and would therefore slip back down towards the ventricular zone. In the reeler mutant, later generated cells do not reach the marginal zone. This could be due to the fact that the earlier born cells do not 'free' the radial glia to allow them to migrate through. In my study, the E14 born cells had probably 'freed' themselves from the radial glia *in vivo*, thus allowing the E16 born cells to reach the top of the cortical plate.

The irregular distribution of CR cells in the cortical slice, is no doubt due in part to the flattening of the slice, which occurs in culture. However, it may also be the result of the loss of cortical afferents to the marginal zone. Synchronised changes in internal calcium have been observed in acute slices of rat cerebral cortex (Yuste *et al*, 1992). It was suggested that this synchronised activity might participate in the formation of cortical columnar organisation. It is possible that synchronised activity might also be responsible for the formation and maintenance of laminar organisation. Thus, if activity becomes desynchronised, due to severing of afferent fibres, for example, this might inhibit the normal formation and preservation of cortical layers. Further, Super *et al.* (1997) have provided evidence for the fact that CR cells migrate tangentially to the site of a lesioned brain area. Since the preparation of cortical slices for culture causes gross injury to the brain, the distribution of the cells might be due in part to their migration to very extensive sites of injury.

The effect of TTX on the ability of *in vitro* born cells to retain their normal position at the top of the cortical wall was also tested. In retrospect, there was no strong reason for doing this given the results of the effects of TTX on CR cells. However, preliminary results on CR cell number did suggest an effect of TTX so these experiments were carried out in conjunction with those on the CR cells. Not surprisingly, given the result on CR cells, TTX had little effect on the ability of cells to maintain their position at the top of the cortical wall.

In summary, this study shows that in the *in vitro* system described in Chapter 4, there is great variability in the number of calretinin positive cells between cultures and the cells that are present have a very diffuse distribution. This cannot be rectified by the addition of TTX to the culture medium. The variability in number, abnormal distribution and the possibility that reelin (which is a secreted molecule) becomes diluted out in culture suggests that there might be a direct link between these findings and the inability of cells that had migrated in culture to maintain their position at the top of the cortical plate and form new laminae.

CHAPTER 6: CORTICAL AND THALAMIC DEVELOPMENT IN THE SMALL EYE MUTANT MOUSE

INTRODUCION

Given the results of Chapter 4 of this thesis that suggest a role for the thalamus in the migration out of the ventricular zone of late born neuronal precursors, I decided to investigate the nature of the thalamus in the Small eye (*Sey*) mutant mouse in which the migration of late born cortical precursors is aberrant.

The Small eye (*Sey*) mouse is a useful model for the study of normal cortical development. The *Pax-6* gene, which is mutated in the *Sey* mouse, encodes a transcription factor that has two DNA binding motifs; a paired domain (Bopp *et al.*, 1986 and Treisman *et al*, 1991) and a paired-like homeodomain (Frigerio *et al.*, 1986). In the forebrain, Pax-6 is expressed throughout the alar diencephalon and telencephalon from embryonic day 8.5 (E8.5; Walther and Gruss 1991; Puelles and Rubenstein, 1993). Its expression becomes more restricted during development (Stoykova and Gruss, 1994; Warren and Price 1997) and by E14.5 it is confined to the telencephalic ventricular zone (Walther and Gruss, 1991 and Stoykova *et al*, 1996), the caudal part of the ventral thalamus, the dorsal midline of the dorsal thalamus and the caudal part of the pretectum (Warren and Price, 1997). The spatiotemporal pattern of expression of Pax-6 in the developing brain suggests that it plays a key role in several developmental processes including cell proliferation, cell differentiaion and cell maintenance (Walther and Gruss, 1991; Mansouri *et al.*, 1994; Stoykova and Gruss, 1994).

Developmental abnormalities

In the *Sey/Sey* mutant used in this study, a point mutation in the *Pax-6* gene produces a 'stop' codon between the paired domain and the homeodomain so that no functional protein is translated (Hill *et al*, 1991). Two mutations of the *Pax-6* gene (*Sey* and Sey^{Neu}) produce similar abnormalities (Hill *et al.*, 1991 and Mastick *et al.*, 1997), therefore studies done on both have been referred to in this chapter. *Sey/Sey*

mice die shortly after birth because they are unable to suckle and breathe at the same time (Hogan *et al*, 1986). *Sey/ Sey* embryos defects of the eye (Hill *et al.*, 1991), olfactory bulb (Hogan *et al.*, 1986), forebrain (Stoykova *et al.*, 1996; Warren and Price, 1997; Mastick *et al.*, 1997), hindbrain and spinal cord (Ericson *et al.*, 1997). In the forebrain these abnormalites include defects of neuronal cell migration in the developing cerebral cortex (Schmahl *et al.*, 1993; Caric *et al.*, 1997), and abnormalities in cell proliferation and regionalisation of the diencephalon (Stoykova *et al.*, 1996; Grindley *et al*, 1997; Warren and Price, 1997).

In the *Sey/Sey* mouse, cortical precursors destined to form the deep cortical layers (born on E13 and E14) migrate normally an d contribute to formation of the cortical plate (Caric *et al*, 1997). However late born precursors do not. Late born (E16 born) postmitotic cells accumulate in the subventricular zone (where they form cell clusters) and never reach the cortical plate (Caric *et al.*, 1997) Caric *et al.* (1997) used a transplant technique to show that if these late born cortical precursors (from *Sey/Sey* brains) were transplanted into wild type E16 rat brains, they were found the cortical wall at the level of layers II and III. Although these authors did not show directly that these cells actively migrated there, since they were injected into the ventricular zone, it must be assumed that this was the case. Thus, it was concluded that the inability of these late born precursors to migrate and form laminae in the *Sey/Sey* mouse is not due to an intrinsic defect in the cells themselves, but is due to a defect in the environment in which they are attempting to migrate. Since the results of the cortical slice culture study (Chapter 4) suggested a role for both Cajal-Retzius cells and the dorsal thalamus in the process of normal migration and lamination of E16 born neuronal precursors, I asked if these might be the environmental factors that are abnormal in the *Sey/Sey* mouse brain. It is not yet known whether Cajal-Retzius cells are present in *Sey/Sey* cortex, and although Schmahl *et al.*(1993), reported that the dorsal thalamus was normal (based on H&E staining), subsequent studies that show abnormalities in proliferation and regionalisation in the diencephalon (Stoykova *et al.*, 1996; Grindley *et al*, 1997; Warren and Price, 1997) point to the fact that it might not be.

I used the calcium-binding protein calretinin to study the development of Cajal-Retzius cells and the dorsal thalamus in both +/+ and *Sey/Sey* brains. Calretinin is a member of the EF-hand family of calcium binding proteins that includes parvalbumin, calbindin-D28K and calmodulin (reviewed by Baimbridge *et al*, 1992). Although its exact function is not yet known, its specific distribution in neuronal subpopulations in the adult (Arai *et al.*, 1994) and developing brain (Vogt Weisenhorn *et al.*, 1994; Abbot *et al.*, 1995; Del Rio *et al.*, 1995; Fonseca *et al.*, 1995; Frassoni *et al.*, 1998) make it a useful marker for anatomical studies. Calretinin is expressed in Cajal-Retzius cells of the marginal zone in the mouse (Del Rio *et al.*, 1995) and in specific thalamic nuclei (Frassoni, *et al.*, 1998) and thalamocortical axons in the rat (Fonseca *et al.*, 1995). I showed that it was expressed in a similar pattern in the mouse thalamus and utilised its expression pattern to investigate the development of Cajal-Retzius cells and thalamic development in the *Sey* mouse.

MATERIALS AND METHODS

Sey/Sey mice die at birth (Hogan *et al.*, 1986), and *Sey/Sey* embryos (easily identified by the absence of eyes and a shortened snout) were obtained from *Sey/+ x Sey/+* matings (heterozygotes were readily identified by their smaller than normal eyes, Hill *et al.*, 1991). Age-matched controls were +/+ littermates from these matings and from +/+ x +/+ matings. E15, E17 and E19 +/+ and *Sey/Sey* embryos were removed from anaesthetised mothers (0.35 ml 25% urethane in saline; i.p.). The number of animals used in this study is shown in Table 1. Whole heads were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (overnight at 4°C) then transferred to 4% paraformaldehyde in 20% sucrose in PBS (overnight at 4°C). Brains were removed and stored in 20% sucrose in PBS until sectioned on the freezing microtome at 35 μm, then rinsed in PBS. Some sections were retained for counter staining in cresyl-violet and adjacent sections were reacted with an antibody against calretinin. Sections were treated in 3% H_2O_2 in methanol (1 in 4; 20 mins.) to remove endogenous peroxidase activity, rinsed in PBS with 0.2% triton X 100 (PBS-T), blocked in 5% swine serum in PBS-T (1 hour), then incubated in rabbit anti-calretinin (Chemicon) diluted 1 in 1000 in blocking solution (overnight at 4° C). After washing in PBS-T, the sections were incubated in biotinylated swine anti-rabbit antibody (Sigma), diluted 1 in 300 in blocking solution (2 hours), washed as before, treated using the ABC system (Vector) and visualised using diaminobenzidine (DAB) as the chromagen. Sections were stored in PBS with 0.2% gelatin and mounted on poly-L-lysine coated slides. Some of the reacted sections were counter-stained in cresyl-violet before coverslipping.

TABLE 1

Age	Sey/Sey	Wild type controls
	Number of animals (litters)	Number of animals (litters)
E15	3 (3)	3 (3)
E17	3 (3)	4 (4)
E19	3 (3)	5 (4)

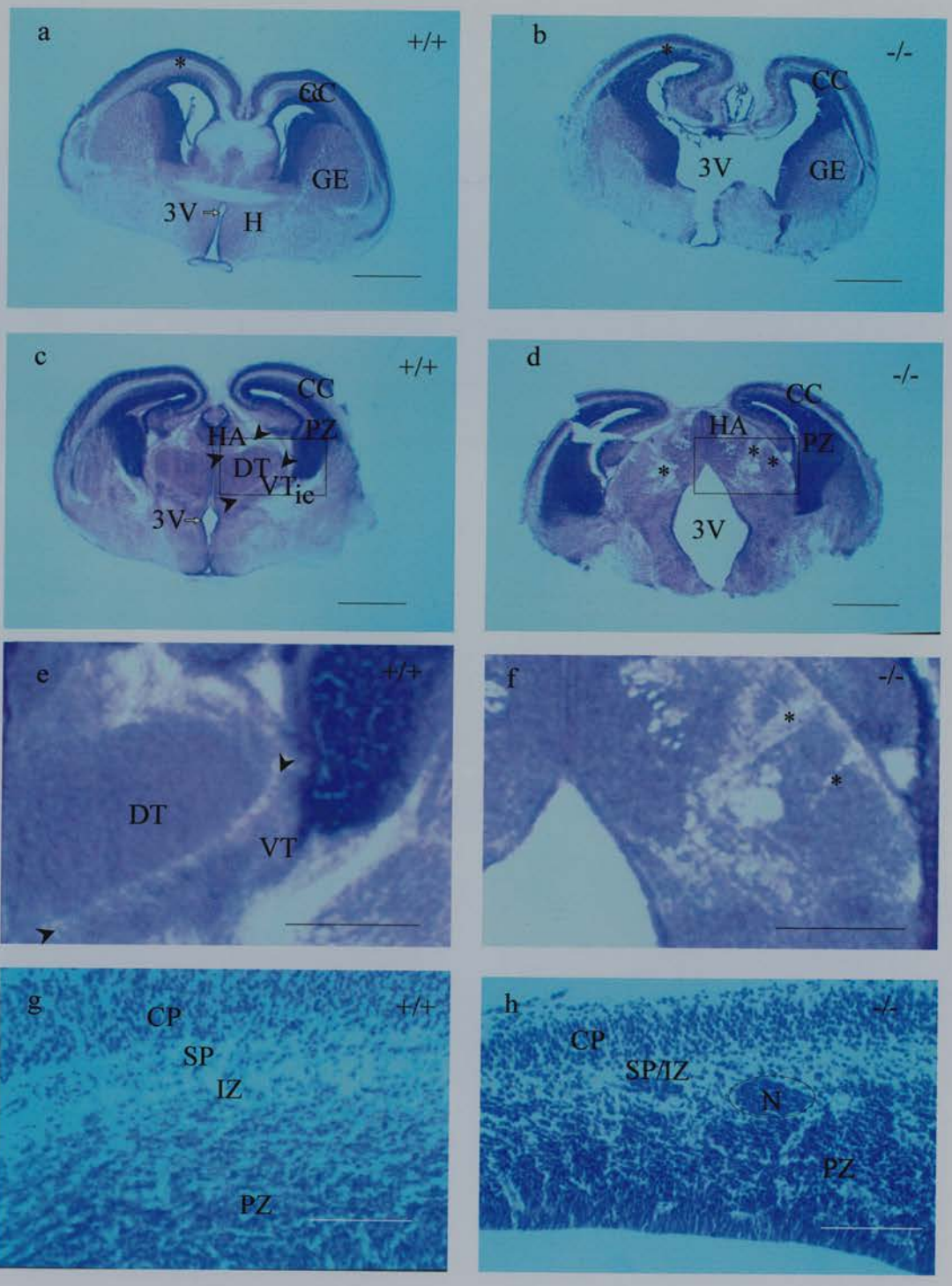
Table 1 shows the number of animal used in this study and the number of litters from which they came. Wild type littermates of *Sey/Sey* mice and age matched brains from +/+ x +/+ matings were used as controls.

RESULTS

Gross morphology

Counter-stained sections of the wild-type and *Sey/Sey* forebrains showed that the gross morphology of the *Sey/Sey* brain was abnormal. This is illustrated in Figure 1 which shows cresyl-violet stained sections of wild-type and *Sey/Sey* brains from littermates, at E17. Figure 1a shows a rostral section of the wild-type brain in which the hypothalamus, ganglionic eminence and cerebral cortex are visible. The cortical subplate is marked with a small asterisk. Figure 1b shows a section of a *Sey/Sey* brain from a similar rostrocaudal level. The third ventricle was enlarged but the stratification of the cortical wall had similar cell dense and cell sparse regions as in the wild type. Figure 1c illustrates a slightly more caudal section of the wild-type brain. Two thin strips of cell sparse tissue separate the habenula from the dorsal thalamus and the dorsal thalamus from the ventral thalamus (see also Fig.1e). The internal capsule (through which fibres pass from the thalamus to the cortex) was clearly visible, as were the cortical zones. In the *Sey/Sey* brain at the same level (Fig. 1d), the habenula was still discernible, but the dorsal thalamus and ventral thalamus were less easy to distinguish from one another. Within the region approximating the dorsal and ventral thalamus, abnormal pathways made by axon tracts were clearly visible as unstained regions (see also Fig.1f). Such thick tracts were never seen in the wild type brains. Although these axon tracts were present in the body of the diencephalon there was no obvious internal capsule. The enlarged third ventricle was evident. In the telencephalon, the proliferative zone was enlarged. Figure 1g shows a higher magnification view of the wild-type cortical wall. The proliferative zone, intermediate zone, subplate and cortical plate can be identified by their location and cell densities. In the *Sey/Sey* brain, at the same magnification (Fig. 1g) the cortical wall was narrower overall. The proliferative zone contained cell dense nodules, the subplate and intermediate zone were thinner and difficult to distinguish and the cortical plate was reduced.

Fig. 1. Nissl-stained coronal sections of wild-type and *Sey/Sey* brains at E17. (a) Rostral section of a wild-type brain showing the hypothalamus (H), ganglionic eminence (GE), cerebral cortex (CC) and 3rd ventricle (3V). The cortical subplate is marked with an asterisk. (b) Rostral section of a *Sey/Sey* brain showing hypothalamus, ganglionic eminence, cerebral cortex and enlarged 3rd ventricle. (c and e) A more caudal section of the wild-type brain showing the habenula (HA), dorsal thalamus (DT), ventral thalamus (VT), 3rd ventricle, internal capsule (IC) and cerebral cortex and telencephalic proliferative zone (PZ). Arrowheads mark the cell sparse zones that separate the habenula from the dorsal thalamus and the dorsal thalamus from the ventral thalamus. (d and f) A similar caudal section in the *Sey/Sey* brain showing the habenula, enlarged 3rd ventricle, enlarged telencephalic ventricular zone, axon tracts (marked with an asterisk) and the cerebral cortex. (g) A high power view of the wild-type cerebral wall showing the proliferative zone (PZ), intermediate zone (IZ), subplate (SP) and cortical plate (CP). (h) A similar high power view of the *Sey/Sey* cerebral wall showing the proliferative zone, intermediate zone, subplate and cortical plate and cell dense nodules in the proliferative zone (N). Scale bars, a-d: 1 mm, e and f: 100 μm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Calretinin immunolabelling

I investigated the differentiated state of the thalamus using an antibody to calretinin. At E15 in the wild-type brain, calretinin staining was found in lateral regions of the developing diencephalon: in the reticular nucleus of the ventral thalamus and the stria medullaris thalami (Fig.2a and c). A few lightly labelled calretinin positive fibres (asterisk Fig. 2a and c) were seen running from just below the reticular nucleus through the internal capsule and the amygdala to the cortical subplate. A few heavily labelled cells could be seen apparently migrating from the neuroepithelium towards the lateral diencephalic wall (Fig. 2e). Calretinin positive cells resembling Cajal-Retzius cells were seen in the cortical marginal zone (Fig. 2g).

In E15 *Sey/Sey* brains, labelling was much more random. Scattered cells and bundles of fibres were seen in the diencephalic wall, mainly on its lateral portion (Fig.2 b, s and f). No calretinin-immunopositive fibres were seen in any part of the telencephalon. Calretinin positive cells, like those in the wild type were seen in the cortical marginal zone (2g).

In E17 (not shown) and E19 (Fig. 3a and c) wild-type brains, calretinin staining was found in the midline nuclei (the paraventricular nucleus, the rhomboid nucleus, the reuniens nucleus) in the intralaminar group (mediodorsal, centromedial, paracentral and centrolateral nuclei), in the dorsal thalamus (the mediodorsal, lateral dorsal, lateral posterior nuclei) in the stria medullaris and bordering the ventral thalamus (along ventral and lateral borders of the reticular nucleus and zona incerta) and in the optic nerve. Thalamocortical fibres were seen in the superior thalamic radiation (asterisk Fig. 3c) and running laterally and ventrally from the midline and intralaminar nuclei (double asterisk Fig. 3c). These fibres entered the telencephalon through the internal capsule and were seen in the cortical subplate at all rostro-caudal levels (Fig.3e). Calretinin positive cells resembling Cajal-Retzius cells were seen in the cortical marginal zone.

In E17 (not shown) and E19 (Fig. 3b and d) *Sey/Sey* brains, calretinin labelling was still random, as seen in E15 *Sey/Sey* brains. There were several heavily

labelled cells in the diencephalon, but individual thalamic nuclei could not be identified. Labelled fibres were seen, but they were heavily fasiculated (asterisk, Fig. 3d). They ran mainly in a latero-ventral direction, but they appeared to terminate within the diencephalon or at the telencephalic-diencephalic junction. They were never seen in the cortical subplate (fig. 3f). Calretinin positive cells resembling Cajal-Retzius cells were seen in the marginal zone, and were similar in terms of their location and morphology to those in the wild type.

These results are summarised Diagrammatically in Figure 4.

Fig. 2. Calretinin stained coronal sections from E15 wild-type and *Sey/Sey* brains. (a) Wild-type brain showing calretinin staining in the diencephalon and telencephalon. (b) A similar section in the *Sey/Sey* brain showing residual calretinin staining in lateral regions of the diencephlaon (c) A high power view of the boxed area in (a) showing the stria medularis (STM) and reticular nucleus (rt). An asterisk marks the thalamocortical axons that enter the telencephalon via the internal capsule and pass along the cortical subplate. (d) A high power view of the boxed area in (b) showing fasciculated fibres (asterisk) in the region approximating the dorsal and ventral thalamus. (e) A high power view of the area marked by a rectangle in (c) showing cells migrating in a medial to lateral direction, from the neuroepithelim to the lateral portion of the diencephalic wall. (f) A high power view of the area marked by a rectangle in (d) showing fasiculated fibres and a few calretinin positive cells. (g) A high power view of the cortical marginal zone showing calretinin positive monopolar cells resembling Cajal-Retzius (CR) cells and axons in the cortical subplate (asterisk). (h) A high power view of the marginal zone in the *Sey/Sey* brain showing similar calretinin positive cells. Scale bars, a and b: 1mm, c and d: 500 μm and e and f: 100 μm: g and h 100 μm and 50 μm (inset).

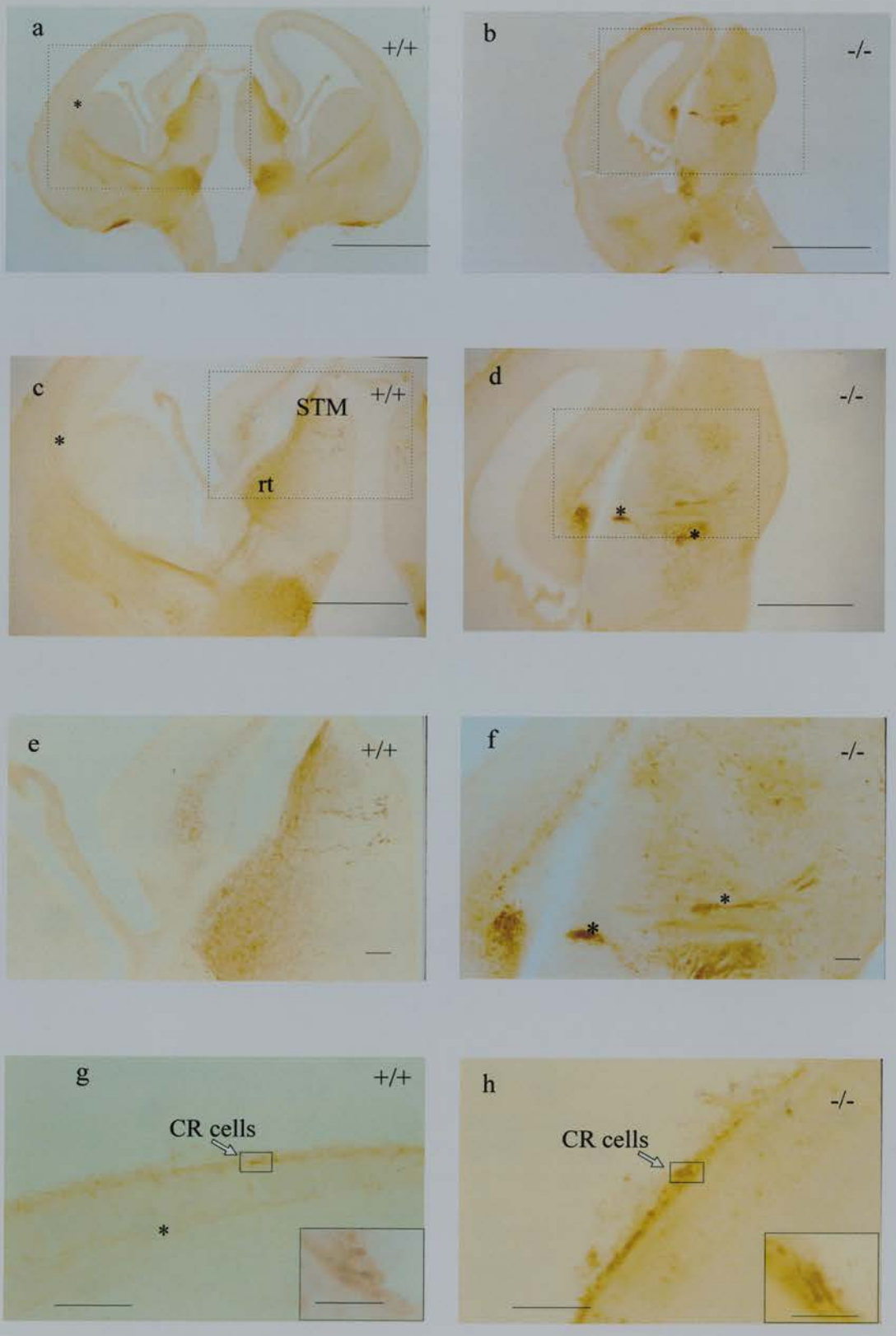
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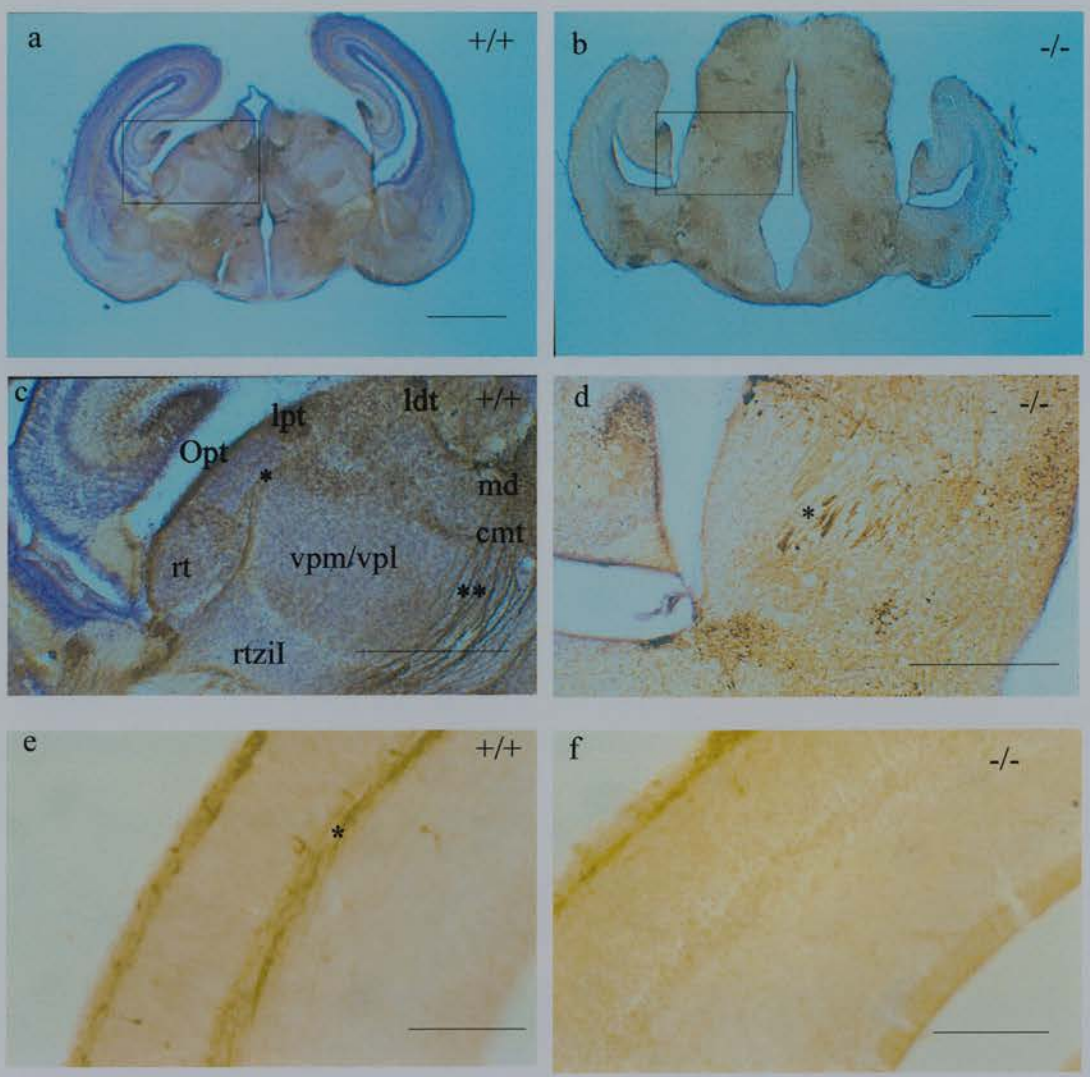
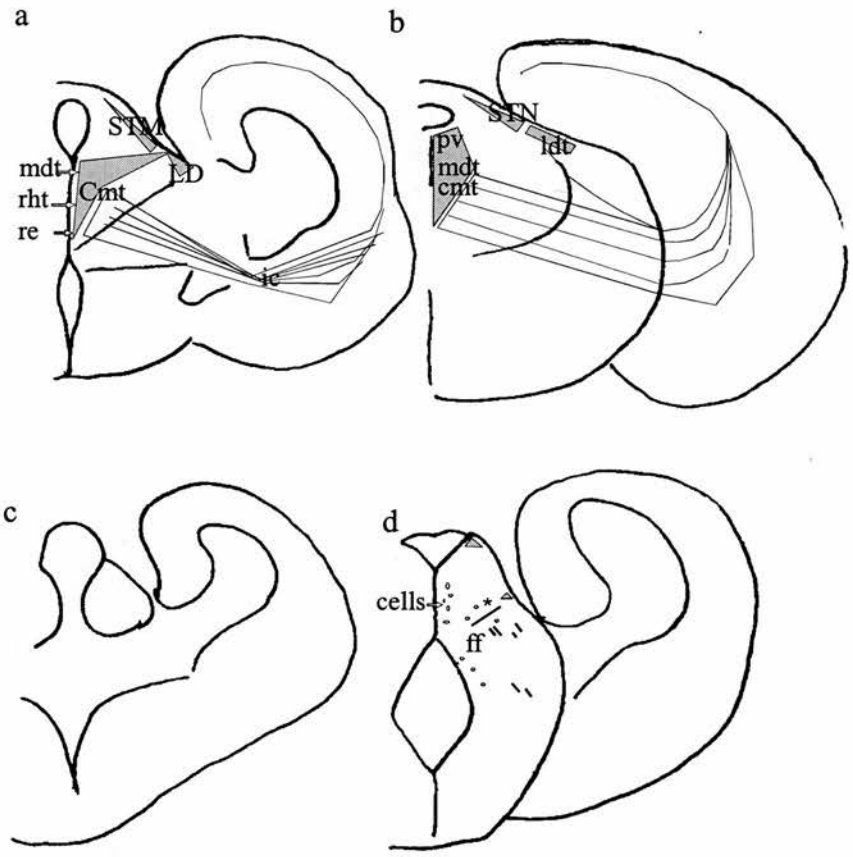
Fig. 3. Coronal sections of Nissl- and calretinin-stained E19 wild-type (a,c,e) and *Sey/Sey* brains (b,d,f). (a)Wild-type brain showing calretin staining in the midline and intralaminar nuclei (asterisk) (b) Higher power view of the boxed area in (a) showing calretinin staining in the mediodorsal (md), centromedial (cm) lateral dorsal (ldt), lateral posterior (lpt) nuclei, the optic tract (Opt), along the borders of the reticular nucleus (rt) and zona incerta (zi) and in thalamocortical axons in the superior thalamic radiation (asterisk) and running ventro-laterally from the midline and intralaminar nuclei (double asterisk). (b) *Sey/Sey* brain showing calretinin stainining in medial and lateral parts of the diencephalic wall (d) A higher power view of the boxed region in b showing fasiculated fibres (asterisk) and calretinin positive cells (e,f) High power views of wild-type and *Sey/Sey* neocortex. (e) Thalmocortical axons (asterisk) in the cortical subplate of the wild-type brain and calretinin positive cells resembling CR cells in the marginal zone. (f) Complete absence of calretinin positive thalamocortical axons in the *Sey/Sey* brain. Calretinin positive cells in the marginal zone similar to those in the wild type. Scale bars, a and b: 1 mm, c and d: 500 μm, e and f: 100 μm.



Scale bar on a and b = 1mm and on c and d = 500 μm

Fig. 4. Diagrammatic representation of calretinin staining in the wild-type and *Sey/Sey* telencephalon, habenula and thalamus at rostral and caudal levels, between E17 and E19. (a) In the wild-type, at a rostral level, calretinin staining was seen in the stria medullaris ateral (stm), the lateral dorsal nucleus (ldt), the paraventricular nucleus (pv), the mediodorsal nucleus (md), the centromedial nucleus (cm), the Rhomboid (rh) and Reuniens (re) nuclei and in thalamocortical axons extending laterally and ventrally from the midline and intralaminar nuclei to the internal capsule (ic) and the telencephalic subplate. (b) At a more caudal level, staining was seen in the lateral posterior nucleus (lp) and in fibres in the superior thalamic radiation (spr), that entered the telencephalon via the internal capsule. (c) In the *Sey/Sey* brain at a rostral level calretinin staining was absent from the telencphalic subplate. (d) At a more caudal levels staining was seen in regions approximating the stria medullaris and lateral dorsal nucleus. The region approximating midline and intralaminar nuclei contained many calretinin positive cells, but the nuclei could not be discerned. Fasiculated fibres (ff) were seen in a region approximating the external medullary lamina (astersik) and extended in a lateral-ventral direction in a region approximating the dorsal thalamus. (a-d) Cajal-Retzius cells were seen in the marginal zone of both wild-type and *Sey/Sey* brains at all levels.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DISCUSSION

This study showed that the development of the dorsal thalamus and thalamocortical axons was abnormal in the *Sey/Sey* mouse at all ages investigated. However, calretinin positive cells resembling Cajal Retzius cells were similar in the *Sey/Sey* brains to those in the wild-type. This discussion deals with the morphological abnormalities seen in the Sey/Sey mice, then discusses calretinin expression in the wild type and *Sey/Sey* brains and proposes mechanisms for the abnormal formation of the dorsal thalamus in the *Sey/Sey* brains. Finally it describes the possible implications for the abnormal thalamic development in relation to the cortical migration defect in the *Sey/Sey* mouse.

The gross morphology of the Sey/Sey forebrain

The gross morphology of the *Sey/Sey* brain was studied in cresyl-violet stained coronal sections and compared to that of the wild-type. The enlarged third ventricle, thinned cortex, enlarged proliferative zone and abnormal ventral thalamus have been reported previously (Schmahl *et al*, 1993; Caric *et al.*, 1997; Stoykova *et al.*, 1996; Grindley *et al.*, 1997). However, the results presented here also showed that the dorsal thalamus was aberrant. In the *Sey/Sey* brains there was no obvious demarcation between dorsal and ventral thalamus and the cells sparse regions that represent axon tracts were highly irregular. Although, it has been shown that there is a slight abnormallity in terms of gene expression (Grindley *et al.*, 1997) and cell density (Warren and Price, 1997), in prosomere 2 (p2) which gives rise to the dorsal thalamus, the thalamus itself had been reported to be normal (Schmahl *et al.*, 1993).

Calretinin expression in the wild-type thalamus

In the wild-type brains, calretinin staining was seen in specific thalamic nuclei and fibre tracts. The pattern of labelling was very similar to that reported previously in the developing rat thalamus (Frassoni *et al.*, 1998). At E15, the thalamic nuclei are just beginning to emerge (Altman and Bayer, 1979) and only the early born structures are present. Of the structures that were labelled with calretinin, the stria medullaris is a fibre bundle which may represent afferents to the lateral habenula

(Altman and Bayer, 1979). The reticular nucleus which is part of the ventral thalamus has reciprocal connections with the dorsal thalamus and receives afferents from the cortex. It is thought to modulate activities of the dorsal thalamus. Cells in the marginal zone, with a morphology similar to Cajal-Retzius cells, were also labelled. This has been shown previously in the mouse cerebral cortex (Del Rio *et al.*, 1995)

By E17 all of the thalamic nuclei were present. Of the nuclei labelled at E17 and E19, the midline and intralaminar nuclei are involved in regulating cortical activity associated with arousal states. The mediodorsal, lateral dorsal and lateral posterior nuclei are association nuclei which project to the association cortex. As at E15, Cajal-Retzius cells in the cortical marginal zone were labelled.

Calretinin staining in the Sey/Sey forebrain

By contrast, in the *Sey/Sey* brains calretinin staining was much more random and although many labelled cells were seen, it was difficult to discern individual thalamic nuclei. A small cluster of cells that may approximate the lateral dorsal nucleus was present in some sections of the E17 and E19 brains. However, the midline and intralaminar nuclei, which stain heavily with calretinin in the wild-type brain, were absent or failed to express calretinin in the normal pattern. Interestingly, since these are the late born thalamic nuclei their precursors are born at a time (i.e. E13 to E14) when *Pax-6* expression is almost completely absent from prosomere 2. Labelled fibres were also seen in the diencephalon, but these were highly fasiculated, appeared to teminate before entering the telencephalon and were never seen in the cortical subplate. Cajal-Retzius cells in the cortical marginal zone were normal in terms of their location and morphology.

Pax-6 expression and the formation of thalamic nuclei

Pax-6 is normally expressed in alar regions of the diencephalon from E8.5 (Puelles and Rubenstein, 1993). By E14.5, it is almost completely absent from P2, except for a narrow strip along the dorsal midline (Warren and Price, 1997). Its expression also

becomes restricted in P1 and 3, but to a far lesser extent, and it continues to be expressed widely in these regions after E14.5 (N. Warren, personal communication). This period of restriction of *Pax6* expression particularly in P2 (the dorsal thalamus), coincides with the birth of thalamic nuclei. From about E11 to E15 the ventricular cells that give rise to the thalamic nuclei are undergoing terminal cell division and migrating to their final destination (Altman and Bayer, 1979). At E15 cell division ends and the last born thalamic nuclei emerge. Thus, down regulation of *Pax 6*, especially in P2 coincides with terminal cell division and the emergence of distinct dorsal thalamic nuclei.

Given the spatiotemporal pattern of expression of *Pax-6*, it seems surprising that the early development of prosomeres 2 and 3, which coincides with ubiquitous *Pax-6* expression, is relatively normal, at least in terms of early regionalisation and gene expression (Stoykova *et al.*, 1996, Warren *et al.* , 1997), in the *Sey/Sey* mouse. Then at a time when the expression is normally down regulated in P2, the dorsal thalamus in the *Sey/Sey* brain exhibits gross abnormalities. This suggests that either the late abnormality is due to (i) the absence of *Pax-6* at early ages affecting downstream gene(s) that are expressed later and are required for normal development of the dorsal thalamus, or (ii) the late development of the dorsal thalamus being dependent upon cues from neighbouring areas, such as the ventral thalamus and pretectum which normally continue to express Pax-6 at later ages.

In support of the first hypothesis, it is well established that cascades of genes play important roles during development. Pax-6 itself has been shown to be involved in such a cascade. In the spinal cord, *Pax-6* interacts with *Shh* and *Nkx2.2* in the generation of distinct classes of motor neurons and ventral interneurones (Ericson *et al*, 1997). Since *Pax6* is normally transiently expressed in P2, (Mastick *et al.*, 1997; Warren and Price, 1997) it is possible that in the absence of a functional *Pax6* protein (in *Sey/Sey* mice) the genetic cascade that specifies dorsal thalamic cells is not activated and, as a consequence late-expressed molecules including calretinin are not expressed in this region. It would be interesting to study early gene markers of the

dorsal thalamus (which might be part of a cascade) in *Sey/Sey* mice to see if their expression is also disrupted.

With regard to an influence from neighbouring tissues, it a also well known that adjacent or target cells and tissue can have important influences in development. This has been shown in a wide range of species including the *Drosophila* (Tomlinson and Ready, 1987), the leech (Torrence *et al.*,1989) and the rat (Nothia *et al.*, 1998). Since the ventral thalamus is abnormal in the Sey/Sey mutant (Stoykova *et al.*, 1996, Warren and Price, 1997) and because it has reciprocal connections with the dorsal thalamus it is a prime candidate. Additionally, its nuclei are among the earliest to arise (Altman and Bayer, 1979), thus it is perfectly reasonable that it could influence the later born nuclei of the dorsal thalamus. This hypothesis could be tested using co-cultures of presumptive dorsal and ventral thalamic tissue, whereby wild-type ventral thalamic tissue is co-cultured with *Sey/Sey* dorsal thalamic tissue and the expression of phenotypic markers us tested.

It seems most likely that both gene expression within P2 and cues from neighbouring tissues play roles in specifying the dorsal thalamus. Although distinct nuclei were not discernible, the presence of calretinin positive cells and fibres in a region approximating the dorsal thalamus, suggests that some degree of specification had taken place.

Thalamocortical innervation

Thalamocortical innervation was shown to be extremely abnormal in the *Sey/Sey* brain. Calretinin positive fibres did not follow their normal trajectory towards the telencephalon and there was complete absence of calretinin positive thalamocortical fibres in the *Sey/Sey* telencephalon at all ages investigated. DiI labelling in the thalamus also indicated the absence of thalamocortical axons from the telencephalon (results not shown). The absence of fibres in the telencephalon provokes the question of whether axons are incapable of entering the telencephalon because late born cortical precursors behave abnormally or if the problem occurs at the level of

the diencephalon or the cortical subplate. As discussed later, the answer to this question has important implications for the possible role of the thalamus in the migration of cortical layer III and IV precursors.

One would expect that if the problem arose at the level of the telencephalon, the fibres would behave relatively normally until they reached that level. The fasiculation of fibres in the diencephalon suggests that the problem occurs before the fibres leave the diencephalon. This could be due to (i) a failure of the axons to grow properly (ii) a lack of or inappropriate positioning of guidance cues for the fibres to follow (ii) an inability of the fibres to follow guidance cues.

Failure of axonal growth is a distinct possibiliy since the calretinin stain reveals that the thalamic nuclei from which the axons project do not develop properly. However, inadequate guidance cues may also play a role. Pax-6 is expressed in the ventral thalamus and in the amygdala (areas over which thalamocortical axons pass) at ages that coincide with thalamocortical invasion of the telencephalon (N. Warren, personal communication). It is therefore possible that these regions may not express the appropriate guidance cues necessary for normal axonal extension, or their morphology is so disorganised that guidance cues are expressed inappropriately. Indeed, Mastick *et al.* (1997) have shown that, in the prosencephalon Pax-6 is required for local navigational information used by axons passing through its domain of expression.

It is also possible that the thalamocortical axons themselves are unable to respond to guidance cues. L1 is a neural cell adhesion molecule that mediates cell interactions in the developing nervous system. It is expressed by thalamocortical axons in the cortical subplate (Fukuda *et al.*, 1997) and in the diencephalon (D. Caric, personal communication). Pax-6 is known to bind to the L1 promoter (Chalepakis, *et al.*, 1994). It is possible therefore that Pax-6 normally regulates the expression of L1 in thalamic axons to enable them to bind to reciprocal molecules, such as neurocan, which they colocalise with in the cortical subplate (Fukuda *et al.*,

1997). It would be interesting to study the expression of L1 in *Sey/Sey* brains in order to see if it is disrupted and may therefore contribute to the abnormal thalamocortical innervation in these mice.

Although, the fasiculation of fibres in the diencephalon suggest that the problem arises at that level, an alternative possibility is that the cortical subplate is not permissive to the ingrowth of thalamocortical axons. This has not been shown to date.

The possible consequences of an abnormal dorsal thalamus and lack of thalamocortical innervation on cortical development

As mentioned previously, postmitotic cortical precursors, which give rise to the superficial cortical layers accumulate in the cortical subventricular zone in the *Sey/Sey* mouse (Caric *et al*, 1997). However, cells born on E13 and E14 migrate normally and contribute to the formation of the cortical plate (Caric *et al.*, 1997). *Pax-6* is normally expressed in the cortical ventricular zone (Stoykova *et al*, 1996) but it is not expressed in postmitotic neurones, therefore its role in the telencephalon is likely to be concerned with cell proliferation. This raises the question of why late born cells don't migrate out of the SVZ and form normal cortical laminae. Caric *et al.* (1997) suggested that the inability to migrate was not a cell autonomous defect.

The present study suggests that Cajal-Retzius cells in the *Sey/Sey* cortical marginal zone are normal, at least in terms of their location and morphology, thus it is unlikely that they are responsible for the migrational defect. However, it was not shown if these cell secreted *reelin*, the transmembrane protein that is important for normal laminar organisation (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995). However, given that early born cortical precursors migrate normally, it seems unlikely that Cajal-Retzius cells and/or *reelin* would be involved. The same argument can be applied to radial glial cells. Although radial glia are disrupted in the Sey/Sey cortex Caric *et al.* (1997), the nature of the disruption, which is to course round the cell nodules made by late born precursors accumulating in the proliferative

zone, suggests that the disruption is secondary to the formation of the nodules. In other respects the radial glia are apparently normal (Caric *et al*, 1997).

The fact that the abnormal migration is restricted to late-born precursors suggests that whatever the environmental problem is, it is likely to be something that acts specifically on the late born cortical precursors. I propose that the abnormal migration of the E16 born precursors might be due, directly or indirectly, to the absence of a normal dorsal thalamus and/or thalamocortical innervation. I showed that in terms of morphology and calretinin staining, both the dorsal thalamus and a subset of thalamocortical axons (from the midline and intralaminar nuclei and the lateral dorsal and/or lateral posterior nuclei) were highly abnormal in the *Sey/Sey* brain. Sensory thalamocortical axons are also completely absent in the Sey/Sey mouse (T. Vitalis, personal communication).

Since the dorsal thalamus and the cerebral cortex are reciprocally innervated, they probably each play a role in the development of the other. Thus the inability of E16 born cells to migrate could affect thalamocortical innervation or the abnormal thalamus and lack of thalamocortical innervation could cause the migrational defect. Since thalamocortical axon trajectory is probably defective at the level of the diencephalon or the subplate, and because cortical layer III and IV cells are born in the ventricular zone after most dorsal thalamic nuclei have emerged, and after thalamocortical axons have invaded the cortical plate, it is more likely that the thalamus influences the early stages in the development of layer II/IV than vice versa. At this stage, it is not possible to differentiate between a permissive and an instructive role for the thalamus. That is, the failure of the late born cells to migrate could be because they have not been specified to a particular laminar fate, or it could be that their environment cannot support their migration. For example, it is possible that thalamic axons that extend radially into the cortical plate from the subplate, might help support radial migration. A transplant technique, similar to the one used by Caric *et al* (1997), could be used to test this. The most direct way to test if the thalamus is instructive or permissive would be to show if cells that are specified to a

laminar position in a normal environment are capable of migrating in the Sey/Sey cortex. However, since this requires that cells are transplanted into the lateral ventricle of the *Sey/Sey* mouse embryo, this would be technically very difficult because of the small size of the mouse embryos. Therefore the best way to test this would be to transplant *Sey/Sey* precursors after they have been allowed to complete the cell cycle *in situ* (that is, in an environment deprived of a normal dorsal thalamus), into a wild type rat brain. This is similar to the experiment carried out by Caric *et al* (1997), except that they transplanted cells immediately after labelling them in S-phase. Therefore, since the cells they transplanted underwent the final stages of the cell cycle in their new environment (in which they would be exposed to normal laminar fate cues), their results do not show if the new environment was merely permissive or also instructive.

In summary, the results of this study show that the dorsal thalamus is highly abnormal in the *Sey/Sey* mouse in terms of its gross morphology, the expression of calretinin positive cells and the projection of calretinin positive thalamocortical axons. The study also shows that CR cells are normal in terms of their location and gross morphology. Taken together, these findings are in concurrence with the results of Chapter 4 of this thesis which suggests a role for the thalamus in the migration (out of the cortical ventricular zone) of late born cortical precursors.

SUMMARY AND FUTURE EXPERIMENTS

In this study, I investigated neurogenesis and gliogenesis in the embryonic mouse forebrain. In particular, the processes of cell migration and cortical lamination were considered.

In Chapter 2 I investigated the distribution of cells born during neurogenesis, in late gestation and at neonatal stages. This study was designed to act as a correlate for the *in vitro* experiments to follow. A strict timetable for BrdU injection was established and the distribution of neuronal precursors during the period between E16 and P1 was characterised.

In Chapter 3 I investigated the distribution and antigenic properties of cells dividing during the transition between neurogenesis and gliogenesis. This investigation of the distribution of BrdU labelled cells was hindered by the proliferative nature of the cells which presumably accounted for their disappearance after short relatively short times. However, the study clearly showed the pattern of distribution of proliferating cells and the initial changes in their distribution. It also revealed the possible course for future experiments to characterise these populations. As described in the discussion to this chapter these experiments might include the use of additional markers of cell proliferation to reveal the nature of proliferation during the few days following the administration of the first marker. For completion, the study requires the used of additional markers of glial precursors and differentiated glial cells. At present there is a lack of specific markers for precursors of the various types of glia cells, however, there are number that are available that could be used. These include markers for O4 and DM20 (an isoform of the proteolipid protein, PLP), which are expressed on oligodendrocyte precursors, and vimentin, which is expressed early in the development of astrocytes. The temporal downregulation of QKI protein by neuronal cells could also be investigated. If this occurred early enough, then it might be possible to differentiate between neuronal and glia precursors at the time when they were proliferating.

In Chapter 4 I attempted to localise cortical laminar fate cues. I showed that the thalamus was important for the initial steps in the formation of cortical laminae. However it became clear that the technique employed could not differentiate between an instructive and a permissive role for the thalamus. In addition, since no new laminae formed *in vitro*, it was difficult to tell if this was because the cells had not received instructive cues or if the environment was simply not supportive of this. Thus, these experiments provide a springboard for the design of more specific experiments to define the role of the thalamus.

For example, BrdU could be injected *in vivo*, and after various short intervals cortical slices derived from treated animals could be cultured in isolation. If cells that were allowed to undergo S-phase and G2-phase *in vivo* migrated and formed normal cortical laminae in isolated cortical slices, this would provide much stronger evidence for an instructive role for the thalamus. However, it would be difficult to rule out the possibility that the role for the thalamus was to enable the cells to attach to the radial glia and it would not overcome the problem that new cortical laminae do not form *in vitro*. Therefore the transplant experiment described in Chapter 6 would also be appropriate. In this experiment the middle layer cortical precursors could be exposed to thalamic tissue or medium conditioned in thalamic tissue during the critical period of the cell cycle, before transplanting them into a normal *in vivo* environment. After at least five days, the distribution of densely and lightly BrdU labelled cells would be analysed to show if those cells that became postmitotic immediately after transplantation could migrate to and contribute to the formation of their normal cortical laminae. Another way of testing the role of the thalamus would be to transplant the same cells into an environment devoid of a normal thalamus, such as in the *Sey* mouse. If these cells migrated and formed new laminae this would provide conclusive evidence that the thalamus is required only in the early stages of the lamination process, that is during the time when the cells receive their instructive cues. However, this would be both technically difficult, given the small size of the

Sey/Sey embryos, and in any case the cells might not be able to migrate through the ectopic cell clusters in the subventricular zone.

If it was found that the thalamus did provide an instructive cue for cortical lamination, the next step would be to isolate and characterise the molecule involved. There are numerous possible candidates including neurotransmitters, growth factors and receptor-ligand systems, that are already know to play important roles in key developmental events such as proliferation and differentiation. To determine the nature of the molecule (secreted or cell attached) if would be sensible to begin by showing if the effect of the thalamus could be mimicked by medium conditioned with E16 thalamic tissue. Then, to isolate the molecule it would be practical to begin with the addition of blocking agents and antibodies, *in vitro*. If, for example, the effect of the thalamus/conditioned medium could be blocked by the addition of a neurotransmitter antagonist, this would provide some evidence for a role for that transmitter.

In Chapter 5 I asked if TTX could improve the survival and/or maintain the distribution of CR cells in cortical slice cultures. It was found that CR cell distribution was very disrupted in cortical slice cultures and the number of CR cells present was highly variable. It would be interesting to investigate the state of CR cells at 2 DIV in cortical slices cultured with and without thalamus to show if the improved migration in the co-culture could be accounted for by differences in the numbers and/or distribution of CR cells in these two conditions. However, given that there was little difference in the numbers or location of cells labelled with BrdU on E12, this seems unlikely. With reference to the question that was being addressed in Chapter 4 which was concerned with the slippage of *in vitro* born cells, I believe that the reason new laminae do not form *in vivo* is partly to do with the disruption of CR cells, but also the damage to the end feet of the radial glia cells when the pial membrane is stripped off. It would be interesting to ask if new laminae will form in cortical slices that retain their pial membrane. However, the pial membrane is a fibrous tissue that grows rapidly in culture, therefore it seems likely that the retention

of the pial membrane would be detrimental to the survival of the nervous tissue. I believe that embryonic cortical slices provide a valuable system for investigating the early processes in neuronal migration, but they not provide a particularly suitable system for studying layer formation. Presumably this is because the factors that are required for layer formation are absent or compromised. Undoubtedly these include the CR cells, the radial glial attachment to the glial limiting membrane, and possibly also cortical afferent connections.

Finally, in Chapter 6 I asked if (1) CR cells were present and (2) if the thalamus was normal in *Sey/Sey* mice in which migration and lamination of late born cortical precursors is aberrant. The CR cell population appeared normal at all ages investigated. However it would be useful to investigate the expression of reelin to show if it was present at normal concentrations in the *Sey/Sey* brains. Western blot analysis could be used to measure concentration of reelin in wild type and mutant brains.

The study showed that the dorsal thalamus and thalamocortical innervation was highly abnormal in the *Sey/Sey* mice. It was not conclusively shown that the failure in migration was directly due to the abnormalities in the thalamus. However, since the migration of early precursors appears normal in this mutant, the aberrant migration correlates strongly with the time point at which thalamocortical axons normally invade the cortex. I began a series of experiments in which cortical slices from *Sey/Sey* brains were co-cultured with wild type thalamus. It had been my intention to investigate the migration of *in vitro* born cells in these slices and to use both DiI and the antibody to calretinin to show if wild-type thalamic axons would invade the *Sey/Sey* cortices in the same way they did in the wild-type. If migration had taken place in these slices as in the wild type, this would have provided further evidence for a role for the thalamus in the normal process of laminar formation. If thalamic axons had invaded the cortical slices, this would have shown that the *Sey/Sey* cortex is permissive to the ingrowth of thalamic axons.

The results of this chapter were included in a comprehensive study of the diencephalon in the *Sey* mouse, with N. Warren, T Vitalis and D. J. Price, which has been submitted for publication.

In summary, this study strongly suggests that the thalamus plays an important role(s) in the formation of the middle cortical laminae. Although it is well established that the thalamus plays a role in regionalisation of the cortex, this is the first study to show an effect on laminar formation. Though the study was not conclusive with regard to the role of the thalamus, it provides a stepping stone for experiments to follow. As molecular techniques evolve, questions that were previously difficult to address can be resolved. The use of the cre-lox technique for example, will be useful in this respect. It is going be employed in this lab to specifically knock out the *Pax-6* gene in the thalamus at various times in development. This will be a particularly valuable in assessing the role of the thalamus in the formation of cortical layers. If cortical layers fail to form properly in an environment in which everything else is normal, it will provide stronger evidence for the role of the thalamus in this process.

Although it remains a distant goal, I hope that this study contributes to the prevention or treatment of neuronal migration anomalies that are associated with both mild and severe clinical features such as intractable epilepsy, mild to severe intellectual impairment, mental retardation, motor dysfunction and possibly dyslexia.

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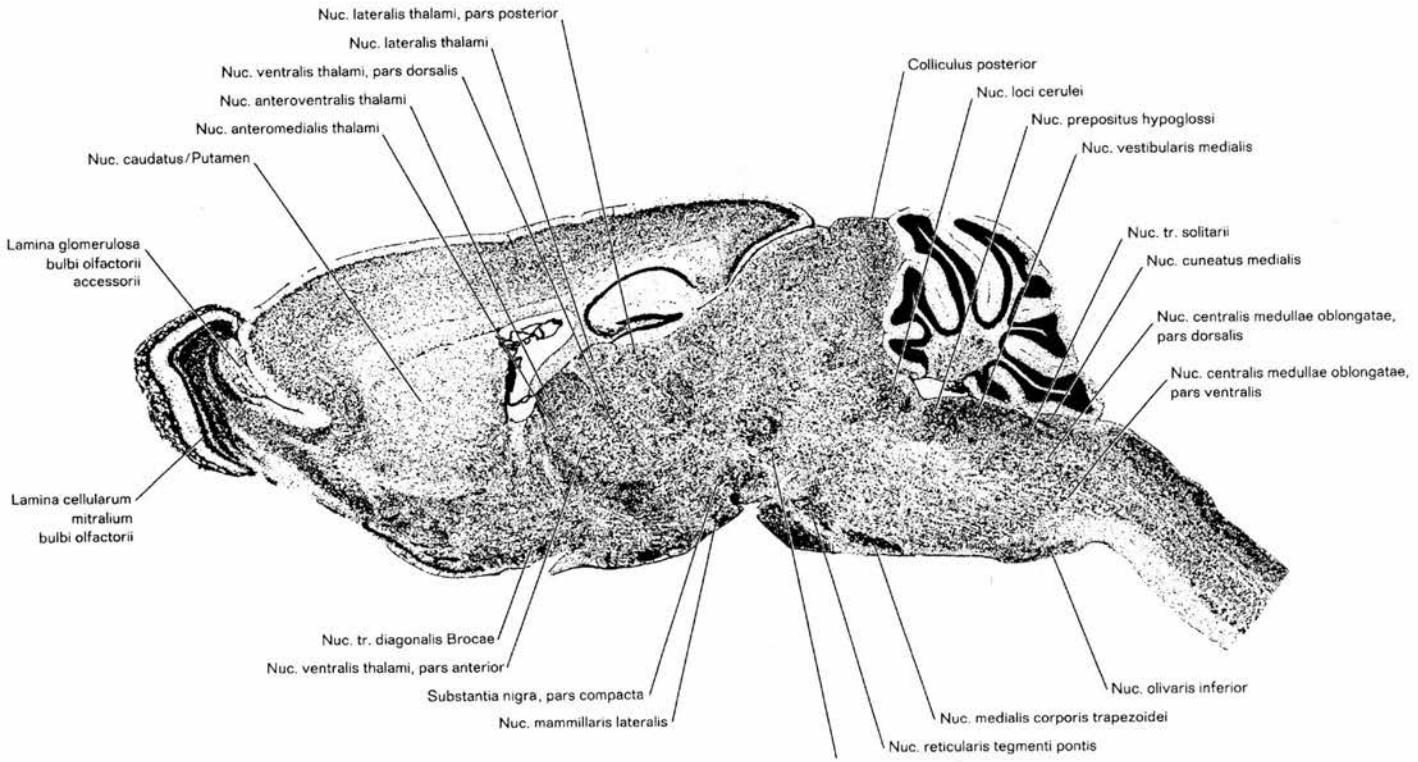

Figure Sagittal section 133 from Sidman et al. 1971

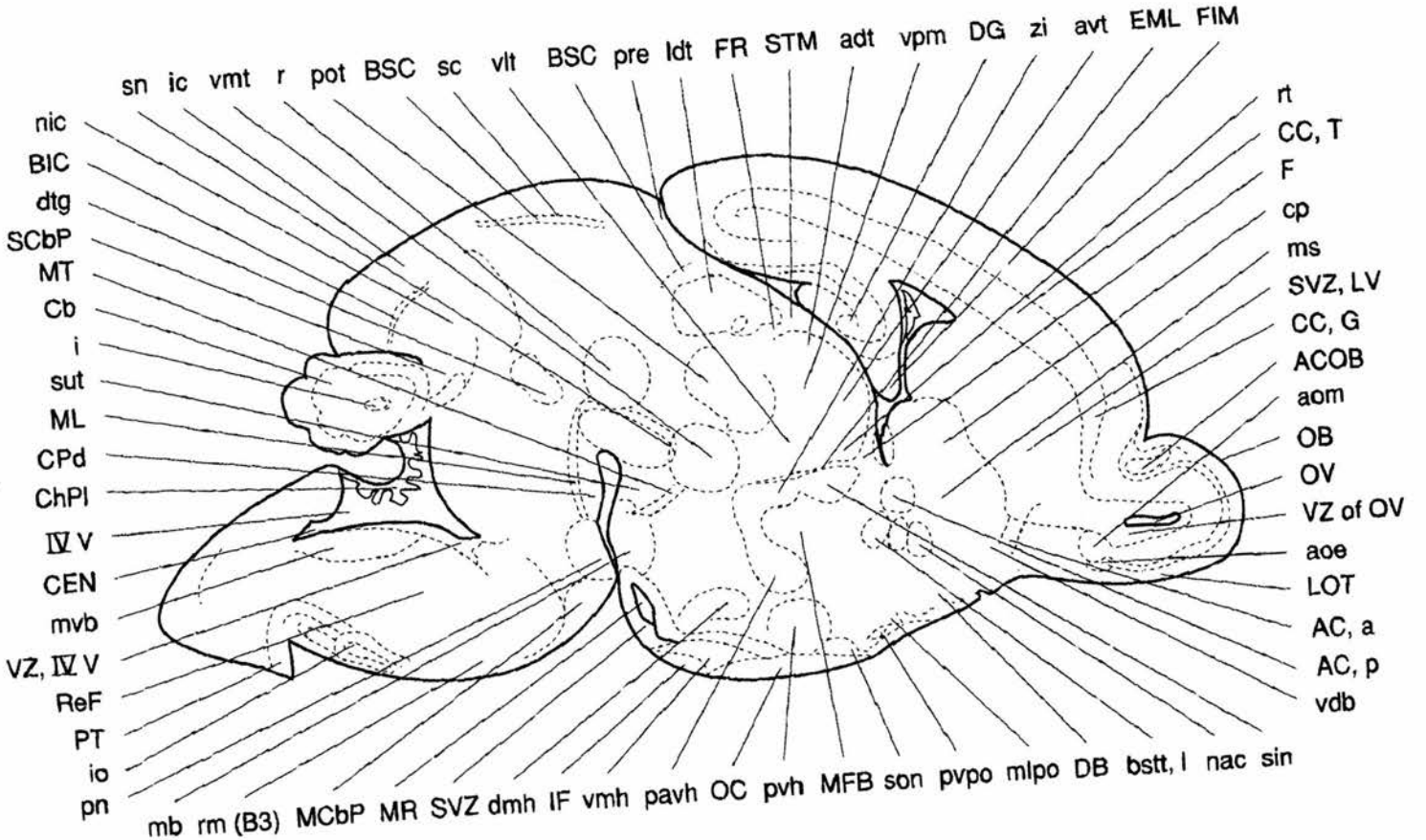

Figure GD18 SAG.7 from Shambra et al ,1992

Appendix

Culture medium

	Sigma Cat. No.	**Final conc.**
Mix together in a sterile beaker		
100 mls F12 (Hams)	N4888	
and		
100 mls Dulbeco's modified Eagle's medium	D5761	

Note: F12 contains more nutrients than DMEM (including amino acids and vitamins)

Add		
1 mg inuslin	I6634	5 μg/ml
2 mg apo-transferrin	T1147	10 μg/ml
3 ml HEPES buffer	H0887	
2 ml antibiotics (gentamycin and kanamycin)	G1264/K1377	
2 ml putrescene	P5780	16.11 μg/ml
20 μl progesterone	P8783	0.00629 μg/ml
20 μl Na_2SeO_3	S5261	0.0052 μg/ml
2 ml L-glutamine	G2128	25 μg/ml

Antibiotics:

Gentamycin sulphate (100 mg)

Kanamycin sulphate (200 mg)

Make up to 20 ml with double distilled water (sterile) then filter sterilise. Store in 1-ml aliquots at –20° C

Supplements:

100 μM **putrescene** (add 161.1 mg to100 ml sterile double distilled water and filter sterilise). Store in 2 ml aliquots at –70 ° C.

20 μM **progesterone** (add 6.29 mg to 100 ml ethanol). Store in 1 ml and then 50 μl aliquots at -70 ° C.

30 μM **Na_2SeO_3** (add 5.2 mg to 100 ml sterile double distilled water and filter sterilise). Store in 1 ml and then 50 μl aliquots at -70 ° C.

0.2 M **L-glutamine** (add 2.5 g to 100 ml sterile double distilled water and filter sterilise). Store in 2 ml aliquots at –70 ° C.

Notes:

Transferrin is a beta glycoprotein (iron transport protein in the blood). It transports iron to (and possibly within the cell) in culture. It may also have a detoxifying role.

Putrescene is the decarboxylated product of the amino acid ornithine. It is needed as a precursor for the synthesis of polyamines which are involved in cell growth and proliferation.

Selenium is a co-factor of glutathione peroxidase which is located in the cytosol and catalyses the reduction of radicals by the antioxidant glutathione.

L-glutamine is a non-essential amino acid which is an important precursor for nucleotide and structural protein synthesis. It is required for L-glutamine and GABA synthesis. At high concentrations is may have neurotoxic side effects.