A STUDY OF CLONAL MIGRATION IN THE DEVELOPING CENTRAL NERVOUS SYSTEM

by

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ABSTRACT

This study addressed several questions relating to the ontogeny of the central nervous system. By using a cell lineage marker, surgical and histochemical techniques coupled with three-dimensional reconstruction, the phenotype and spatial distribution of individual clones of cells in the rat somatosensory cortex were examined. This area contains a unique arrangement of cells, termed barrel/columns, which are thought to be morphological examples of the modular processing units in the neocortex. A retroviral vector was used as the lineage marker and was introduced into the lateral ventricle of embryos *in utero*. After birth the cerebral cortices of infected animals were processed histochemically in order to visualize barrel/columns and retrovirally labelled cells. Using computer aided three-dimensional reconstruction, clonal relationships were inferred by propinquity. The criteria used to define clones were probably correct as clones were found to be oriented non-randomly. Furthermore both the proportion of clonal types and their size were similar to those reported in previous experiments. A total of 112 clones of neurones were found in the reconstructions although only 28 of them were within barrel/columns. Twelve of these 28 clones were multicellular, yet in only one of these 12 cases were all the cells of a clone in the same barrel/column. The remaining clones spread across barrel/column boundaries. Although my findings were unexpected they were accounted for by several known features of cortical development.

In addition an attempt was made to produce an amphotropic retroviral vector capable of infecting cells in the early avain neural tube. Unfortunately all attempts at infection *in ovo*, with this or other retroviral vectors, were unsuccessful.

DEDICATION

To my parents and teachers

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ABBREVIATIONS

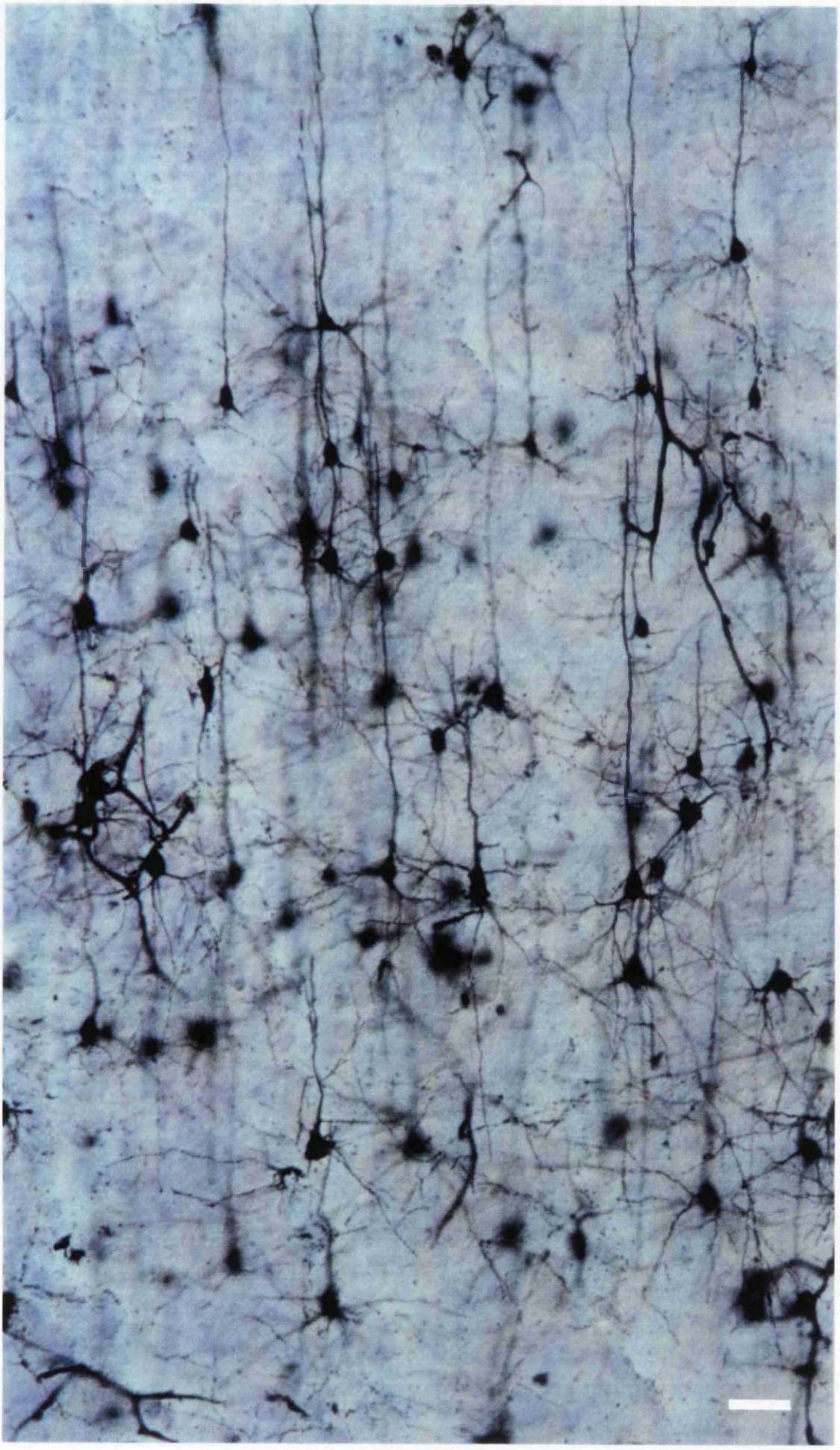
A	Adenosine
ACh	Acetylcholine
ALBSF	Anterolateral barrel sub-field
BrdU	5-Bromo-2'-deoxyuridine
bp	Base pairs
CEE	Chick embryo extract
cfu	Colony forming units
CP	Cortical plate
DAB	3,3'-Diaminobenzidine
DiI	1,1' Dioctadecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMEM	Dulbecco's modified Eagle medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
E	Embryonic day
EC	Embryonal carcinoma
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis (ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
FCS	Foetal calf serum
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
[^{3}H]-TdR	Tritiated thymidine
HAT	Hypoxanthine aminopterin thymidine
HH	Hamburger Hamilton
HIV	Human immunodeficiency virus
hr	Hour(s)
HS	Horse serum
IZ	Intermediate zone
kDa	Kilo Daltons
kbp	Kilobase pairs
LGN	Lateral geniculate nucleus
LIF	Leukaemia inhibitory factor
min	Minute(s)
MoMLV	Moloney murine leukaemia virus
MZ	Maginal zone
NBT	Nitro blue tetrazolium
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartic acid
NP40	Nonidet P40
NPY	Neuropeptide Y
O2A	Oligodendrocyte-type 2 astrocyte precursor
P	Postnatal day
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDL	Poly(D-lysine)
PEG	Polyethylene glycol
PIPES	Piperazine-NN'-bis-2-ethane sulphonic acids
PMBSF	Posteromedial barrel sub-field
PPL	Primordial plexiform layer
rpm	Revolutions per minute
SEM	Scanning Electron Microscope
Sm1	Primary somatosensory cortex
SSPROF	Serial section data entry program
SSRCON	Serial section reconstruction program
SVZ	Subventricular zone
SV40	Simian virus 40
T	Thymidine
VBN	Ventrobasal nucleus
VIP	Vasoactive intestinal polypeptide
VZ	Ventricular zone
X-Gal	5-Bromo-4-chloro-3-indolyl ß-D-galactopyranoside

FRONTISPIECE

A Coronal Section of a Three Week Old Rat Neocortex Stained by the 'Golgi' Preparation.

The Golgi stain was discovered accidentally over 100 years ago by Camillo Golgi and reveals with unsurpassed clarity the somata and processes of individual cells. Indeed the resolution of the technique has allowed impregnated cells to be classed into different morphological groups (Parnavelas *et al.*,1977). The stain was extensively used in the pioneering work of Ramón y Cajal and is still commonly used to this day (e.g. Braak and Braak,1985). It is particularly useful as only a very small randomly selected proportion of cells in a section are impregnated with reaction product. In the figure the superficial neocortical layers are towards the top of the picture. The scale bar (bottom right) represents 100μm.



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'Since the full grown forest turns out to be impenetrable and indefinable, why not revert to the study of the young wood, in the nursery stage.....?'

S.Ramón y Cajal (1852-1934)

1.1 Introduction

The adult CNS contains a bewildering array of cells that have been the subject of extensive anatomical study over the past one hundred years. The cells differ not only in morphology but also transmitter and receptor phenotype, serological type and a plethora of other features. Attempts to classify this diversity have centred around the fact that some cells share a number of common typological traits. For example neurones and glia are two broad classes of cells that can be distinguished by certain specific typological characteristics and physiological properties. Each cell in these two classes can be further subdivided into more specialized categories. For example CNS neurones can be classed as either pyramidal or non-pyramidal and even further sub-divided by neurotransmitter content.

In addition to typological classification cells can also be defined topologically. Two neurones for example may be indistinguishable biochemically but because they occupy different positions in the CNS they are positionally different, and perhaps will have different physiological functions. For example a motoneurone located caudally in the spinal cord will innervate a different target from a phenotypically identical motoneurone located rostrally.

Thus each cell comprising the adult CNS can be defined both typologically and topologically. These two features constitute a cell's fate. To the developmental neurobiologist an obvious question arising is how is this diversity generated in the embryo? Perhaps this problem is most acute in the CNS where there is such an enormous diversity of phenotypes. Theoretically there are two mechanisms via which fate determination may be specified during development (although they may not be mutually exclusive). First there is the influence of cell lineage, the geneological relationships of progenitor cells and their progeny. In this scenario cells inherit molecules from their parent that direct them on a specific pathway of differentiation. For example a progenitor cell may produce only cells that will adopt a neuronal phenotype or cells that share the same positional characteristics. In the former case for example the presumptive neurones inherit some molecules from the parent which direct the expression/repression of genes present/absent in the neuronal phenotype.

Alternatively fate determination could involve the influence of the local environment that a cell occupies (transiently or permanently). For example an individual progenitor may produce several phenotypes or cells that occupy positionally different parts of the body. In this case the fate of a cell is not directly related to lineage but is governed by epigenetic interactions within the local environment. This mechanism fate determination involves signal transduction at the cell membrane which via second messages directs the expression/repression of specific genes.

The mechanism responsible for fate determination in a tissue can be studied by an examination of cell lineage. Classically a cell lineage

study involves the introduction of a vital, cell autonomous marker of some kind (several have been used with different degrees of success, see Price,1989) into an individual progenitor cell. The test is based on the fact that if several fates are generated by a single progenitor then it clearly was not determined at the time of labelling. For example if a single progenitor cell generated several phenotypes or cells that crossed a physiological boundary (see figure 1.1A) then fate was not determined at the time of labelling. Thus as the progeny had different fates, fate was either determined later in development (see figure 1.1C) or determined by environmental influences. However if only a single phenotype was generated or if the progeny all shared common positional characteristics then the progenitor *may* have been determined at the time of labelling. It is important to appreciate that this result suggests, but does not conclusively prove, that a cell was determined at the time of labelling. Perhaps the cells in a clone all shared the same positional characteristics simply because a positional boundary was not challenged (figure 1.1B). Furthermore perhaps only one phenotype was generated by a progenitor simply because a clone was small in size. The fate of such a small clone could be determined environmentally but the cells by chance could share a common fate.

Cell lineage studies do not address if cells were irreversibly committed to a fate or subjected to some extrinsic influence that directed them on a specific pathway of differentiation. The state of commitment is addressed by transplantation of cells to ectopic sites or transposition to *in vitro* culture. If they were committed, then in their new environment the cells will continue on their presumptive pathway of differentiation (e.g. McConnell,1988b) but if not they will adopt a fate characteristic of their new location (e.g. O'Leary and Stanfield,1989) or follow an ectopic pathway (e.g. Omlin and Waldmeyer,1989).

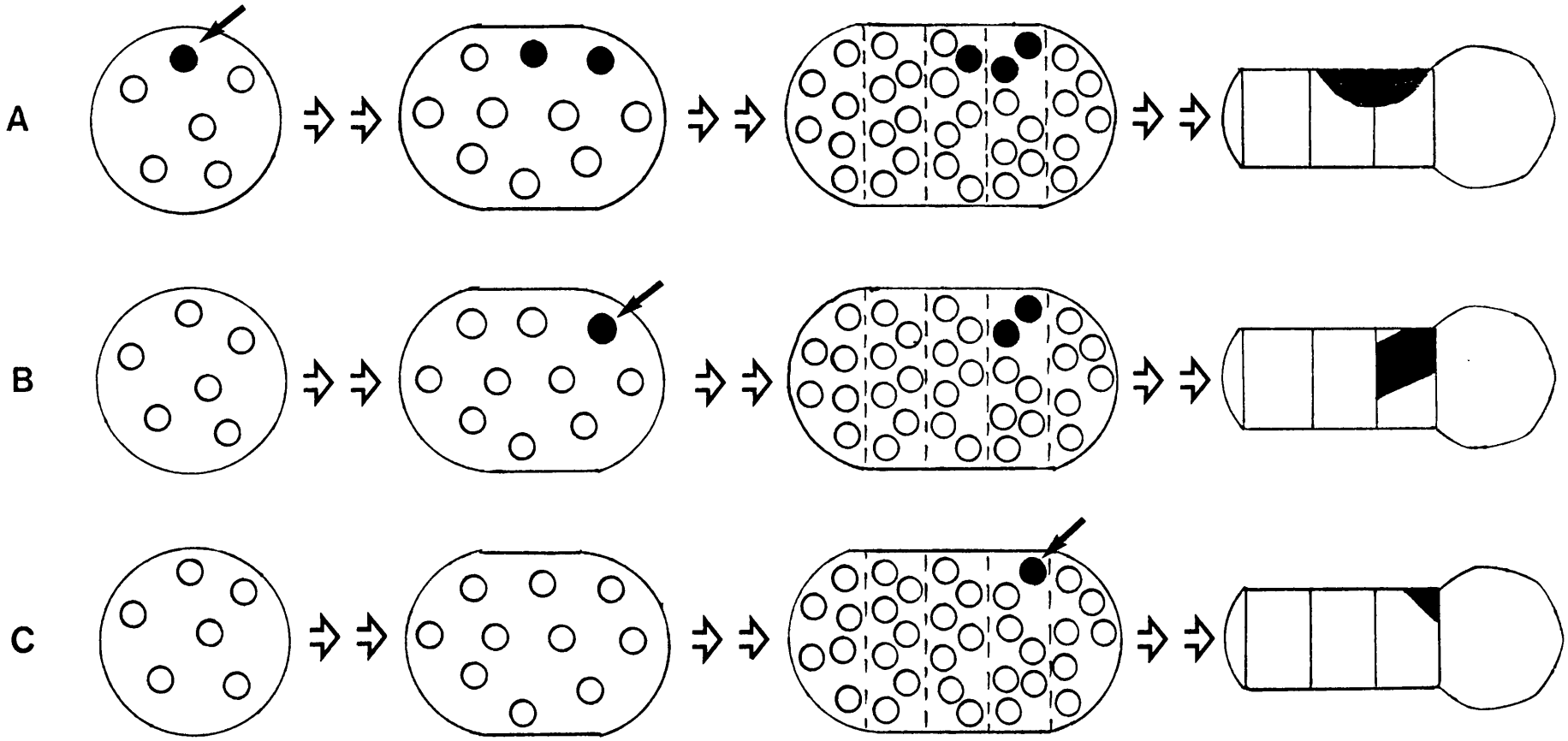
1.2 Cell Lineage in the Vertebrate Neural Tube

Unfortunately, principally for technical reasons, the majority of cell lineage or transplantation studies in the past century have been restricted to invertebrates or large vertebrate embryos (e.g. Weisblat *et al.*,1980; Sulston *et al.*,1983; Technau,1987). However two techniques have recently emerged which have greatly increased the number of cell lineage studies performed in vertebrate embryos. The first is the introduction of the ionotophoretic injection technique (Wetts and Fraser,1988). The strength of the technique is that one can select particular cells for injection. Thus, theoretically at least, small target cells can be labelled with dyes and the lineage examined. After ionotophoretic injection of a vital dye into individual progenitor cells in the chick hindbrain, Fraser *et al.*,(1990) examined cell lineage in relation to morphological structures termed rhombomeres. Preliminary data suggested that all the cells of a clone shared the same phenotype (A.G.S.Lumsden, personal communication). This is a unique observation as data presented below for chick tectum and spinal cord suggests that the

Figure 1.1. An Illustration of How a Study of Cell Lineage Can be Used to Examine Positional Specification in the Developing Embryo.

Cells that have been labelled with a lineage marker are coloured black. The time at which individual cells were labelled is indicated by an arrow. If the progeny of a cell labelled very early in development were not restricted to a particular part of the embryo then the progenitor was not positionally specified. This is the case shown in A. However if a cell is labelled after it is positionally specificied then its progeny will be found within a particular unit in the animal (as in C). Note that the converse of the above does not always apply. In B the progenitor was not positionally specified at the time of labelling although progeny were found within a unit in the adult.

Positional determination does not necessarily imply phenotypic determination or *vice versa*. Thus the progeny of a positionally determined cell, such as that in figure 1.1C, could be composed of several phenotypes.


A
B
C

progenitors are multipotential. However although typologically determined early in development, the progenitors were not topologically determined. When progenitors were labelled prior to the appearance of rhombomeres, their progeny were able to spread freely to such an extent that when analyzed in the more mature embryo some labelled cells were found in a neighbouring rhombomere. However this spread did not occur when cells were labelled after rhombomere formation; clones often abutted a neighbouring rhombomere but did not spread into it (Fraser *et al.*,1990). Thus a cell lineage study demonstrated that progenitors in the hindbrain were topologically determined when labelled after rhombomere formation. Unfortunately, although promising, the ionotophoretic injection technique is expensive and difficult to perform technically and not universally used. Certainly a cheaper, more frequently used technique involves the use of virus lineage markers.

The application of retroviral vectors to cell lineage, has recently emerged and been applied to lineage problems in different germinal regions in the CNS by several independent groups (the advantages of using retroviral vectors are discussed further in 1.12). Perhaps the most striking facts to emerge from lineage studies using retroviral vectors have centred around the development of the mammalian retina. Here a single multipotential progenitor cell can generate up to six different cell types, including neurones and Müller glia (Turner *et al.*,1990). Only retinal astrocytes appear to arise from a separate cell lineage (Watanabe and Raff,1988). Virtually all proliferating cells in the mouse retina at E13 and E14 are multipotential (Turner *et al.*,1990) and even the last division of such a cell can result in the generation of two different types of cell (Turner and Cepko,1987). Consequently it was proposed that cell fate was determined in the retina at or just after division of the multipotential precursor cell, by interactions between the cell and its environment (Turner *et al.*,1990). A second feature to emerge from these retina studies was that all of the clones observed were found in a strict radial traverse of the retina (Turner *et al.*,1990) implying that the labelled progenitors were topologically determined. This finding is particularly interesting in view of the somatotopic relationships between retina and tectum. Whether the progenitors were committed to a particular fate, i.e. to a particular somatotopic projection, is not yet known however. This issue will need to be addressed by transplantation studies of progenitors to ectopic locations in the retina.

Retroviral vectors have been applied to the study of lineages in the early CNS of birds. Avian embryos are particularly useful as a model system for early lineage studies as the neural tube, in particular the optic tectum, in the early chick embryo is particularly large, well characterized and accessible at all developmental stages (Summerbell and Hornbruch,1981; Hamburger and Hamilton,1952) and hence an easy target for injection of retroviral vectors. Individual progenitors in the optic tectum were multipotential as retrovirally labelled clones contained a

number of neuronal phenotypes and astrocytes (Gray *et al.*,1988; Galileo *et al.*,1990; Gray and Sanes,1991). A particularly striking feature of the data was that the daughter cells were strongly radially oriented during migration to and within the mature tectal plate (Gray *et al.*,1988; Gray and Sanes,1991). This radial distribution was apparently mediated by radial glial guides (Gray and Sanes,1991) and as the tectum has a physiological radial organization the progenitors could have been determined at the time of labelling. However the radial distribution was not totally precise. Some cells were spread tangentially several hundred microns from their siblings (Gray *et al.*,1988; Gray and Sanes,1991). The spread of these cells occurred exclusively within the mediolateral plane and was possibly mediated by fascicles of axons (Gray and Sanes,1991). These outlying cells appeared to differentiate exclusively into the 'multipolar' cells present in the major tectal efferent cell layer (layer 13). However it was not clear from this study if the multipolar cells were committed to their phenotype after they were born. Alternatively the multipolar cell fate could be dictated by environmental influences e.g. contact with fascicles of axons. Furthermore later in development more cells that appeared to be astrocytes, deviated from the precise radial distribution. This second dispersion may be mediated by the penetration of retinal afferents into the tectum (Gray and Sanes,1991).

The developing chick spinal cord has been the subject of a recent lineage study using retroviruses (Leber *et al.*,1990; Gray *et al.*,1990). Individual clones in the spinal cord contained a variety of phenotypes. The majority of clones contained both motoneurones and glia, although clones consisting exclusively of motoneurones were present, but in a minority (Leber *et al.*,1990). There was also little evidence of topological determination of spinal cord progenitors. Clonally related motoneurones were found in different motoneurone pools and hence innervated different targets. All clones initially migrated radially from the germinal region (Gray *et al.*,1990) although some clones subsequently migrated either ventrally or dorsally, perhaps by following an axonal substrate (Gray *et al.*,1990). Furthermore although clonal spread appeared to be restricted to the mediolateral plane a rostrocaudal dispersion of oligodendrocytes was observed at more mature stages.

Thus the data generated from the spinal cord to date have some striking features in common with that from the developing optic tectum. This is surprising as although the spinal cord has both laminar and nuclear features, unlike the optic tectum it does not have a radial organization. During the development of both chick tectum and spinal cord migrating cells were radially aligned, possibly by radial glial guides. Subsequently however a tangential migration orthogonal to the radial arrays occurred and was probably dictated by axon fascicles. This dispersion, for both tectum and cord, appeared to be restricted to the mediolateral axis although later in development there was a rostrocaudal

dispersion of glial cells. Finally in both tectum and spinal cord the precursor cells showed no obvious restriction of developmental fate to the neuronal or glial phenotype. Thus different parts of the CNS, in the chick at least, appear to be constructed using very similar principles.

Cell lineage in the mammalian cerebral cortex has also been studied and the emerging story suggests that lineage is similar to the retina, optic tectum and spinal cord in some respects, but also has some distinct differences (the precise details of the lineage are discussed in 1.13). The subject of the spatial distribution of clones has been the matter of some controversy, possibly because two earlier studies in the optic tectum and retina demonstrated a strict radial distribution of clones. All three groups using retroviruses to study cortical development found a radial migration, as suggested by classic studies, but also found some non-radial displacement of cells. One group (Luskin *et al.*,1988) noted a predominant radial arrangement whereas the others (Price and Thurlow,1988; Walsh and Cepko,1988) stressed the existence of non-radial dispersal. These discrepencies apparently reflected differences in emphasis as all three groups agreed that migration was much more strongly radial in the optic tectum. However none of the studies examined if clonal boundaries were restricted within the functional boundaries of the cerebral cortex.

No study to date has extensively examined clonal spread or phenotype in the early avian forebrain. The developing chick forebrain has a number of differences and similarities with the mammalian counterpart (see Gray *et al.*,1990). For example radial glial guides are present in the chick forebrain (Gray *et al.*,1990) and mammalian forebrain (see 1.3.5) and may prevent a lateral dispersion of clones (see 1.8). Indeed for part of my study an examination of cell lineage in the chick forebrain was attempted with retroviral vectors (see Chapters Two and Four). Possibly the chick forebrain may be constructed via the lineages outlined above for the optic tectum and spinal cord. However a considerable part of my study also involved an examination of cell lineage in the late mammalian CNS, specifically the developing cerebral cortex.

1.2.1 The Anatomy of the Cerebral Cortex

In the human the cerebral cortices contribute two-thirds of total neuronal mass, contain about three-quarters of all synapses (Rakic,1988a) and play a pivotal role in most complex functions of the human nervous system such as language, cognition and memory (Geschwind,1965).

Over the past 100 years the cerebral cortices have been well studied anatomically, particularly by use of the Golgi and Nissl stains. Each cerebral cortex is partly composed of a six layered structure, unique to mammals, termed neocortex. The neocortex is also parcelled into 'areas', whose boundaries run perpendicular to the neocortical layers. The six neocortical layers are continuous throughout these areas; however there

are subtle differences between areas in the morphology and functional characteristics of the cells. These local idiosyncrasies constitute the 'cytoarchitectonic' map of the cortex (Caviness,1975). For example, the rodent somatosensory cortex contains an anatomically and functionally distinctive arrangement of neurones devoted to the processing of sensory input from the vibrissae (see 1.5). Furthermore each cortical area has distinctive interconnections with other cortical areas and subcortical structures, which lead to a functional role. For example area 17 (the primary visual cortex) is interconnected with the lateral geniculate nucleus (LGN) and the primary somatosensory cortex with the ventrobasal nucleus (VBN). Specifically in each cortical area thalamocortical input terminates mainly in layer IV and neurones in layer VI of that area send projections back to the same thalamic nucleus (reviewed by McConnell,1988a).

Only in the past 20 years, principally for technical reasons, has the development of the neocortex been the subject of experimental study. One specific aspect of cortical development of which we are ignorant is cell lineage. For example, developmental neurobiologists are interested in the lineage relationships between the many different types of cortical cells and in how individual clones of cells disperse during the development of the cerebral cortices.

A study of the spatial relationships of individual clones of cells in the developing neocortex occupied a considerable part of my research. Hence part of this Chapter is devoted to a discussion about neocortical development to provide a background to the topic. Then discussed are the tools and techniques used to study cell lineage in both the rat cerebral cortices and the early avian neural tube.

1.3 The Development of the Cerebral Cortex

Cortical development, like the development of most of the CNS, can be considered to be two distinct phases.

(i) First, there is a period of cytogenesis and histogenesis in which the cortical cells are generated and assembled into the appropriate laminar order (discussed in 1.3.1). In the human this is a monumental achievement considering that some 20 billion cortical neurones are generated within the space of a few weeks. However the timing and length of this phase in development varies between different species (e.g. Saunders *et al.*,1989; McConnell,1988b; Rakic,1977). In the rat it is accomplished during the final (third) week of gestation (Berry and Rogers,1965). This phase has a number of similarities with epidermal cytogenesis (Watt,1988; see 1.3.1). This similarity is particularly interesting in view of the fact that neuroectoderm, like epidermis, is ectodermally derived.

(ii) Cytogenesis and histogenesis are followed by a protracted period of neuronal differentiation during which dendritic and axonal processes are

elaborated (see 1.3.6). Synapses and characteristic cytoarchitectonic areas are formed during this phase. These events occur during the first postnatal month in rodents (Blue and Parnavelas,1983).

Both neocortical cytogenesis and histogenesis are disrupted by a wide variety of heritable factors (Caviness *et al.*,1988,1989) and teratogens (Miller,1986). Indeed both genetical studies and teratogens have been used experimentally to generate some insight into normal cortical development (e.g. Jensen and Killackey,1984; Caviness *et al.*,1988).

1.3.1 Neocortical Cytogenesis and Histogenesis

The neocortical neurones and macroglia are not generated at random positions throughout the developing neocortex. Instead the germinal region, a pseudostratified neuroepithelium termed the ventricular zone (VZ) (Boulder Committee,1970), surrounds the lateral ventricles in the forebrain (figures 1.2 and 1.3; Seymour and Berry,1975; Hinds and Ruffett,1971; Sturrock,1982). In common with its epithelial nature the VZ shows desmosomal adhesion (Shoukimas and Hinds,1978; Hirst *et al.*,1991), gap junctional communication (Dermietzel *et al.*,1989; Keane *et al.*,1988) nuclear interkinetic migration (Sauer,1935; Fujita,1963) and migration of progeny superficially (Rakic,1972). However it is not known how the VZ cells divide. They could divide in a proliferative symmetrical mode whereby two equal daughters are produced e.g. like the $O2A^{perinatal}$ cell (Temple and Raff,1986). However they could also divide asymmetrically in a stem cell mode whereby two unequal daughters are produced (Cairns,1975; Stent,1985). Such an asymmetric mode of division is quite feasible in the developing CNS and does occur with the $O2A^{adult}$ (Wolswijk and Noble,1989) progenitor cell (Noble *et al.*,1990). In the developing retina however, some progenitors undergo a phase of symmetrical division (Turner *et al.*,1990).

Whatever mode(s) of mitosis occur in the VZ, several features of the kinetics of division are known. First, the VZ cells divide asynchronously (Sauer,1935). Second the cell cycle lengthens throughout the neural tube during development (Kauffman,1968) including in the cerebral cortex (Hoshino *et al.*,1973; Korr,1980). This is due to a specific lengthening of the G1 phase. Third, in the rat VZ there are two co-existing populations of VZ cells with different cell cycle times (Waechter and Jaensch,1972; Altman and Bayer,1990). It is not known if these two types of VZ cells correspond to cells committed to different fates. In addition to these known features of division at the VZ, a model has been postulated which suggests that it is possible to predict how the progeny of a mitotic event at the VZ will distribute themselves with respect to one another (Martin,1967; Tuckett and Morriss-Kay,1985). The model suggests that the plane of cleavage of the VZ cells dictates the fate of the progeny. For example after a horizontal plane of cleavage the two progeny will remain at the VZ, but after a radial cleavage they will separate from one another. The model is attractive

Figure 1.2. A Coronal Section of Part of the Developing Telencephalon of an E16 Rat Embryo.

The section is stained by the Holmes stain (see Hirst *et al.*,1991). See figure 1.3 for a full explanation of the anatomy. Scale bar 100μm.

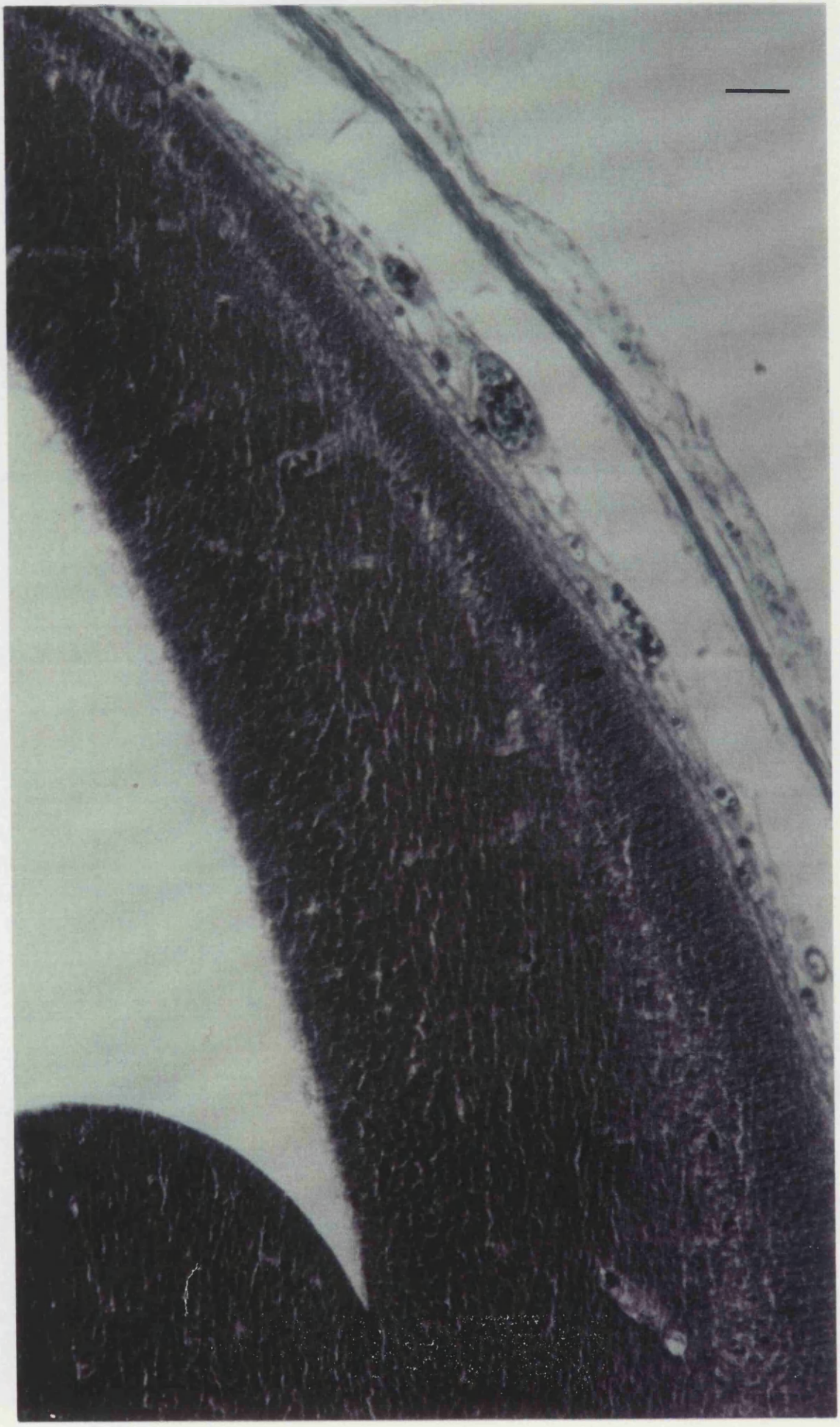
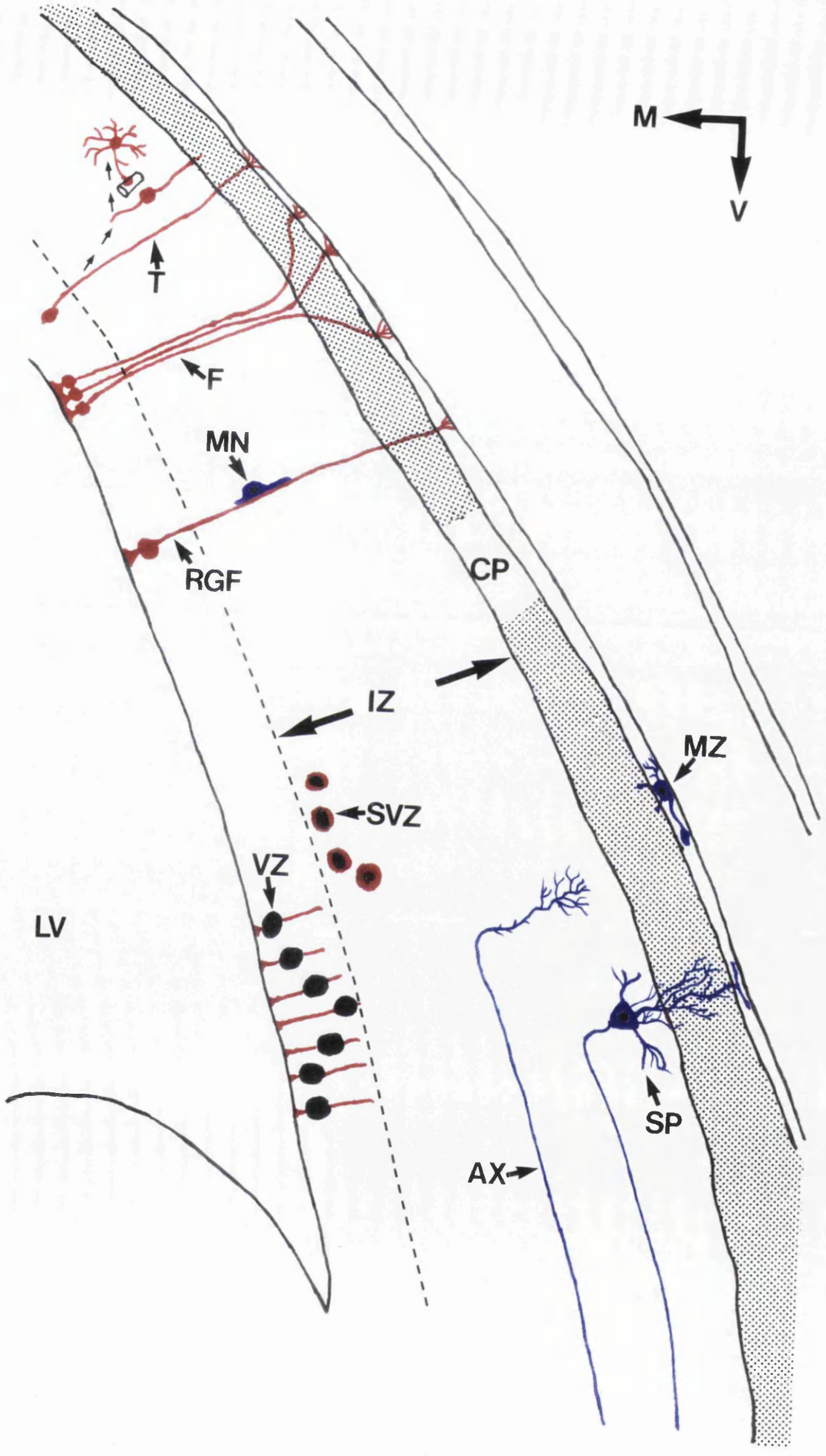


Figure 1.3. A Cartoon Depicting the Different Cells and Zones Within the Developing Rat Telencephalon.

The figure is a drawing based on the photograph shown in figure 1.2. However for clarity the cells are not drawn to scale. MN denotes a neurone migrating along a radial glial fibre (RGF), through the intermediate zone (IZ) *en route* to the cortical plate (CP). Migrating cells move superficially at rates between 10-20μm/hr (Hicks and D'Amato,1968). The two germinal regions are shown: the ventricular zone (VZ), with the nuclei undergoing interkinetic migration (see text), and the subventricular zone (SVZ). The CP is sandwiched in between cells in the marginal zone (MZ) and the subplate (SP). A thalamocortical afferent (AX) is shown 'waiting' in the SP region. Prior to this waiting period the afferents grow at speeds up to 40μm/hr (Blakemore and Molnár,1990). The VZ borders the lateral ventricle (LV). A population of radial glia are shown fasciculating (F) within the IZ and defasciculating within the CP. Radial glia are also shown transforming (T) into astrocytes.


M
V
T
F
MN
RGF
CP
IZ
MZ
SVZ
VZ
LV
SP
AX

and has also been proposed in other developmental systems (e.g. Hyman and White,1987). Indeed a transition from horizontal to vertical cleavage planes is associated with the initiation of neurogenesis in the neural tube (Martin,1967). However it is not clear if this has any relevance to the development of the cerebral cortices. In the rodent VZ 90% of the cleavage planes are oriented radially and the figure remains approximately constant throughout development (Smart,1973).

The nature of the molecules, either soluble or membrane bound, controlling cell proliferation at the VZ are not known. However some soluble molecules are mitogens for VZ cells albeit not in the cerebrum (Cattaneo and McKay,1990). Cell-cell interactions have been very successfully studied in the *Drosophila* neurogenic region (Campos-Ortega and Knust,1990). One of the cell surface molecules involved in transducing a proliferative signal in the *Drosophila* neurogenic region has a vertebrate homologue expressed in the VZ (Coffman *et al.*,1990). This suggests that some cell surface molecules involved in cell cycle control during development may be common to both vertebrate and invertebrate Phyla.

A variety of techniques have been applied to study the fate of individual VZ cells (e.g. Luskin *et al.*,1988; Temple,1989; Levitt *et al.*,1981). The data obtained to date suggest that some VZ cells appear to produce neurones only, some only glia and some generate both neurones and glia (discussed further in 1.13).

The second embryonic zone to be formed, the marginal zone (MZ) appears at the pial surface of the VZ and establishes a visible polarity in the neuroepithelium (figure 1.3). The MZ was initially defined as being composed of the outermost cytoplasmic processes of the VZ cells (Boulder Committee,1970). However Marín-Padilla (1978) subsequently renamed the MZ the primordial plexiform layer (PPL) as it is not cell sparse but is occupied by a population of horizontally oriented cells, that appear to be neurones (Chun *et al.*,1987; Marín-Padilla,1990) (see 1.3.2).

The intermediate zone (IZ) is formed next between the VZ and MZ by the migrating progeny of VZ cells (Boulder Committee,1970) and subsequently expands due to invasion by axons (figure 1.3).

After IZ formation, at approximately E15 in the rat (Raedler *et al.*,1980; Altman and Bayer,1990), a second germinal region the subventricular zone (SVZ) is formed between the VZ and IZ (Boulder Committee,1970) (figure 1.3). This zone is occupied by small round mitotic cells (Sturrock and Smart,1980), which unlike VZ cells remain both static, without showing interkinetic nuclear migration (Smart,1961; Rakic *et al.*,1974), and proliferative into adulthood (Sturrock,1979; McDermott and Lantos,1990). The SVZ is less dense than the VZ and the cells are more randomly oriented (Van Eden *et al.*,1989). The exact nature of the cells produced by this zone i.e. neuronal, glial or both is not known, although in the macaque some of the SVZ cells are immunoreactive for an astrocyte specific protein, glial fibrillary

acidic protein (GFAP) (Levitt *et al.*,1981). This suggests that some SVZ cells are produce astrocytes (although some intermediate filament proteins, such as GFAP, are expressed ectopically during development).

At the junction of the IZ and MZ migrating cells stop migrating and form the cortical plate (CP) (Boulder Committee,1970) (figure 1.3). The CP in the rat is formed first in the lateral aspect of the telencephalon at E15 (Raedler *et al.*,1980; Valverde *et al.*,1989).

All of the embryonic zones, with the exception of the SVZ, mentioned above are ephemeral. After neocortical histogenesis has ceased, the VZ transforms into an essentially post-mitotic (Korr,1980) ciliated ependymal layer (Gould *et al.*,1990). The IZ ultimately transforms into the cortical white matter, the CP into the grey matter and the MZ into neocortical layer I.

One histogenetic feature often overlooked is the development of the vasculature of the cerebral cortex. This is surprising as in addition to providing a circulatory supply, the vasculature may directly influence neuronal development. Initially the forebrain is avascular but is invaded radially on E12 by endothelial cells from the leptomeninges (lying above the MZ in figure 1.3) (Strong,1964; Duckett,1971; Risau and Wolburg,1990). The vasculature certainly influences the morphology of cortical astrocytes which project end-feet to the endothelial cells (Peters *et al.*,1976) and induce a permeability barrier termed the blood-brain barrier (Janzer and Raff,1987).

Two tentative pieces of evidence suggest endothelial cells are directly involved in histogenesis. First, blood vessels are oriented in the cortex in a non-random manner (Patel,1983) suggesting that they could act as a regular scaffold within which histogenesis takes place. Second, endothelial cells have been shown to stabilize glial development in the optic nerve (Lillien *et al.*,1990). It is possible, although unproven to date, that they could have an analogous role in the cerebral cortex.

1.3.2 The Generation Sequence of the Neocortical Layers

After they are generated in the VZ, neurones never divide again (Rakic,1985a). However gliogenesis continues postnatally. This has enabled birth-dating studies of neurones employing either [^{3}H]TdR (Angevine and Sidman,1961) or BrdU (Miller and Nowakowski,1988). Experiments using these labels have demonstrated two important points:

(i) Cortical neurogenesis in the rat begins at E13 (Raedler and Raedler,1978) and is complete by the time of birth (E21/22) (Berry and Rogers,1965).

(ii) The neocortical layers are formed sequentially (Angevine and Sidman,1961; Berry and Rogers,1965; Rakic,1974). The first generated neurones lie in the deepest cortical layers, while later-born neurones occupy successively more superficial layers.

This 'inside-out' manner of migration occurs in all mammals examined (see references cited by Sanderson and Weller,1990), but adherence to this rule is more strict in primates than in rodents. In the latter there is a considerable spread of neurones generated at one particular time point throughout the neocortical layers. This spread is more pronounced at later developmental ages when the more superficial layers are generated (Cavanagh and Parnavelas,1988). The inside-out gradient is a characteristic feature of the mammalian telencephalon, since in reptiles neurones settle in an opposite 'outside-in' manner (Goffinet *et al.*,1986).

The gradient of neurogenesis in mammals mentioned above is not synchronous throughout the entire mediolateral arc of cortex. Superimposed upon the inside-out gradient is a mediolateral gradient (Smart and Smart,1982). Neurogenesis is about 24 hrs more advanced in lateral cortex relative to medial cortex. Consequently cells in any given lamina are generated earlier in more lateral cortex than the equivalent cells of more medial cortex. This means that during neurogenesis the CP is thicker laterally than medially (see figures 1.2 and 1.3; Smart and Smart,1982; Van Eden *et al.*,1989). This mediolateral gradient is not an exclusive feature of CP development as it is also seen with the generation of the neurones within the PPL (Bayer and Altman,1990) and generation of SVZ cells (Raedler *et al.*,1980). Cortical development is further complicated by a slight anteroposterior gradient (Smart,1983; Sanderson and Weller,1990). Hence overall the neurogenic gradient in the cerebral cortices is from anterolateral to posteromedial. Such gradients of development are common and occur in a wide variety of species and organs. For example *Drosophila* ommatidial development proceeds from posterior to anterior (Tomlinson,1988), vertebrate retinal development proceeds from central to peripheral (Young,1985) and spinal cord development proceeds from ventral to dorsal (Nornes and Das,1974). The gradients presumably reflect a graded distribution of signalling molecule(s).

The cortical layers are not formed below or above the PPL. On the basis of anatomical evidence Marín-Padilla (1978) suggested a mechanism whereby the CP forms *within* the PPL, dividing the latter into a superficial (the MZ) and deep layer (the subplate, SP) (figure 1.3). According to this hypothesis the CP is positioned between the SP and MZ in a 'cocoon or sandwich-like' fashion. The CP neurones are then assembled within this 'sandwich' in the 'inside-out' fashion described above. However the early generated PPL neurones differ from those in other neocortical layers in several respects. In the rat, MZ neurones are generated before the SP neurones, and thus the generation sequence is like that found in reptiles, i.e. 'outside-in' (Raedler and Raedler,1978; König *et al.*,1977; Valverde *et al.*,1989). Also although most, but not all, of the MZ (Parnavelas and Edmunds,1983) and SP (Valverde and Facal-Valverde,1987; Valverde *et al.*,1989) neurones subsequently die postnatally, they have a function. Both receive

functional synaptic inputs before neurones in the CP (Friauf *et al.*,1990; Kostović and Rakic,1990; Chun and Shatz,1988) and possibly could provide trophic support for afferents before their target cells are generated. Furthermore both SP and MZ neurones pioneer projections to cortical and subcortical (McConnell *et al.*,1989; Bradford *et al.*,1977) targets before many CP neurones are born. McConnell *et al.*,(1989) speculated that this pioneer pathway may guide the subsequent thalamocortical innervation by selective fasciculation. This is supported by the fact that SP neurones appear to be required for the growth of thalamocortical afferents into the CP *in vivo* (Ghosh *et al.*,1990). However *in vitro* some thalamocortical efferents can enter the CP without contact with the SP region (Blakemore and Molnár,1990). Furthermore it is not clear if the SP projection is positionally ordered as at least *in vitro* the SP cells project equally well to explants from different thalamic locations (Blakemore and Molnár,1990). However the *in vivo* experimental data and a variety of anatomical observations (e.g. Kostović and Rakic,1990) suggest that the SP and MZ neurones are not ancient relics of evolution with no function but provide a transient neural scaffold within which cortical development takes place (reviewed by Shatz *et al.*,1988; Kostović and Rakic,1990).

1.3.3 Naturally Occurring Cell Death in the Developing Neocortex

Cell death is found in most parts of the developing vertebrate and invertebrate nervous systems. Degenerating cells have been found in the postnatal neocortex showing that cell death occurs in this region too (Finlay and Slattery,1983; Wahle and Meyer,1987; Al-Ghoul and Miller,1989; Ferrer *et al.*,1990). The classical view is that cell death is responsible for the refinement of connections, by eliminating neurones that are incorrectly connected, positioned or that are excess to requirements, although it is unclear if this is the case for cortical development. The proportion of neurones eliminated from the mouse neocortex has been estimated at 30% (Heumann *et al.*,1978). This is less than many areas of the nervous system (Oppenheim,1989). More common in the neocortex is the selective elimination of inappropriate projections (Cowan *et al.*,1984; Stanfield *et al.*,1982; Ivy and Killackey,1982). Cortical neurones often project to a number of different targets initially but as development proceeds certain projections are selectively stabilized and others retracted.

1.3.4 Neuronal Migration During Neocortical Development

As mentioned in 1.3.1 all neocortical neurones are generated in the VZ, some distance from the CP. The 'inside-out' manner of deposition of migrating neurones described above suggests that neurones are not passively displaced superficially by newly generated progeny. Instead they are believed to actively migrate from the VZ to the CP. This distance can be several hundred microns in the rodent (figures 1.2 and 1.3) and millimetres in the developing primate. They do so at speeds

estimated to be of the order of 10-20μm per hr (Hicks and D'Amato,1968; McConnell,1988b). However these are average values and it is not known if migration is uniform or proceeds in bursts followed by resting periods, as it does *in vitro* (Gasser and Hatten,1990). Migration is an important feature of cortical histogenesis and is central to any study of cell lineage. For example, VZ cells may be determined to forming a particular part of the cortex if their progeny are prevented from dispersing laterally. Indeed three independent models have been proposed to account for the migration of neurones from the VZ to the CP (figure 1.4).

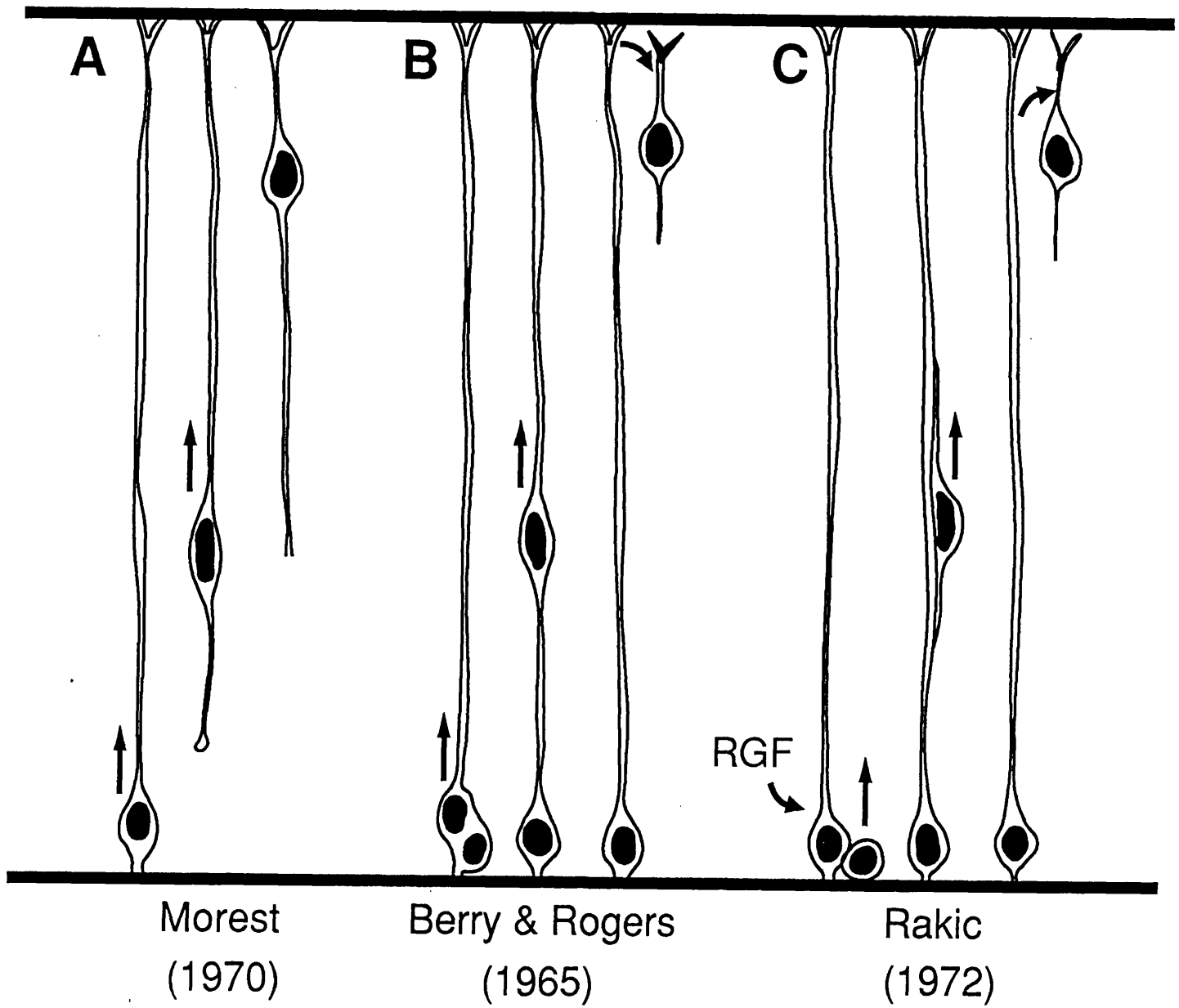
Morest (1970) proposed that young neurones maintained an attachment to both limiting surfaces and that they did not migrate *per se*, but simply translocated their nucleus to the CP and withdrew their ventricular process (figure 1.4A). A very similar mechanism of migration was proposed by Berry and Rogers (1965) (figure 1.4B). They also suggested that VZ cells maintained connections to ventricular and pial surfaces, but they speculated that cytoplasmic division of these cells did not occur immediately after nuclear division. While one nucleus remained near the ventricular surface the other was proposed to be translocated *through* the pial process towards the pial surface. Cytokinesis, it was proposed, occurred in the superficial layers. However neither of these hypotheses were supported by subsequent electron microscopical studies. All anatomical studies demonstrated that cells migrated with a stereotypic amoeboid posture, were mononuclear with an ovoid shaped nucleus, and fusiform in appearance with radially extended leading and trailing processes (Rakic,1972; Shoukimas and Hinds,1978; Gadisseux *et al.*,1990). In the macaque cerebrum, the leading process of migrating cells extends for 70μm towards the pial surface but the trailing process is typically longer (Rakic,1972). Three-dimensional reconstructions reveal that the leading process is a very complex structure which extends numerous filopodia and lamellipodia (Rakic *et al.*,1974) much like a growth cone (Landis,1983; Gasser and Hatten,1990). The leading process is believed to project towards the presumed trajectory of the cell which Austin and Cepko (1990) suggested may not be strictly radial. However no study, other than that of Morest (1970) has demonstrated that the leading or trailing process remain attached to both ventricular and pial surfaces (e.g. Rakic,1972). Perhaps the cells Morest (1970) observed were radial glia transforming into astrocytes (see 1.3.5.3). Hence the migration mechanisms proposed by Morest (1970) and Berry and Rogers (1965) may not occur for neurones during neocortical histogenesis.

1.3.5 Migration Along Radial Glial Fibres

On the basis of anatomical evidence in the macaque, Rakic (1972) proposed that migrating neurones are guided to the CP by an interaction with a specialized astrocyte, the radial glial cell (Levitt and Rakic,1980; Rakic,1990) (figures 1.3 and 1.4C). Rakic (1972) observed

Figure 1.4. Three Putative Mechanisms of Neuronal Displacement From the Ventricular Zone.

Neurones generated within the VZ find their way to the CP. Three models (A,B and C) have been proposed to account for this (see text for details). The VZ is located at the bottom and the CP at the top of the figure. The distance between the two surfaces, unlike that shown in the figure, can be several millimetres in some animals. Furthermore, because of the 'inside-out' generation sequence of neurones, the distance neurones migrate within an individual animal increases with time.


A
B
C
RGF
Morest
(1970)
Berry & Rogers
(1965)
Rakic
(1972)

that during migration to the superficial layers, migrating cells were directly apposed to the radial glial fibres, even when the glial fibres followed a curved trajectory. Rakic (1972) suggested that because of this very close association, radial glia guided migrating neurones to the CP. Indeed the close association between migrating cells and radial glia observed in the Golgi analysis of Rakic (1972) has also been observed after a SEM study (Meller and Tetzlaff,1975).

The anatomical evience supporting this mechanism of migration is strong (Rakic,1972; Gadisseux *et al.*,1990). Certainly radial glia have an anatomy required for a role in neuronal migration and are present only transiently during development (see below). Furthermore between the neuronal soma and the glial cell a specialized 'migration junction' is formed. This is believed to be the site where the force required to propel the neurone is generated (Hatten,1990). However a role for radial glia in neuronal migration is not universally accepted. For example Shoukimas and Hinds (1978) did not find any close association between migrating neurones and radial glia in the developing mouse neocortex. Perhaps the strongest evidence supporting glial guided neuronal migration is the success in reproducing the phenomenon in an *in vitro* microculture system (reviewed by Hatten,1990). In this system migrating neurones adopt the stereotypic posture, speed and mode of movement as seen *in vivo* (Edmondson and Hatten,1987; Gasser and Hatten,1990). However such studies to date have only demonstrated such migration in cultures from the cerebellum, rather than cerebrum.

In the macaque telencephalon radial glia do not divide during the period of neuronal migration but remain dormant in the G1 phase for two months (Schmechel and Rakic,1979a) as if they are a 'sitting target' to which migrating cells attached. Presumably if radial glia did divide then they would retract their pial process (Seymour and Berry,1975) and would not be able to guide neurones. This dormancy however does not occur in the rodent neocortex where at least 30% of radial glia divide coincident with neuronal migration, with their nuclei undergoing the interkinetic migration typical of VZ cells (Misson *et al.*,1988). The significance of this finding to neuronal migration is unclear.

Atypically, in birds neurogenesis occurs in the adult telencephalon (Goldman and Nottebohm,1983; Paton and Nottebohm,1984). Stem cells located in the VZ generate progeny which migrate as much as 5mm to their final position. Again this migration appears to be mediated by radial glia cells, as a close apposition of neurones and radial glia is observed and rates of migration are greater in the areas of the forebrain rich in radial glia (Alvarez-Buylla and Nottebohm,1988). Again, in accord with observations in the rodent reported above, the radial glial cells do divide during this phase of migration (Alvarez-Buylla *et al.*,1990).

As radial glia are found throughout the CNS (Edwards *et al.*,1990) it is possible that they are the exclusive substrate for neuronal migration. Indeed a role in neuronal guidance has also been suggested

for radially oriented glia in different parts of the CNS, e.g. Bergmann glia in the macaque cerebellum (Rakic,1971; Sidman and Rakic,1973), radial glia in the mesencephalon (Shults *et al.*,1990), optic tectum and spinal cord (Gray and Sanes,1991), and Müller cells in the retina (Raymond and Rivlin,1987).

It is not known if radial glia present the exclusive substrate for migration throughout neocortical histogenesis. Rakic (1972) only studied material from the late embryo when cells were migrating to the superficial layers. It is quite feasible that another mechanism of migration occurred during earlier stages. Indeed McConnell (1988a) has suggested that early in development when the telencephalon is thin, cells migrate by the mechanism suggested by Morest (1970). At least in the chick optic tectum some, but not all, cells appear to migrate tangentially along axon fascicles (Gray and Sanes,1991). Furthermore there is a gradient of GFAP staining in the cerebral cortex complementary to the mediolateral gradient of neurogenesis (Woodhams *et al.*,1981). This study suggested that migration could not have exclusively occurred along GFAP positive radial glia as they had not differentiated in lateral aspects of cortex. However this observation appears not to be a gradient of radial glial differentiation, but one of GFAP expression. The RC1 antibody demonstrated radial glia uniformly in the E10 mouse forebrain (Misson *et al.*,1988) suggesting that practically all neuronal migration can indeed occur along radial glia.

1.3.5.1 The Anatomy of Neocortical Radial Glia

Radial glia are bipolar cells whose soma are located in the VZ or SVZ (Rakic,1972; Peters and Feldman,1973). Uniquely, they have a radially directed process, some of which span the entire width of the cerebral wall terminating in conical end-feet at the pial surface.

Rakic (1972) originally reported, on the basis of Golgi impregnations, that radial glia were not clustered in any fashion and the processes were unbranched. However immunohistology using radial glia specific antibodies has shown in the rodent (Misson *et al.*,1988; Edwards *et al.*,1990) and human (Kadhim *et al.*,1988) that radial glia are grouped into regularly spaced fascicles which defasciculate upon entry of the CP. Furthermore individual fibres sometimes branch or terminate on blood vessels. How fasciculation and branching affects neuronal migration is unclear. Perhaps the fascicles provide regularly spaced channels along which neurones migrate. This is supported by the fact that migrating cells are in close apposition with several glial fibres (Gadisseux *et al.*,1990). However presumably the migrating neurones need to choose which fibre along which to migrate when they reach the CP.

1.3.5.2 The Mechanism of Migration Along Radial Glial Fibres

If migrating neurones use radial glia as a migratory substrate then there must be a molecular mechanism by which they achieve both selective binding and locomotion. The precise details are unknown but several

models have been postulated. Any model needs to take account of three features of migration. First, there needs to be a mechanism favouring neurone-glial (as opposed to neurone-neurone) adhesion to provide specificity for the interaction. Second, there needs to be a locomotive generator to propel the migrating cell, presumably unidirectionally. Finally there must be some mechanism that allows a migrating neurone to detach from the glial guide and cease migration at the appropriate time. Indeed transplant experiments suggest that neurones do not simply migrate to the end of a radial glia fibre (McConnell,1988b) as they do *in vitro* (Hatten,1990; Gasser and Hatten,1990), but actively stop migrating and detach from the radial glia fibre upon reaching the appropriate lamina position.

Rakic (1981) postulated a mechanism to account for the migration of the neurone along the radial glial cell based on the fact that neuronal processes preferentially grow along the surfaces to which they most strongly adhere. According to this model, the binding of the two cells is so strong that the membranes of the a radial glial cell and a migrating neurone are fixed along their interphase. The migrating cell moves by adding new membrane components to the leading process. The cell, by moving the nucleus superficially, can then progressively extend along the radial glial fibre in an amoeboid fashion. This model is attractive as it reconciles an apparent contradiction; how cells migrate but still remain strongly bound to radial glia. However this model does not explain how migration is unidirectional, nor how it ceases.

Binding between neurones and glia in the microculture system developed by Hatten (1990) is inhibited by neutralizing antibodies against a novel neuronal antigen termed astrotactin (Edmondson and Hatten,1987; Stitt and Hatten,1990). Hatten (1990) favours a model where neurone-glial binding is facilitated by astrotactin, migration involves the integrin molecules (Hynes,1987), and detachment is the result of neurone-neurone adhesion mediated by NCAM and L1 adhesion molecules. Furthermore Hatten (1990) proposes that the mechanism of migration is generic throughout the CNS (e.g. Gasser and Hatten,1990).

Exactly how a migrating cell penetrates densely packed neuropile *in vivo* is not known. Rakic (1985b) suggested that proteolytic enzymes released from the growing process aid migration. Indeed the serine protease, plasminogen activator, is abundant during the period of CNS (Moonen *et al.*,1982) and PNS (Valinsky and Le Douarin,1985) migration. However, the electron microscope shows that at least during early developmental stages there is plenty of extracellular space in the telencephalon (König *et al.*,1977) which may not be the result of proteolytic enzyme action. Hence proteolytic enzymes may not be needed to aid tissue penetration.

1.3.5.3 The Fate of Radial Glia

Radial glia are present only transiently in the embryonic nervous system (Schmechel and Rakic,1979b; Choi and Lapman,1978; Kadhim *et al.*,1988;

Takahashi *et al.*,1990), coincident with the phase of neuronal migration. Again this is consistent with a role they play in guiding migrating neurones. Some radial glial cells may be eliminated by cell death. However some radial glia undergo a transformation into astrocytes (Schmechel and Rakic,1979b; Takahashi *et al.*,1990). This transformation can be demonstrated *in vitro* and appears to be neurone dependent (Culican *et al.*,1990), and/or under the control of thalamocortical afferents (Crandall *et al.*,1990). Perhaps the most convincing evidence for a transformation has been provided by Voigt (1989) who labelled radial glia *in vivo* with the vital dye, DiI (Honig and Hume,1989), and found that in the mature animal the dye was exclusively restricted to cells with an astrocyte morphology.

Several studies have shown that radial glia often extend a process to a blood vessel (Schmechel and Rakic,1979b; Misson *et al.*,1988). This is a feature characteristic of astrocytes (Peters *et al.*,1976) and suggests that such cells are in the process of transformation. Furthermore Kadhim *et al.*,(1988) observed an increase in the abundance of lysosomes and autophagic vacuoles in radial glia at the time of the proposed transformation. It was suggested that this was cytological evidence for a cellular transformation (Kadhim *et al.*,1988).

However it is not known what proportion of radial glia transform into astrocytes, nor what proportion of astrocytes are derived from radial glia. Indeed some cells in the VZ which are not radial glia contain GFAP (Levitt *et al.*,1981) and could produce cortical astrocytes.

1.3.5.4 The Role of Glial-Guided Migration in Neocortical Development

Glial-guided neuronal migration appears to be unique to the developing CNS. A 'glial scaffold' may be necessary in development in view of the large distance between the site of generation and the site of eventual deposition of a migrating neurone. This distance may be several millimetres in a primate telencephalon, and the terrain traversed by a migrating cell may be quite complex. However neural crest cells migrate a considerable distance, over several different terrains in the embryo without the aid of any specific cellular guide (reviewed by Bronner-Fraser,1988; Bronner-Fraser and Fraser,1989). A glial guide may not be necessary as chick/quail chimeras have demonstrated that the fate adopted by the neural crest depends upon the position of neural crest cells within the embryo and not upon lineage relationships (Le Douarin,1986).

Possibly radial glia are present in the telencephalon to preserve an exact topological correspondence between migrating neurones and their progenitors in the VZ. This would imply that the future areas of the neocortex are pre-specified in the VZ. Indeed this idea has been incorporated into a model of neocortical development (see 1.8). However before an outline of this hypothesis and how it was tested, a discussion of the anatomy and physiology of the adult neocortex is needed.

1.3.6 Neuronal Differentiation in the Neocortex

Prior to entry to the CP a migrating neurone loses the bipolar morphology (Caley and Maxwell,1968), enlarges slightly (Rakic *et al.*,1974) and grows a primitive axonal process from its basal pole (Shoukimas and Hinds,1978). Apart from these features, the cell shows few visible signs of differentiation. However subsequently it will undergo three further aspects of differentiation, morphological, chemical and physiological; all of which are briefly discussed below. In general, but not exclusively (e.g. Lund *et al.*,1977), the differentiation of the different kinds of neurones present in the cortex occurs concurrently, but follows an 'inside-out' gradient where the differentiation of deeper neurones precedes that of more superficial cells (Miller,1988). For example, some neurotransmitters are synthesized in an 'inside-out' chronological sequence (e.g. Alvarez-Bolado *et al.*,1990). Furthermore there is also a mediolateral gradient of differentiation where neurones located laterally often differentiate prior to those located more medially (e.g. McCandlish *et al.*,1989).

1.3.6.1 The Morphological Differentiation of Neocortical Neurones

During the first two postnatal weeks individual neurones enlarge (Miller,1981) and acquire a characteristic morphology (Parnavelas *et al.*,1978; Lund *et al.*,1977). Mature neurones are divided into two principal morphological classes: pyramidal and non-pyramidal. The majority (70%) of neurones are pyramidal (Winfield *et al.*,1980) with their axons projecting either outside of the cortex or to another cortical area (Parnavelas *et al.*,1977). Pyramidal neurones have a triangular shaped perikaryon and a prominent apical dendrite directed towards the pia. Pyramidal neurones occur in all layers, except layer I (Bradford *et al.*,1977), and although they vary considerably in size they have the common basic structure described above wherever they are found. Non-pyramidal neurones are a heteromorphic population, present in all cortical layers (Feldman and Peters,1978). However they all have axons that only project within the immediate cortical region (Parnavelas *et al.*,1977). The formation of the projections characteristic of pyramidal and non-pyramidal neurones can be reproduced *in vitro* (Bolz *et al.*,1990) and may occur by chemotropism (a phenomenon discussed further in 1.6.1).

1.3.6.2 The Chemical Differentiation of Neocortical Neurones

Neurones characteristically synthesize, store and release neurotransmitters (Parnavelas *et al.*,1988). The time when neurones start synthesizing individual neurotransmitters varies in development but for each neurone it generally coincides with the period of morphological differentiation (McDonald *et al.*,1981). Pyramidal neurones are thought to use the amino acids aspartate and glutamate as excitatory neurotransmitters (Donoghue *et al.*,1985; Fonnum,1984). However this has been difficult to confirm unequivocally as the two amino acids are found in all cells. The majority of non-pyramidal neurones synthesize and

secrete the inhibitory neurotransmitter GABA (Ribak,1978; Hendrickson *et al.*,1981). However a smaller population of non-pyramidal neurones not containing GABA synthesize ACh (Eckenstein and Thoenen,1983). A subpopulation of non-pyramidal neurones also contain a variety of neuropeptide neurotransmitters (Jones and Hendrey,1986).

Some neurotransmitters, e.g. VIP (Cavanagh and Parnavelas,1989), NPY (Wahle and Meyer,1987) and monoamines (Berger *et al.*,1985), are only synthesized transiently by neocortical neurones during early development. It is not clear whether this is transient expression by cells that survive into adulthood, as is thought to be the case with certain cells in the PNS (Jonakait *et al.*,1984), or whether it is the cells themselves that are transient. However if these neurones die this would represent a unique type of cell death in the nervous system in which all the cells with a particular phenotype, irrespective of their position in the neocortex, are removed. This may nonetheless be the case. Wahle and Meyer (1987) have suggested that neurones of this type are removed after playing a part in the establishment of connectivity in the CNS, possibly by exerting a neurotrophic effect. There is tentative evidence for this; VIP added to *in vitro* cultures rescues neurones that would have died as a result of tetrodotoxin activity blockade (Brenneman and Eiden,1986).

1.3.6.3 Physiological Differentiation in the Neocortex

Mountcastle (1957) was the first to describe a physiological organization of neurones in the somatosensory cortex of the cat, in which a 'column' of neurones, oriented perpendicular to the neocortical layers, tended to exhibit similar functional properties, and responded, in relation to the somatotopic body surface map, in a place specific fashion. This discovery led to the suggestion that the neocortex is organized into a mosaic of elementary functional units of 'columns' or cylinders of cells oriented perpendicular to the cortical surface (Mountcastle,1957). However Mountcastle (1957) was unable to correlate these physiological units with specific cytoarchitectonic features of the neocortex.

Following this pioneering work, the columnar organization of the neocortex was extensively studied, most prominently in the primary visual cortex (area 17) of the cat and the macaque (Hubel,1982; Wiesel,1982).

1.4 The Columnar Organization of the Neocortex

The columnar organization of the cortex described above can be subdivided into two catergories.

(i) Columns that are the result of the intrinsic circuitry of neocortical neurones, such as 'orientation columns' of the macaque (see 1.4.1).

(ii) Columns that are based upon afferent input to the neocortex such as 'ocular dominance' columns of the macaque (see 1.4.2) and the 'barrel' columns of the rodent (see 1.5).

Initially orientation and ocular dominance columns were defined in electrophysiological rather than anatomical terms. However methods were subsequently developed that demonstrated histologically both orientation (Hubel *et al.*,1977a; Löwel *et al.*,1987) and ocular dominance columns (Hubel and Wiesel,1972; Wiesel *et al.*,1974; LeVay *et al.*,1975). In order to provide a suitable background to the columnar characteristics of the neocortex both orientation and ocular dominance columns are discussed below.

1.4.1 'Orientation' Columns

In the primary visual cortex, area 17, of the cat or macaque, neurones only respond when an appropriate stimulus (a bar or edge) is presented in the visual field in a specific orientation (receptive-field axis orientation). Hubel and Wiesel (1968) found that all the cells within one column had the same receptive-field axis orientation. Long oblique penetrations almost parallel to the cortical surface showed a slow anticlockwise or clockwise change of receptive-field orientation of 7-10° every 25-50μm (Hubel and Wiesel,1974). Hence the visual cortex is subdivided into a 'checkerboard' of 'orientation' columns, perpendicular to the cerebral surface, where each neighbouring column differs very slightly in receptive-field orientation.

1.4.2 'Ocular Dominance' Columns

Superimposed onto this pattern is the phenomenon of ocular dominance. Neurones in one 'ocular dominance' column are more strongly responsive to stimuli presented to one eye rather than the other (Hubel and Wiesel,1968). Tangential electrode penetrations revealed an alternating pattern of responses specific for one eye or the other. Anterograde transport of tritiated amino acids from the eye demonstrated that ocular dominance columns form a series of alternating parallel stripes (which are not entirely straight and occasionally coalesce with neighbours) approximately in 0.5mm width, separated by an unlabelled interphase (the terminals from the other eye) of approximately the same width (LeVay *et al.*,1975).

The two columnar systems described above are related, but independent. For example, when an electrode crossed from a left-eye region to a right-eye region there was no noticeable disturbance in the systematic sequence of orientation columns (Hubel and Wiesel,1974). Indeed Hubel and Wiesel (1972) termed a complete set of orientation columns (a full 180° receptive-field axis rotation) and two ocular dominance columns, a hypercolumn. They suggested that hypercolumns were a fundamental unit of visual cortex organization, with each point of visual space represented by one.

The rodent neocortex also has a physiological columnar organization. This property is typified by the rodent 'barrel' columns. As 'barrel' columns were the subject of a substantial part of this study, their anatomy, somatotopic relationship to mystacial vibrissae, and columnar properties are discussed below.

1.5 The 'Barrel' Columns of the Rodent Somatosensory Cortex

1.5.1 Anatomy and Phylogenetic Distribution of 'Barrel' Columns

In 1970 Woolsey and Van der Loos reported a 'barrel' arrangement of cell bodies in layer IV of the mouse primary somatosensory cortex (Sm1) (see figure 1.5). Unlike most neocortical cytoarchitectonic features the 'barrels' were best appreciated anatomically in the tangential plane (e.g. figure 1.5). However coronal sections revealed that individual barrels spanned all of layer IV, that they were radially arrayed, and that they were indeed barrel-shaped i.e. their width was greatest at mid-height (Woolsey and Van der Loos,1970). This seminal study initiated others directed towards a better understanding of barrel anatomy and physiology.

Each barrel consists of a hollow which is surrounded by septum (Welker and Woolsey,1974). The hollows are the roughly circular figures in figure 1.5 which are stained more strongly than surrounding septum. Individual hollows receive thalamic input (Killackey and Leshin,1975) whereas the septa have a very different connectivity (Koralek *et al.*,1988; Olavarria *et al.*,1984). Collectively the barrels form five parallel rows that arc roughly posteriomedial to anterolateral (Woolsey and Van der Loos,1970; Welker and Woolsey,1974). These parallel rows compose the PosteroMedial Barrel Sub-Field (PMBSF) or 'barrelfield' for short (Woolsey and Van der Loos,1970). Anterior and lateral to the PMBSF is another subfield of smaller, circular barrels (Woolsey and Van der Loos,1970) termed the AnteroLateral Barrel Sub-Field (ALBSF) (Olavarria *et al.*,1984).

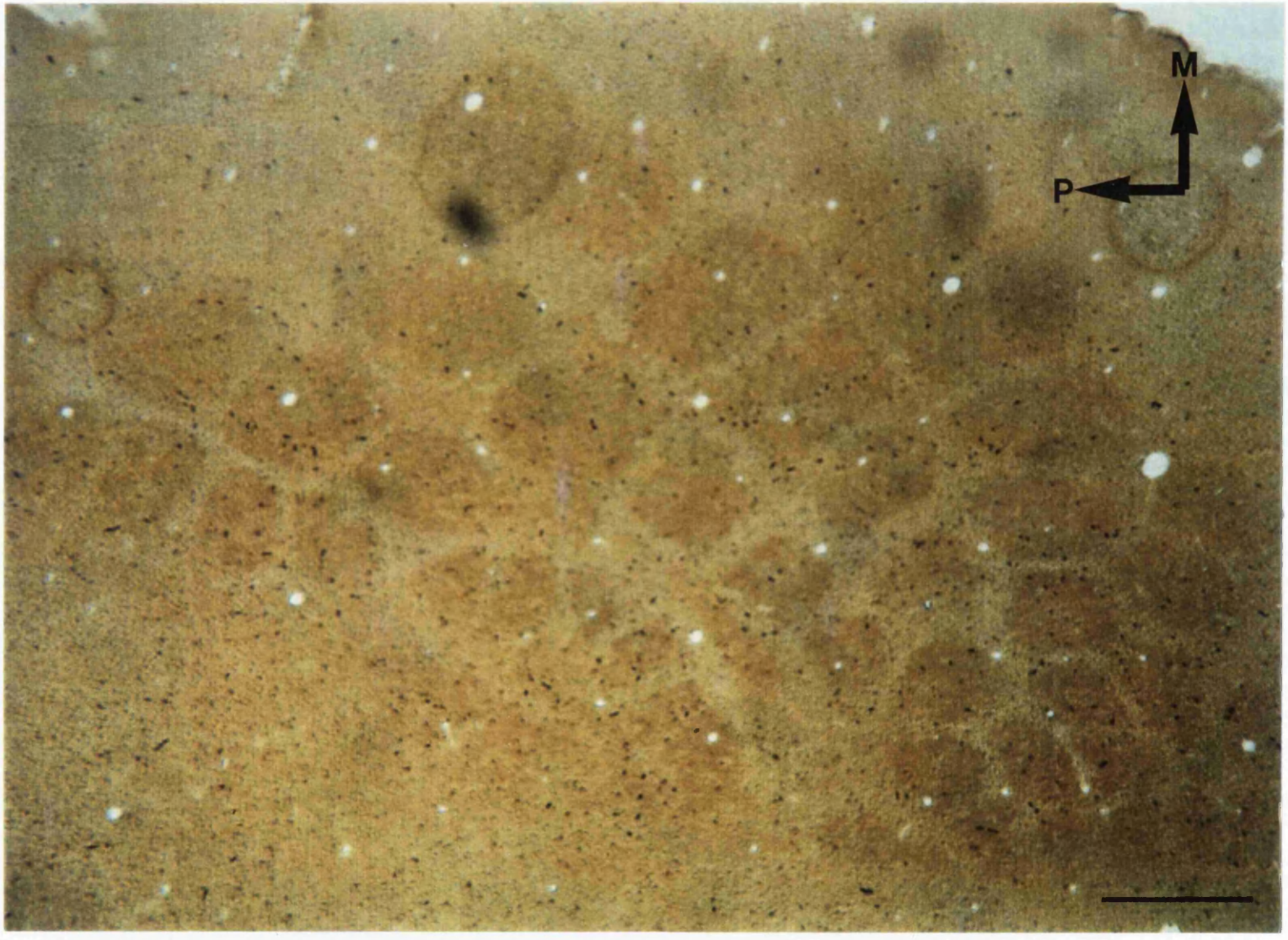
The aggregation of neurones into a barrel is unique to Sm1 as they are not present in the secondary somatosensory cortex (Welker and Sinha,1972) or any other neocortical area in the rodent. However they are found in a number of other mammals (Woolsey *et al.*,1975; Weller,1972) including the rat (Welker and Woolsey,1974) but not the cat (Rice,1985a). In general the barrels are less prominent in those species that have relatively larger cortices (Woolsey *et al.*,1975). The reasons for the interspecies variations, i.e. the differences between rat and mouse (Welker and Woolsey,1974; Rice,1985b) or absence of the barrels, are not known.

1.5.2 Somatotopic Relationship of 'Barrel' Columns With Mystacial Vibrissae

Woolsey and Van der Loos (1970) speculated that the PMBSF barrels formed a somatotopic relationship with the large mystacial whiskers (vibrissae) on the contralateral muzzle of the mouse (the 'whiskerpad') (figure

Figure 1.5. The Appearance of 'Barrel/Columns' in a Tangential Section of Rat Sm1 Stained for Cytochrome c Oxidase.

Heightened cytochrome c oxidase activity is found within the barrel/column hollows. The white 'dots' are radially oriented blood vessels. The barrels are arranged in rows that represent the rows of vibrissae present on the mystacial whisker pad (see text). Scale bar 300μm.


M
P

1.6). They suggested that the PMBSF vibrissae were represented by barrels in a 1:1 fashion and the barrels in the ALBSF were the cortical representation of sinus hairs on the upper lip. This proposal of a somatotopic relationship was subsequently confirmed by both metabolic (Kossut *et al.*,1988; McCasland and Woolsey,1988) and electrophysiological (Simons,1978; Armstrong-James and Fox,1987) studies. The relationship is such that the dorsal row of vibrissae is represented by the most posterior barrels and the ventral row of vibrissae by the most anterior barrels. Vibrissae positioned most anteriorly connect to the most lateral barrels in a row, and posterior vibrissae connect to medial barrels (figure 1.6).

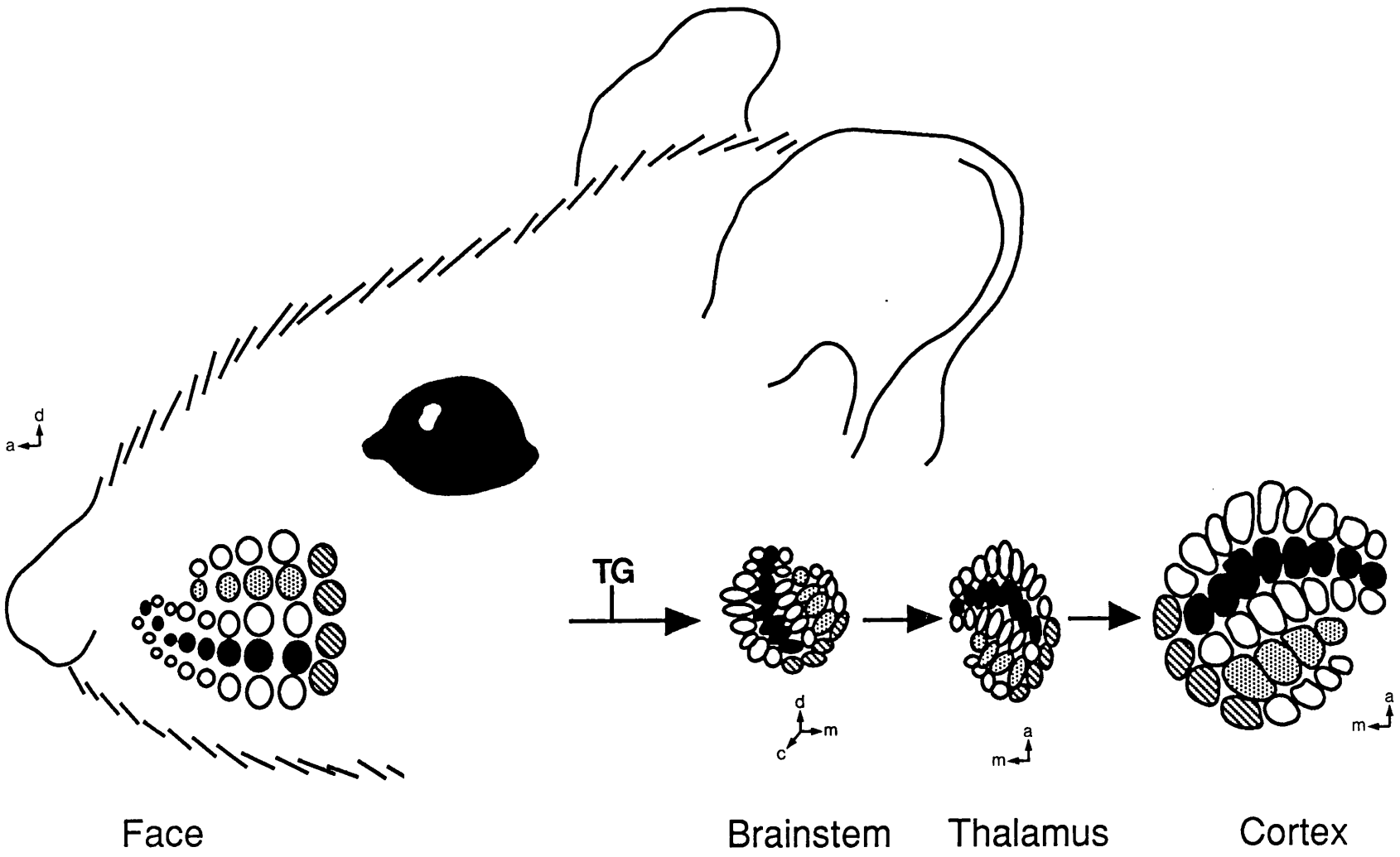
Vibrissae are very conspicuous in rodents and occupy five rows running from dorsal to ventral; the largest vibrissae are found most posteriorly in all five rows. Vibrissae in rats and mice are actively moved back and forth (termed 'whisking' by Van der Loos) during exploratory behaviour (Welker,1964; Carvel and Simons,1990). Indeed a vibrissal deflection of only a few degrees is sufficient to elicit a maximal response of neurones in different parts of the pathway (Armstrong-James and Fox,1987; Lichtenstein *et al.*,1990). Furthermore trimming the muzzle vibrissae in rodents results in a variety of profound behavioural deficits which are only corrected once the vibrissae re-grow (Vincent,1912; quoted in Woolsey and Van der Loos,1970; Guić-Robles *et al.*,1989). Indeed in view of the poorly developed visual sense of rodents, the vibrissae have been termed the 'blind man's stick' (Van der Loos,1977).

As outlined in 1.5.1 several mammals have barrels in Sm1. Collectively these animals occupy a variety of habitats (e.g. terrestrial, subterranean and arboreal) and not all appeared to have the 'whisking' behaviour characteristic of rodents (Woolsey *et al.*,1975). Hence the existence of barrels is not precluded by certain behavioural patterns. Furthermore some animals with barrels (e.g. squirrel) could supplement the sensory information from their vibrissae with their well developed visual sense.

The somatotopic relationship between the vibrissae and the whisker barrels is not a simple projection of vibrissal sensory neurones to Sm1. The projection is third order, involving synapses between neurones located at three different levels of the sensory pathway. The sensory neurones in the trigeminal ganglion project to the trigeminal nucleus in the brainstem. Neurones in the brainstem project to the ventrobasal nucleus (VBN) of the thalamus which in turn projects to Sm1. At each stage in this pathway it is possible to detect histochemically the somatotopic pattern of the vibrissae: 'barrelettes' in the brainstem (Ma and Woolsey,1984; Bates and Killackey,1985), 'barreloids' in the VBN (Van der Loos,1976; Land and Simons,1985; Sugitani *et al.*,1990) and 'barrels' in Sm1 (Woolsey and Van der Loos,1970) (figure 1.6). The entire sensory pathway from vibrissae to cortex has been termed the rodent trigeminal pathway (Woolsey,1990).

Figure 1.6. The Somatotopic Relationship Between the Mystacial Vibrissae and the Barrel/Columns in the Rat Neocortex.

The relationship of the mystacial vibrissae is reproduced in an aggregation of cell bodies in the brainstem trigeminal complex ('barrelettes'), the thalamus ('barreloids') and the neocortex ('barrel/columns'). Collectively these neural structures are devoted to the processing of sensory input from the vibrissae. The connection from the vibrissae to the cortex is third order involving somatotopic connections between neurones in separate parts of the CNS. Like most input to the cortex the projection from the vibrissae is contralateral crossing the midline between the brainstem trigeminal complex and the thalamus. The input from the vibrissae on the muzzle of a rat is conveyed *via* the infraorbital nerve to the trigeminal ganglion (TG). All parts of the pathway are shown according to their relative size and orientation.


d
a
TG
d
m
c
a
m
a
m
Face
Brainstem
Thalamus
Cortex

1.5.3 Columnar and Somototopic Properties of Rodent Sm1

Using anatomical criteria Woolsey and Van der Loos (1970) speculated that Sm1 barrels were a 'morphological manifestation, in layer IV, of functional columns defined by electrophysiological means'. This was subsequently confirmed electrophysiologically (Simons,1978; Armstrong-James and Fox,1987). In physiological experiments radial penetrations of the recording electrode normal to the pia and through individual barrels revealed a columnar structure where all layers had the same receptive field, i.e. they were all activated by one principle vibrissa. The barrel activated was the somatotopic homologue of the vibrissa stimulated. Thus although barrels can only be seen histochemically in layer IV, they have the classical columnar property of extending the entire thickness of the grey matter. A barrel also has a considerable number of horizontal connections such that each barrel is activated by more than the principle vibrissae (Simons,1978). Such horizontal connections are not unique to Sm1 and have also been described for the visual system (Gilbert,1985).

However the barrels are the *only* known example of a cortical column which can be detected in the rodent by very simple histological techniques. Furthermore rat Sm1 contains the only example of a histologically discernable somatotopic map (a 'rattunculus') with 'clusters' of cells corresponding to lower/upper lips, forepaw, hindpaw and trunk (Dawson and Killackey,1987; Welker,1976). The PMBSF representation occupies approximately 20% of the rattunculus.

In the text below each individual cortical barrel has been termed a barrel/column. This is a recent innovation used in several publications (e.g. Bernardo *et al.*,1990) to emphasize the fact that although the barrels are only found in layer IV their columnar properties extend radially through all neocortical layers.

1.6 The Development of the Vibrissal Sensory System

1.6.1 The Development of the Trigeminal Pathway

The development of precise somatotopic connections (reviewed by Undin and Fawcett,1988) such as that seen in the rodent trigeminal pathway remains enigmatic. It appears that during the development at least one of the connections, between the trigeminal ganglion and the maxillary epithelium (in which the vibrissae develop), is mediated by a graded distribution of a diffusible (and as yet uncharacterized) molecule originating from the target; a phenomenon termed chemotropism (Lumsden and Davies,1983; Lumsden,1988). Furthermore upon reaching the maxillary epithelium the trigeminal neurones are prevented from dying by a neurotrophic factor (nerve growth factor, NGF) derived from the maxillary epithelium (Harper and Davies,1990).

However chemotropism may not account for the development of *all* the connections in the trigeminal pathway. This contention is supported by experiments in the visual system. In co-culture, LGN efferents project to their correct target, layer IV of the visual cortex. However, they do

not show positional specificity and project equally well to layer IV of other cortical areas (Blakemore and Molnár,1990). Although not examined to date, it is possible that in such co-cultures VBN efferents would incorrectly project to visual as well as Sm1. Presumably cell surface molecules are also involved in guidance. Indeed guidance involving cell surface molecules has been demonstrated in the visual system (Walter *et al.*,1987; Stahl *et al.*,1990).

1.6.2 The Development of the Barrel/Columns in the Rodent Neocortex

Barrel/columns cannot be found in Sm1 of newborn rodents by Nissl staining, as layer IV is of uniform cell density (Rice and Van der Loos,1977). A very immature barrelfield can be detected by postnatal day 3-4 (P3/4) in the rat and P2/3 in the mouse (Rice and Van der Loos,1977). However the area destined to form the entire rattunculus, the barrelettes and barreloids, can be detected earlier than this by several histochemical techniques. These structures are transiently demarcated by radial glia (Cooper and Steindler,1986b; Crandall *et al.*,1990) that are rich in glycoconjugates (Cooper and Steindler,1986a; McCandlish *et al.*,1989) and adhesion molecules (Steindler *et al.*,1989). These demarcated or 'cordoned off' areas have been termed 'cordones' (Steindler *et al.*,1989).

The formation of the characteristic barrel/column anatomy and the barrelfield is preceeded by the arrival of the afferents from the VBN (Wise and Jones,1978). Recently however Erzurumlu and Jhaveri (1990), using the fluorescent tracer DiI, found that VBN afferents arrive in Sm1 earlier than previously reported. Furthermore these studies demonstrated that upon arrival in layer IV the afferents are diffusely arranged but over the next few hours they segregate into the specific somatotopic pattern. The rate of ingrowth of these afferents is not uniform as they appear to 'wait' in the IZ (Wise and Jones,1978). Again a very similar phenomenon occurs with ocular dominance columns; eye specific afferents, after waiting for some time in the IZ (Rakic,1977), project into layer IV uniformly and then segregate into the mature pattern of alternating parallel bands (Hubel *et al.*,1977b; LeVay *et al.*,1980).

The formation of a barrel/column seems to depend on this afferent input. The removal of vibrissa(e), during a restricted period in early postnatal life (i.e. the 'critical period'), results in the malformation of the corresponding contralateral barrel/column(s) (Woolsey and Van der Loos,1973; Kaas *et al.*,1983). Moreover 'barreloids' in the VBN (Woolsey *et al.*,1979) and the 'barrelettes' in the brainstem are also malformed (Durham and Woolsey,1984). However removal of vibrissae after the PMBSF has formed does not alter the morphology of the corresponding, barreloid, barrelette or barrel/column (Weller and Johnson,1975; Woolsey and Wann,1976; Jeanmonod *et al.*,1980), although a decrease in metabolic activity can be demonstrated in the denervated barrel/column using cytochrome c oxidase histochemistry (Wong-Riley and Welt,1980).

These results are not exclusive features of Sm1. Analogous experiments in the visual system give a similar result. Monocular deprivation leads to a reduction in the size of ocular dominance columns for the deprived eye and an expansion of those from the normal eye (Hubel *et al.*,1977b). In the visual cortex this plasticity only occurs in a critical period early in postnatal life (LeVay *et al.*,1980). In fact it seems to be dependent on an immature cortical environment as plasticity can be restored in the adult by re-introducing dissociated visual cortex astrocytes from newborn animals (Müller and Best,1989). It is not known if Sm1 astrocytes can restore plasticity to adult Sm1 (C.Müller, personal communication), although it is an attractive idea as glia are known to be involved in outlining barrel/columns during the immediate postnatal period as cordones (see above).

1.7 'Minicolumns' and the Modular Hypothesis of Neocortical Organization

Before a discussion of a hypothesis which suggests how the columnar and areal organization of the neocortex is generated I need to discuss a subunit that is proposed to be a modular structure found in all cortical columns. This structure has been termed a 'minicolumn' (Mountcastle,1979) and is a radial array of neurones spanning the entire width of the adult cortex. Minicolumns are believed to have the same basic anatomy in different cortical areas. They are proposed to have a diameter of 30μm and contain 110 cells regardless of area or species, with the exception of area 17 of the macaque where they contain 260 neurones (Rockel *et al.*,1980). They are believed to be the simplest functional unit in the cortex. The diameter of a minicolumn is approximately the same as the diameter of orientation columns (see 1.4.1) (Hubel and Wiesel,1974). Hence presumably orientation columns are the smallest column of neurones with physiological properties. However both individual ocular dominance and barrel/columns are considerably larger than orientation columns and presumably are composed of several adjacent minicolumns (Szentágothai,1978).

This modular organization of the cerebral cortex is appealing (Leise,1990). Indeed examples of repetitive structural/functional elements are frequently found in neural structures e.g. the *Drosophila* ommatidium (Tomlinson,1988) and in electronic circuits e.g. the microprocessor. However this modular view of neocortical organization is not universally accepted (Swindale,1990).

1.8 The Radial Unit Hypothesis of Neocortical Development

Rakic (1978,1988a,1988b) has postulated a model, the radial unit hypothesis, which seeks to explain how the columnar and areal organization of the neocortex are generated during ontogeny. The hypothesis has also been elaborated to account for the expansion of cortical areas during phylogeny and to account for various birth defects (Rakic,1988a; Rakic,1988b).

Specifically Rakic suggested that neurones originating from a VZ progenitor or a group of neighbouring VZ progenitors (both termed 'a proliferative unit' by Rakic) will migrate along a single radial glial fibre or fascicle to form an 'ontogenetic column' in the CP (figure 1.7). An ontogenetic column is a radially oriented cohort of cells spanning the entire width of the CP. According to the radial unit hypothesis a single ontogenetic column found in the embryo is the progenitor of one minicolumn found in the adult (Mountcastle,1979). The radial glia scaffold (see 1.3.5) is proposed to maintain an exact one to one correspondance between the VZ progenitor(s) and a single ontogenetic column in the CP. According to the hypothesis, this association is strong enough to prevent a lateral dispersion of individual clones of migrating neurones, for which there is otherwise ample opportunity. In the macaque cortex the various *gyri* and *sulci* cause the CP to convolute and bend in relation to the VZ and in lissencephalic cortex there is a change in the relationship of the CP and VZ during development (Smart and McSherry,1982). Both of these distortions could disperse clones laterally.

So in summary the radial unit hypothesis suggests that a particular part of the VZ is essentially pre-specified to forming a particular part of the CP, prior to neocortical histogenesis. Hence, the formation of the CP is simply an expansion of the VZ in the third dimension (figure 1.7). Thus, a single barrel/column would originate from a discrete grouping of proliferative units, much like the rhombomeres in the hindbrain or polyclonal compartments in insects (see 1.2 and Crick and Lawrence,1975).

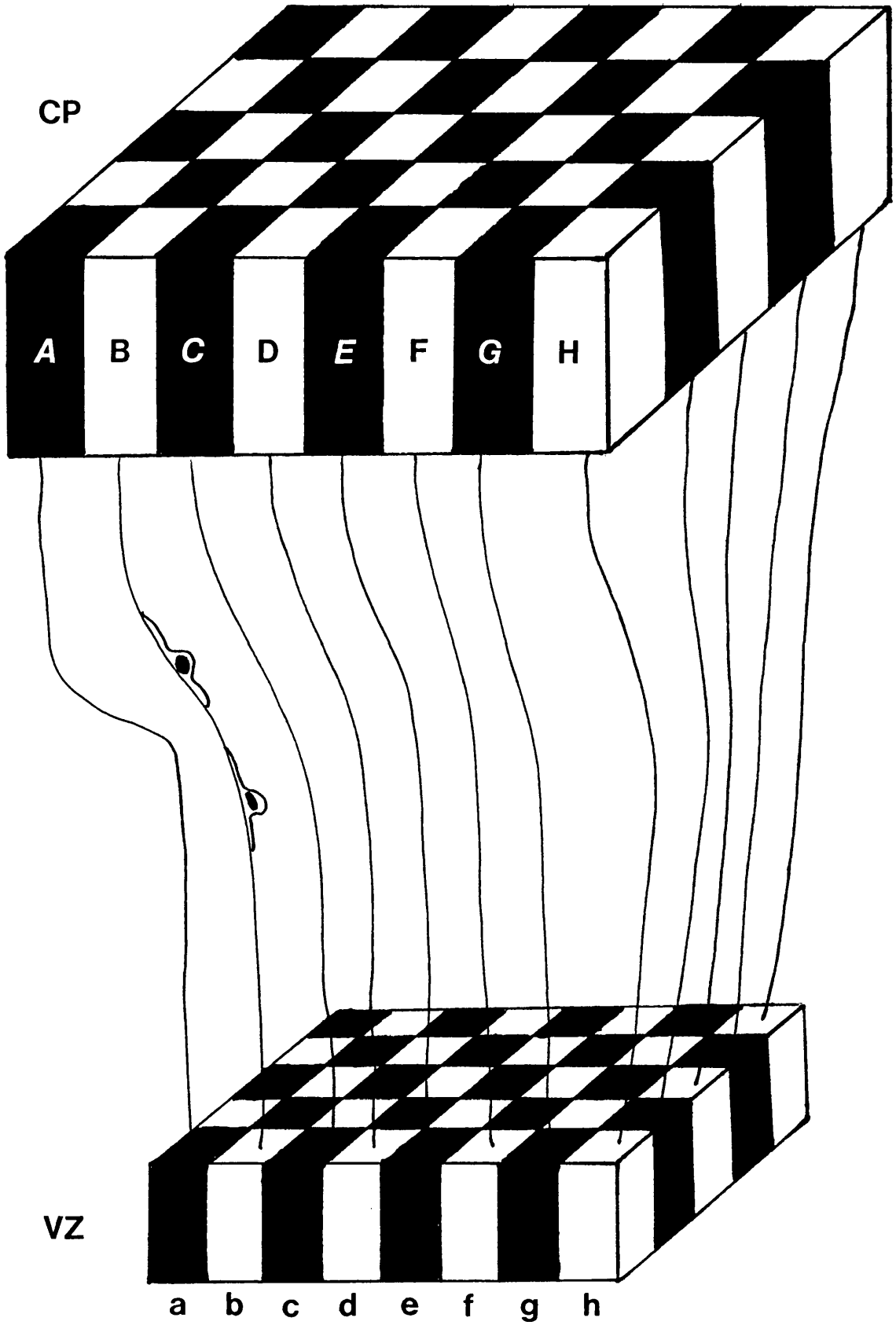
The radial unit hypothesis also addresses the question of how, having established a columnar arrangement throughout the neocortex, separate areas take on an areal identity. The ontogenetic columns are proposed to be partially programmed to an area specific fate. The radial unit hypothesis suggests, ontogenetic columns are subject to epigenetic maturation by specific cortical or thalamic afferents which, by molecular and/or electrical factors, specify areal identity.

Some circumstantial evidence supports the radial unit hypothesis. In the macaque Rakic (1988a) has estimated the number of both ontogenetic columns and proliferative units and found them both to be 15-20 million. This stoichiometry is in accord with a direct one to one relationship between the two. Second, some migrating cells in the IZ express an antigen that is characteristic of mature neurones in the CP above them (Horton and Levitt,1988). Furthermore if neurones are prevented from migrating to the CP they still form their normal projections (Jensen and Killackey,1984). Both of these observations suggest that areal fate is specified prior to reaching the CP.

Some experiemnts have suggested that the neocortex can develop normally in the absence of extrinsic influences. If macaque embryos are binocularly enucleated *in utero*, area 17 develops a basically normal laminar pattern of neurotransmitter distribution and synaptic density

Figure 1.7. The Proposed Relationship Between a Small Patch of Proliferative Ventricular Zone and a Corresponding Area in the Developing Cortical Plate as Suggested by the Radial Unit Hypothesis.

The radial unit hypothesis of cortical development (Rakic,1988a; Rakic,1988b) proposes that individual ontogenetic colunms in the CP (a black or white rectangle in the CP) originates from an individual proliferative unit in the VZ (a black or white square in the VZ). Migrating neurones from an individual proliferative unit are prevented from dispersing laterally by the radial glial guide. Upon maturation an ontogenetic column forms a minicolumn (see text).


CP
A
B
C
D
E
F
G
H
VZ
a
b
c
d
e
f
g
h

(Rakic,1988a). These enucleated animals also develop normally in other ways, for example the callosal connections of area 17 develop normally (Dehay *et al.*,1989), and the cytochrome c oxidase 'blobs', another structural feature of area 17 in the macaque, are normal (Kuljis and Rakic,1990; Kennedy *et al.*,1990). However these enucleated animals are not totally normal. The surface area, and hence number of ontogenetic columns occupying area 17, is less than half the control value (Rakic,1988a). Rakic (1988a) speculated that the remainder of area 17 has taken on hybrid area 17/18 characteristics due to expansion of area 18 efferents.

The radial unit hypothesis is attractive as it requires a minimum amount of genetic information for the assembly of the cortex. Theoretically only two sets of genes are needed; one controlling the instructions for a specific protomap at the VZ and another controlling progenitor cell proliferation. Specification at the VZ is prudent as the VZ is small and has only two dimensions. Indeed this situation is analogous to the specification of the *Drosophila* blastoderm (Ingham,1988).

The radial unit hypothsis is attractive for its simplicity. Indeed although first suggested in 1978 (Rakic,1978) a similar model was briefly suggested by Morest (1970) after histological analysis of the early telencephalon. However despite the popularity of the radial unit hypothesis (e.g. Leise,1990; Smart and McSherry,1982) it has not been tested by a direct study of clonal migration in the developing mammalian embryo. In the majority of cases neocortical histogenesis occurs *in utero*, and in general any attempts at foetal manipulations *in utero* lead to absorbtion or abortion of the foetus. Moreover the VZ cells are not only inaccessible but small and fragile, so that physical injection of lineage tracers would be very difficult. Furthermore during development there is an extensive number of cell divisions and embryonic growth such that any lineage tracer would be diluted below a detection threshold. Collectively these difficulties, until recently, prevented the study of clonal migration during neocortical histogenesis.

However a lineage labelling technique has recently been developed, which circumvents all of the problems mentioned above. The technique, briefly discussed in 1.2, involves the transfer of a marker gene to individual progenitor cells by a retroviral vector. The virus is inherited by all the progeny of an infected cell and labelled cells can be detected by their expression of the marker gene.

In order to explain how retroviral vectors can be applied to study clonal migration, both retroviruses and retroviral vectors (and their differences from one another) are discussed below.

1.9 Retroviruses

A retrovirus is essentially a mobile genetic element that can travel from cell to cell (reviewed by Varmus and Brown,1989; Varmus,1988; Mason *et al.*,1987). Much scientific interest has centred on this family of

viruses as they have a unique replication strategy. An obligatory step in their lifecycle is the 'reverse transcription' of their single stranded RNA genome into a double stranded DNA intermediate upon entry into the host cell (Baltimore,1970; Temin and Mizutani,1970; Baltimore,1985). This DNA intermediate is then covalently integrated into the host genome as a provirus.

1.9.1 Retroviral Anatomy

In their extracellular form retroviruses are spherical particles 90-100nm in diameter, termed virions. A virion consists of a nucleoprotein core harbouring the genome surrounded by a lipid envelope derived from the membrane of the host cell. This lipid envelope contains glycosylated viral proteins that recognize specific cell surface receptors. The virions are diploid (Bender and Davidson,1976) containing two identical single stranded, positive sense, genomic RNA transcripts with 5'cap and 3'poly(A) structures. The two RNA genomes are joined together near the 5' end.

The genomic transcripts have the potential to code for at least three polyproteins needed for replication: *gag*, *pol* and *env* which are most commonly arranged: 5'-*gag*-*pol*-*env*-3' (e.g. Shinnick *et al.*,1981; Varmus and Brown,1989). Each of these polyproteins is proteolytically cleaved to form structural nucleocapsid or genome associated glycoproteins (*gag*), viral replication or polymerase enzymes (*pol*), and exterior envelope glycoproteins (*env*, proteins often denoted by the prefix gp). All of these proteins act in *trans* and are flanked at either end by identical sequences termed long terminal repeats (LTRs). The LTRs contain the DNA sequences in *cis* needed for integration and regulation of transcription and are generated during reverse transcription. In between the LTRs and the viral *trans* acting genes are *cis* sequences required for viral replication. These are the sites from which DNA synthesis is primed. A cellular $tRNA^{Pro}$ binding site near the 5' end of the genome is the priming site for the synthesis of minus sense DNA (Taylor,1977). In the course of minus sense DNA synthesis the genomic RNA is degraded (Collett *et al.*,1978) so only one provirus is synthesized from one genomic RNA. Initiation of the second, positive sense strand DNA begins from a polypurine tract near the 3' end of the genome (Taylor and Sharmeen,1987).

1.9.2 The Retroviral Life Cycle

A typical retroviral lifecycle is shown in figure 1.8. The cycle can be divided into several phases: adsorption, internalization, reverse transcription and integration, expression and packaging. The time involved from receptor binding to integration is of the order of only a few hours.

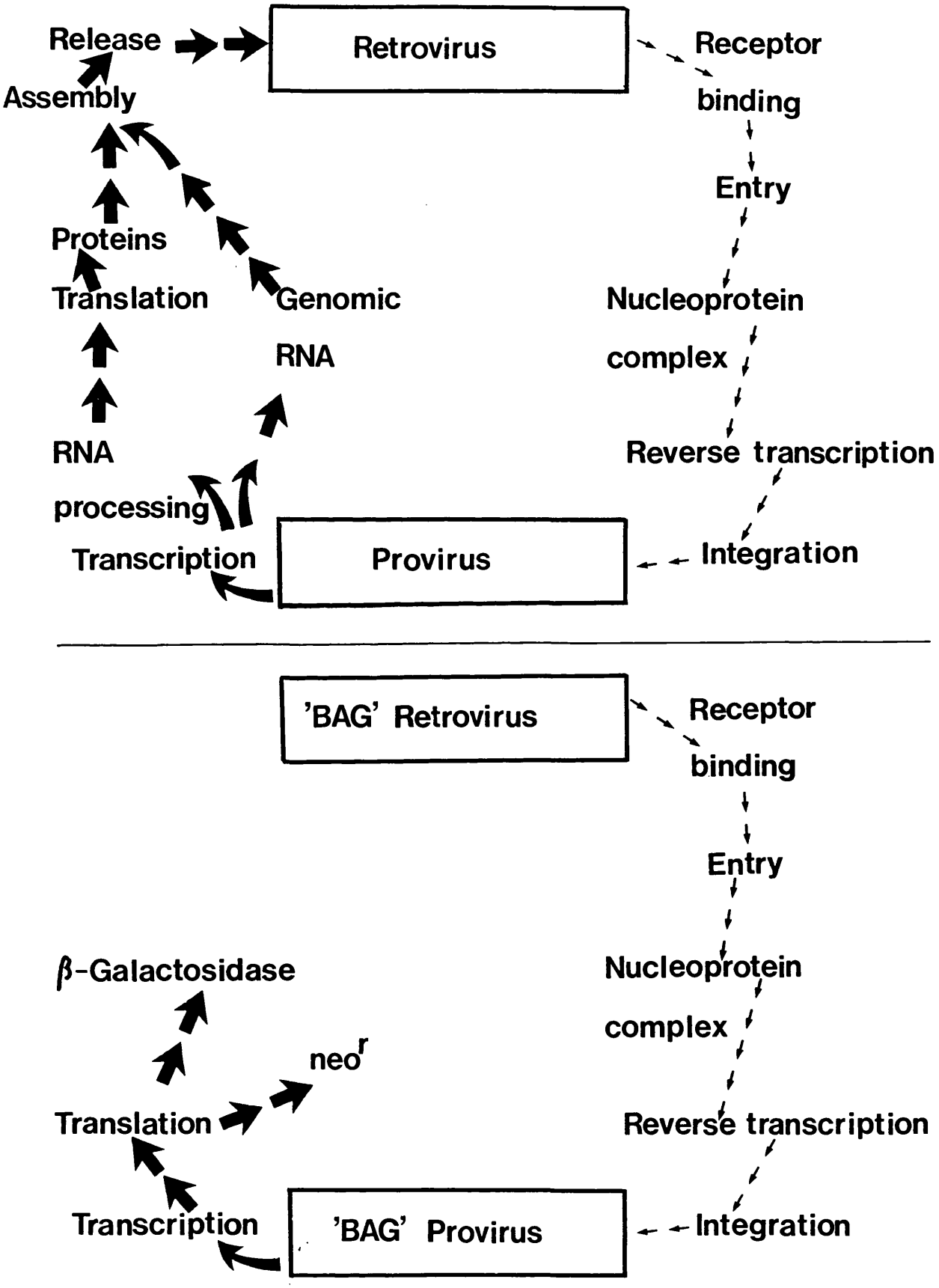
Figure 1.8. The Life Cycle of a Retrovirus and the 'BAG' Retroviral Vector.

Upper figure.

The circular lifecycle of a retrovirus (see text for discussion). The thicker arrows on the left side of the diagram denote an amplification of the genome.

Lower figure.

The 'BAG' retroviral vector has an non-circular lifespan (see text for discussion).


Release
Assembly
Retrovirus
Receptor
binding
Entry
Proteins
Translation
Genomic
RNA
Nucleoprotein
complex
RNA
processing
Transcription
Provirus
Reverse transcription
Integration
'BAG' Retrovirus
Receptor
binding
Entry
β-Galactosidase
neo^r
Nucleoprotein
complex
Translation
Reverse transcription
Transcription
'BAG' Provirus
Integration

1.9.3 Retroviral Adsorption to Host Cells

The host receptor is the first cellular molecule that a virion encounters *en route* to cellular infection (Lentz,1990; White and Littman,1989; Marsh and Helenius,1989; Dimmock,1982; Meager and Hughes,1977). The binding of the virion to the cellular receptor is mediated by the viral *env* protein. However despite their importance in the maintenance of the retroviral life cycle surprisingly little is known about the host receptors and how they mediate internalization. Indeed the host receptor may be the sole agent responsible for tropism (Albritton *et al.*,1989).

Recently a retroviral receptor, termed ERR has been characterized (Albritton *et al.*,1989; Kozak *et al.*,1990) which confers susceptibility to infection by ecotropic retroviruses (see below). The ERR molecule appears to be ubiquitously expressed in all adult tissues (J.Cunningham, personal communication) and there is only one report of cells being refractory to infection, apparently due to a lack of the receptor (Wolff *et al.*,1987). However it is not known if ERR is the only molecule which can serve as a receptor for its cognate virus. Indeed the ERR belongs to a family of proteins (MacLeod *et al.*,1990), but currently it is not known how many of them are viral receptors. HIV has been shown to infect different cell types via different, but uncharacterized, receptors (Clapham *et al.*,1989; Weber *et al.*,1989).

1.9.4 Retroviral Internalization

Upon binding to the cellular receptor the virus is internalized by direct fusion with the membrane and/or by receptor mediated endocytosis (Sommerfelt and Marsh,1989). The virus looses its membrane in the lysosomal compartment (Kabat,1989) to reveal a nucleoprotein complex. The biochemical events underlying the internalization remain largely unknown although, uniquely amongst retroviruses, entry of MoMLV is pH dependent (McClure *et al.*,1990). This pH dependence is probably because a postulated proteolytic cleavage of the *env* protein by host proteases is pH dependent (McClure *et al.*,1990). For HIV, phosphorylation of the HIV receptor is an obligatory step for entry (Fields *et al.*,1988).

1.9.5 Reverse Transcription and Integration of the Retroviral Genome

Reverse transcription of the RNA genome occurs in a nucleoprotein complex in the cytoplasm of the infected cell (Bowerman *et al.*,1989; Ellison *et al.*,1990). A model of how the RNA genome is reverse transcribed into DNA has been proposed (Gilboa *et al.*,1979), which uniquely in viral replication involves two nucleic acid strand transfer reactions. These strand transfers seem to occur non-randomly between the two genomic RNAs (Panganiban and Fiore,1988; Hu and Temin,1990). Unfortunately relatively little is known about how the nucleoporotein complex arrives at the nucleus or which host proteins are associated with it.

Three different forms of viral DNA can be detected in the nuclei of infected cells and only one of them, the linear form, is the exclusive substrate for the integration reaction (Fujiwara and Mizuuchi,1988; Brown *et al.*,1989). Integration (Skalka,1988; Grandgenett and Mumm,1990) occurs via an intermediate in which both 3' ends of the LTR are joined to the target whereas the 5' ends remain free. Cellular enzymes repair the single strand gaps in this integration intermediate and complete the integration reaction.

Integration of the viral DNA occurs essentially at random in the target DNA (Seiki *et al.*,1984), although recent studies have shown that some preferred sites exist (Rohdewohld *et al.*,1987; Shih *et al.*,1989; Gama Sosa *et al.*,1989; Bushman and Craigie,1990). Such preferred loci are perhaps associated with transcribed regions of DNA (Sandmeyer *et al.*,1990). In view of the thousands of transcribed genes the overall probability of repeatedly perturbing specific genes must be very low. However retroviruses are well characterized as germ line insertional mutagens (Hartung *et al.*,1986; Stoye *et al.*,1988; Jaenisch and Soriano,1987).

Retroviruses almost without exception, only integrate into the genomes of mitotic cells during or after the S phase of the cell cycle (Varmus *et al.*,1977; Springett *et al.*,1989; Miller *et al.*,1990). Hence postmitotic cells, e.g. neurones, cannot be infected by retroviruses. Why this is so remains enigmatic as integration can be accomplished, regardless of the replication state of the target DNA, in a cell free system (Brown *et al.*,1987). Perhaps the host nuclear envelope serves as a barrier to the nucleoprotein complex and only when it is removed during mitosis can integration occur. Data from preliminary experiments suggests that after infection only one daughter of the mitotic event inherits the provirus (Austin and Cepko,1990).

1.9.6 Expression of the Retroviral Genome

The retroviral LTR is composed of DNA from the 5' end of the genomic RNA (regions R and U5) adjacent to sequences from the 3' end (U3) of the genome in the order U3-R-U5. The promoter and enhancer sequences, which direct initiation of transcription, are located within the U3 region. Enhancers are *cis* acting sequences which are not unique to viruses and have been described for a wide variety of cellular genes (reviewed by Müller *et al.*,1988). They often consist of tandemly repeated sequences in a bipartite arrangement. For Moloney murine leukaemia virus (MoMLV) each of the two sequences are 75bp long (Laimonis *et al.*,1984), and are themselves composed of a patchwork of conserved sequence motifs (Speck *et al.*,1990). Equivalent motifs occur in different permutations and combinations in other cellular and viral enhancers. Typically enhancers strongly activate transcription, independent of their orientation or position, and in some cases can perform this from a position several hundreds of bases upstream or downstream of the transcription initiation

site. The MoMLV enhancer, for example, is located approximately 160bp from the transcription initiation site.

Enhancers from both cellular and viral genes are often tissue specific, such that transcription is only activated in certain specific cell types. In fact, the disease specificity of retroviruses maps to the enhancer region (Li *et al.*,1987). Exactly how the enhancer of MoMLV confers a leukaemogenic phenotype to the virus is not known as it can infect and express in nearly all rodent cells examined (see below).

Enhancers contain the binding sites for specific protein factors and it is assumed that the association of these proteins must somehow mediate the transcriptional enhancement. The MoMLV 75bp enhancer binds six distinct nuclear proteins of host origin (Speck and Baltimore,1987). Expression from the MoMLV LTR occurs in most cell types examined, and the level of RNA transcribed from the MoMLV LTR in mouse cells has been estimated to comprise 5-10% of the total cellular RNA (Fan,1977). However there is one notable exception where expression from the MoMLV LTR does not occur. MoMLV and several other viruses are transcriptionally inactive in the preimplantation rodent embryo (Jaenisch *et al.*,1975). However, near the time of implantation, the LTR is activated in some of the embryonic cells (Savatier *et al.*,1990).

The block to productive infection has been studied *in vitro* using murine embryonal carcinoma (EC) stem cells (Martin,1980). EC cells are a model system, representing cells of the preimplantation mouse embryo. Like the early embryo they are refractory to viral infection (Teich *et al.*,1977; Hasegawa *et al.*,1990). Results obtained using EC cells suggest that transcriptional inactivity is achieved by three independent mechanisms:

(i) Enhancer elements in the MoMLV LTR are not functional in undifferentiated EC cells (Feuer *et al.*,1989).

(ii) A negative regulatory factor specific to EC cells interacts with the $tRNA^{Pro}$ primer binding site in the MoMLV genome (Feuer *et al.*,1989; Loh *et al.*,1988; Loh *et al.*,1990).

(iii) Finally over a period of several days, the LTR becomes heavily methylated and never expresses again (Gautsch and Wilson,1983), even if the stem cells subsequently differentiate (Niwa *et al.*,1983). However some of these 'silent' genomes can be reactivated by treatment with 5-azacytidine (Jaenisch *et al.*,1985).

EC cells *in vitro* are maintained in an undifferentiated state by a soluble molecule termed leukaemia inhibitory factor (LIF) (Williams *et al.*,1988; Gough *et al.*,1989). The LIF mRNA is present in the extraembryonic tissues of mouse embryos (Conquet and Brûlet,1990) suggesting that EC-like cells could be present within the developing embryo.

1.9.7 Packaging of the Genome into a Virion

A genomic packaging or psi (ψ) sequence (Mann *et al.*,1983; Lever *et al.*,1989; Aldovini and Young,1990; Derse and Martarano,1990) located near the 5' *gag* region is necessary and sufficient (Adam and Miller,1988) for the packaging of the RNA genome into the budding viral capsid. It is possible that this psi sequence is recognized by one of the *gag* proteins containing a 'zinc finger-like' (Klug and Rhodes,1987) protein motif (Gorelick *et al.*,1988).

1.10 Gene Expression Techniques and Vectors

For several experiments, including an examination of cell lineage using genetic markers, it is necessary to introduce genes into cells in which they are not normally found. Both chemical (e.g. $CaPO_4$ transfection (Wigler *et al.*,1978), DEAE-Dextran mediated uptake (Milman and Herzberg,1981)) and physical (e.g. microinjection (Capecchi,1980; Wolff *et al.*,1990), electroporation (Potter *et al.*,1984)) methods have been extensively and successfully used as an efficient means of gene transfer. However in the past ten years viruses have been employed very successfully as efficient delivery vehicles (vectors) for a variety of genes. Viral vectors have a suitable part of their genome deleted and replaced by heterologous gene(s). This recombinant genome can then be packaged normally to form an infectious particle. Expression of the heterologous gene(s) within the host cell can be very efficient.

Many DNA animal viruses have served as vectors e.g. papovaviruses (Hamer and Leder,1979), herpesviruses (Geller and Freese,1990; Geller and Breakfield,1988) and baculoviruses (Pennock *et al.*,1984). However perhaps the most versatile group of viral vectors are derived from retroviruses.

1.11 Retroviral Vectors

Retroviral mediated gene transfer has been the subject of several reviews (Morgenstern and Land,1990a; McLachlin *et al.*,1990; Miller and Rosman,1990; Donehower,1987; Gilboa *et al.*,1986; Temin,1986). The advantages of this technique, both theoretically and practically, are as follows:

(i) Retroviral vectors infect cells both *in vitro* and *in vivo*, with efficiencies sometimes near 100% (e.g. Palmer *et al.*,1987).

(ii) Proviral DNA can be cloned and manipulated like any piece of DNA.

(iii) The *cis* and *trans* elements needed for viral replication are physically segregated in the wild type viral genome. This has enabled the construction of simple complementation systems in which a gene of interest can be easily and efficiently packaged into a viral particle (see 1.9.7 and 1.11.1).

(iv) The proviral DNA is stably inherited by sibling cells and is never excised by retroviral enzymes.

(v) Infection by retroviruses or retroviral vectors is usually not harmful to the host cell.

(vi) The vector integrates into the host nuclear DNA and is not episomal.

(vii) Usually only one copy of a gene is transferred into a single cell and is integrated in a predictable manner.

(viii) At least MoMLV based vectors are stable over quite a large range of pH values (McClure *et al.*,1990) that may be encounted *in vivo* or *in vitro*.

The usefulness of the retroviral mediated gene transfer is most aptly illustrated by the number of vectors which have been constructed to date (23 are quoted in one publication; McLachlin *et al.*,1990) and the fact that they are considered the suitable agents for human gene therapy (Anderson,1984; Drumm *et al.*,1990; Weatherall,1991). However the technique suffers from one inherent problem, which fortunately can be monitored. This is the danger of 'helper virus' (see 1.11.1).

The lifecycle of a retrovirus and how it is curtailed in a retroviral vector is illustrated in figure 1.8. The principle difference between a retrovirus and a retroviral vector is that the latter does not have a circular lifecycle. After infection of an individual cell, a retroviral vector cannot spread 'horizontally' and infect neighbouring cells.

1.11.1 Packaging Systems for Retroviral Vectors

With the knowledge of the *cis* and *trans* elements involved in the retroviral lifecycle (figure 1.9), several groups have constructed packaging cell lines. These cells provide all of the proteins required in *trans* i.e. *gag*, *pol* and *env*, such that by complementation, practically any RNA containing the psi sequence (in *cis*) can be packaged into an infectious virion (figure 1.10). The virion is secreted non-lytically in the supernatant. Typically these packaging lines contain a wild type virus lacking a psi sequence. Hence, although they are able to package any RNA containing the psi sequence they are unable to package their own RNA genome (figure 1.10). The design of packaging cell lines is continually evolving and a large number of different packaging lines are available today. Examples of packaging cell lines include psi-2 (Mann *et al.*,1983), psi-am (Cone and Mulligan,1984), PA317 (Miller and Buttimore,1986), Q2bn (Stoker and Bissell,1988), ΩE (Morgenstern and Land,1990b), *Isolde* (Cosset *et al.*,1990), CRE and CRIP (Danos and Mulligan,1988). Different packaging lines contain different *env* genes which confer different host tropisms. Hence it is possible to package

Figure 1.9. Proviral Sequences Required in a Retroviral Vector.

A MoMLV provirus, with its genomic RNA is shown at the top of the figure. The minimum sequences required for a retroviral vector in *cis* are shown enlarged below. These include the long terminal repeats (LTRs), and the psi sequence. The latter is required for packaging of the genomic RNA into a budding virion. The '-' and '+' sequences are the sites of initiation of '-' and '+' sense DNA respectively. The position of the two 75bp enhancers are shown in the LTR. The 'TATA' sequence is believed to be involved in efficient and accurate initiation of transcription (Breathnach and Chambon,1981). The 'CAAT' sequence is thought to increase the efficiency of transcription initiation (Benoist *et al.*,1980).

One or more heterologous genes can be inserted in a variety of arrangements in the region previously containing the coding sequences of the parental virus. The heterologous genes may or may not have their own promoter-enhancer (P-E) elements.

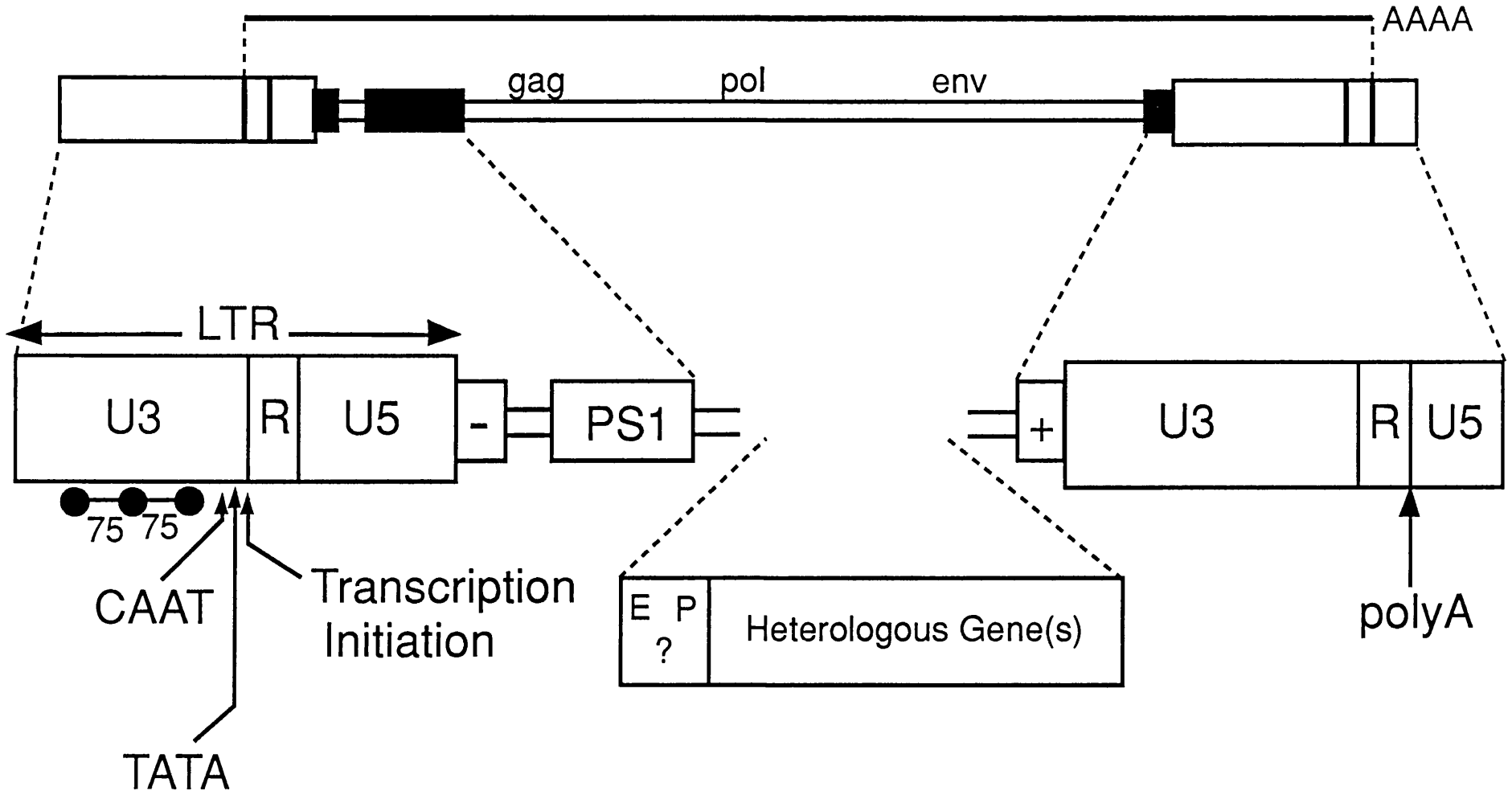
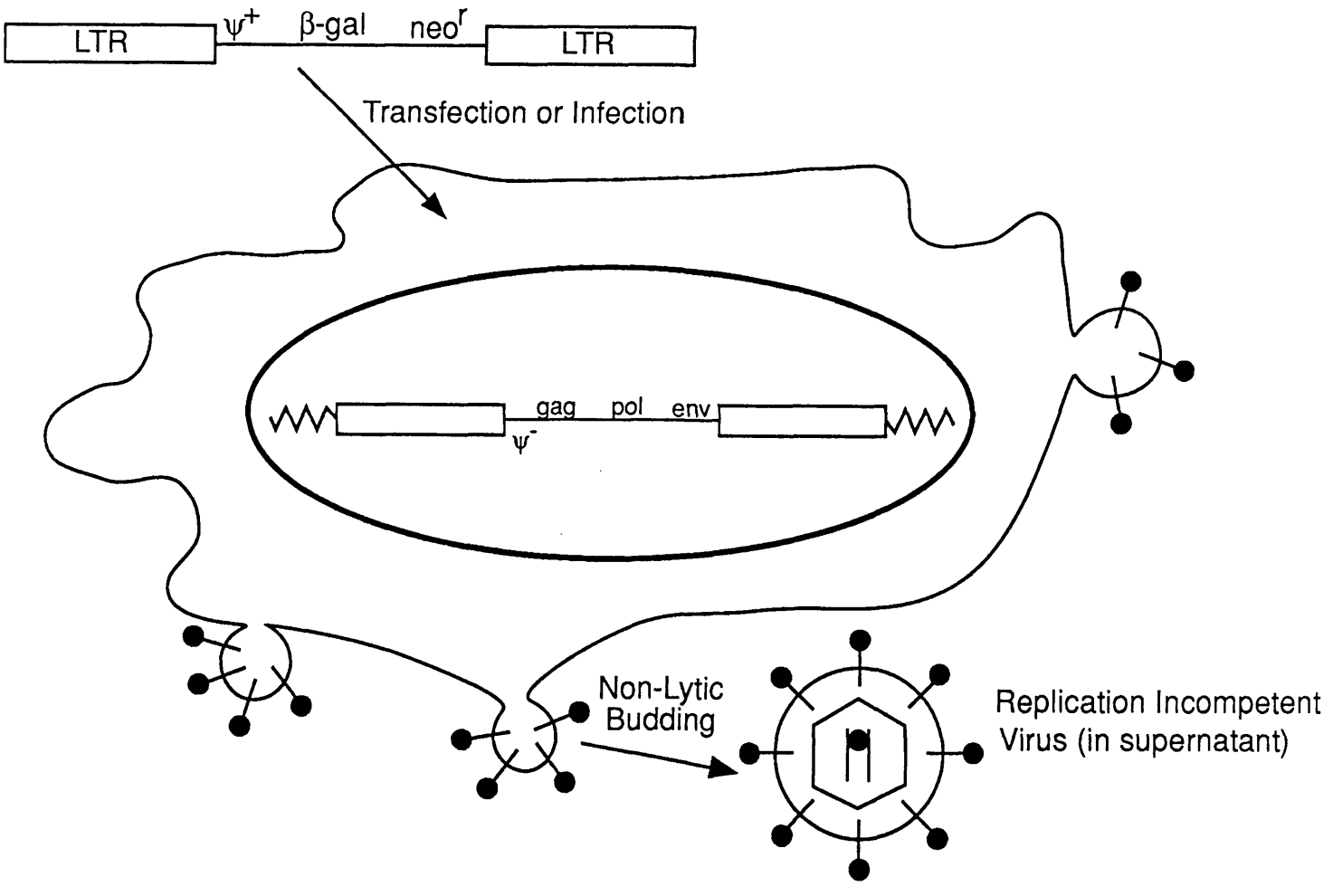

AAAA
gag
pol
env
LTR
U3
R
U5
-
PS1
+
U3
R
U5
75
75
CAAT
Transcription
Initiation
TATA
E
P
?
Heterologous Gene(s)
polyA

Figure 1.10. Production of a Retroviral Vector in Vitro.

Packaging lines are cells containing the *gag*, *pol* and *env* genes as a result of a stable transfection. These genes direct the synthesis of the *gag*, *pol* and *env* proteins. The genomic RNA produced by this virus cannot be encapsidated within a budding virion as it does not contain a psi sequence. However, these cells can package any genome containing the psi sequence into a budding virion. The vector genome can be introduced into the cells by infection or transfection. Retroviruses, containing the vector genome are secreted non-lytically into the supernatant.


LTR
ψ+
β-gal
neor
LTR
Transfection or Infection
gag
pol
env
ψ-
Non-Lytic
Budding
Replication Incompetent
Virus (in supernatant)

practically any RNA into a virion, *in lieu* of the wild type genome, which can then infect any permissive cell. For example, the *env* gene encoded by the psi-2 cell line is of ecotropic specificity and can bind to the cellular receptor present on rodent cells (Mann *et al.*,1983). Superinfection of packaging lines by the virus they produce is prevented by the phenomenon of viral interference (Sommerfelt and Weiss,1990).

As mentioned above one problem associated with retroviral vectors is the potential for 'helper virus'. If a cell infected by a retroviral vector acquires or already has inherited an endogenous provirus then it can potentially become a producer cell as all viral functions needed for infectivity are provided in *trans*. A similar phenomenon can occur by recombination between the vector and the packaging constructs. In order to counteract these problems several modifications have been made to packaging lines so that the wild type virus is crippled in several ways. Perhaps the most promising approach is to physically isolate different parts of the viral genome so that they *trans* complement (Markowitz *et al.*,1988). This is the case with the most of the recently constructed packaging cell lines such as CRIP and CRE (Danos and Mulligan,1988), ΩE (Morgenstern and Land,1990b) and *Isolde* (Cosset *et al.*,1990). This procedure drastically reduces the frequency of helper virus, although does not eliminate it (Bosselman *et al.*,1987; Muenchau *et al.*,1990). Recombination between the vector and packaging genomes can be obviated by reducing the ammount of homology between the two genomes (Bosselman *et al.*,1987; Dougherty *et al.*,1989).

1.12 Retroviruses and Retroviral Vectors as Tools in Developmental Biology

1.12.1 Retroviruses as Cell Lineage Markers

Retroviruses have become an increasingly useful tool with which to probe the mechanisms of mammalian development (Price,1987; Cepko,1988; Cepko,1989a; Cepko,1989b). As mentioned above in some cases they can be used as germ line insertional mutagens to create novel mutations. Retroviral vectors can also immortalize progenitor cells via oncogene transduction (Cepko,1988; Cepko,1989b). However they can also be used as benign markers of cell lineages, where individual lineages are identified by a unique proviral integration site (Korczak *et al.*,1988; Soriano and Jaenisch,1986; Lemischka *et al.*,1986).

1.12.2 Retroviral Vectors as Cell Lineage Markers

A more feasible method of tracing cell lineages involves the use of retroviral vectors encoding proteins or enzymes that can be detected histochemically or immunohistochemically (Herrup,1987; Price,1987; Sanes,1989; Sanes *et al.*,1986; Vaysse and Goldman,1990). Histological detection allows the exact location and identity of individual cells to be established. To date, all such vectors used for cell lineage work have expressed *E.coli* ß-galactosidase (Wallenfels and Weil,1972) as theoretically it is an ideal lineage marker i.e. it is cell autonomous,

developmentally neutral (e.g. Fitt *et al.*,1989; Beddington *et al.*,1989), can be clearly distinguished from endogenous activity (Nanba and Suzuki,1990; Shimohama *et al.*,1989; Gatt and Rapoport,1966), easily assayed (Asp and Dahlqvist,1971; Lim and Chae,1989) and detected histochemically (Messer and Vielmetter,1965; Lojda,1970; Gossrau,1973; Holzmann and Johnson,1983).

One retroviral vector used in lineage analysis is the 'BAG' retroviral vector (see 1.12.3 and figure 1.11) (Price *et al.*,1987). All progeny of a single 'BAG' infected cell will inherit the proviral DNA, and provided gene expression occurs in all decendents, infected cells can be detected histochemically. Furthermore the expression of ß-galactosidase from the 'BAG' retroviral vector does not appear to be negatively regulated or cause perturbations in developing cells as results obtained with it have been reproduced using conventional lineage tracing techniques (Holt *et al.*,1988; Wetts and Fraser,1988) and different retroviruses (Turner *et al.*,1990; Cepko,1989a).

However retroviral vectors suffer from one limitation that is a particularly problem for lineage studies. The position of an infected progenitor cell is not known *a priori*. Hence if progeny cells migrate away from one another they need to be inferred as belonging to a particular clone. This limitation is not too severe when the technique is used to study lineage in a two-dimensional system such as dissociated tissue cultures *in vitro* (e.g. Vaysse and Goldman,1990). Here the number of infective events can be more carefully controlled than *in vivo* and any clonal spread is restricted to two dimensions. However not all of the features of cortical development can be reproduced *in vitro*, for example the barrel/columns are unique three-dimensional histological entities and only form *in vivo*.

1.12.3 The 'BAG' Retroviral Vector

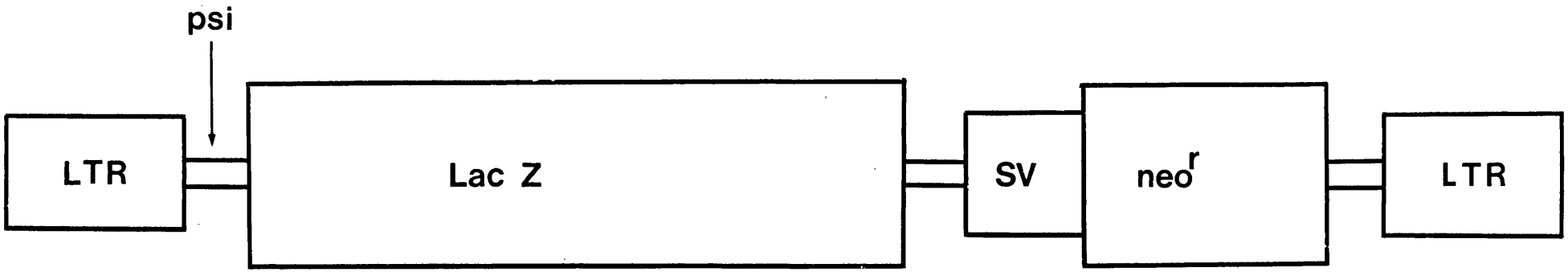
The 'BAG' vector (Price *et al.*,1987) (figure 1.11) encodes two proteins: ß-D-galactosidase (lactase; E.C. 3.2.1.23) and neo^r. The former is used to detect 'BAG' infected cells histochemically. All of the *cis* elements of the vector (e.g. psi sequence, LTR etc.) are derived from MoMLV (Shinnick *et al.*,1981). Expression of the ß-galactosidase is driven from the enhancer and promoter elements in the MoMLV LTR whereas the neo^r gene is expressed from a SV40 promoter. The latter confers neomycin or G418 resistance (Bar-Nun *et al.*,1983). Sufficient ß-galactosidase is transcribed from the LTR for histological detection within 1-2 cell cycles postintegration (Austin and Cepko,1990).

1.13 Retroviral Vectors and Cell Lineage in the Developing Rodent Neocortex

Several independent research groups have applied retroviral vectors, including 'BAG', to the study of cell lineage in the neocortex (Walsh and Cepko,1988; Luskin *et al.*,1988; Price and Thurlow,1988; Austin and Cepko,1990). They have attempted to address two specific questions.

Figure 1.11. The 'BAG' Retroviral Vector.

The 'BAG' retroviral vector (Price *et al.*,1987) has been applied to a myriad of *in vivo* and *in vitro* studies since it was constructed four years ago. This is partly because of the ease with which infected cells can be specifically detected by ß-galactosidase histochemistry. *LacZ* encodes *E.coli* ß-galactosidase (Kalnins *et al.*,1983), neo^r is a gene conferring neomycin (or G418) resistance (Southern and Berg,1982) and SV denotes the SV40 promoter. The position of the psi sequence in the genome is shown by an arrow. Proviral 'BAG' is approximately 7kb in size.


psi
LTR
Lac Z
SV
neo^r
LTR

First, at what point do undifferentiated VZ cells commit themselves to generate neurones or glia (for a review of evidence before the use of retroviral vectors see McConnell,1988a)? The data from both *in vivo* and *in vitro* experiments using retroviral vectors to date indicates that there are at least two distinct types of progenitor cell co-existing in the VZ during the peak period of neurogenesis (Price and Thurlow,1988; Luskin *et al.*,1988; Temple,1989; figure 1.12). One generates only grey matter astrocytes and as previously discussed (see 1.3.5.3) may be a radial glial cell. The other, termed the multipotential precursor cell (J.Price and B.P.Williams, personal communication), gives rise to neurones and some types of glial cell. The neurones and glia are generated via intermediate precursors, a neuronal precursor (N^* in figure 1.12) and the cortical equivalent of the O2A cell (Raff,1989) termed the 'horizontal cell', respectively. The O2A cell from the optic nerve, at least *in vitro*, has the ability to generate either oligodendrocytes or a type of astrocyte (reviewed by Raff,1989). Possibly the horizontal cell is a source of cortical oligodendrocytes and astrocytes, as is the O2A cell *in vitro*. This multipotential precursor bears a striking similarity to the progenitor cell found in the chick optic tectum (discussed in 1.2). In both cases it is not clear if progeny are committed to a phenotype after they leave the VZ or if fate is dictated by environmental influences. For example the horizontal cell fate could be dictated by chance interactions within the IZ, with say axon fascicles.

Thus, according to the model outlined in figure 1.12, during the peak of neurogenesis lineage restrictions for different cell types have occurred. This is in contrast with the situation in the retina where different cell types are generated from a multipotential precursor cell (Turner and Cepko,1987; Turner *et al.*,1990).

The second point of interest relates to when the columnar organization of the neocortex is specified (for a review see McConnell,1989b). Are progenitor cells positionally specified in the VZ as suggested in the radial unit hypothesis? Although the research groups have differed in the extent to which they emphasized clonal spread (see 1.2; Price and Thurlow,1988; Luskin *et al.*,1988), none have analyzed if the lateral spread was constrained within a physiological cortical column i.e. none have directly tested the radial unit hypothesis. It is clear however that progenitors in the VZ are not positionally specified in one respect; individual VZ cells generate neurones that occupy several neocortical laminae (Walsh and Cepko,1988; Luskin *et al.*,1988; Price and Thurlow,1988). However it is not clear if a VZ cell can generate neurones present in *all* layers (as is the case in the retina and tectum).

1.14 Outline of the Present Studies

The work presented here constitutes an attempt to examine if the embryonic VZ was positionally specified as suggested in the radial unit

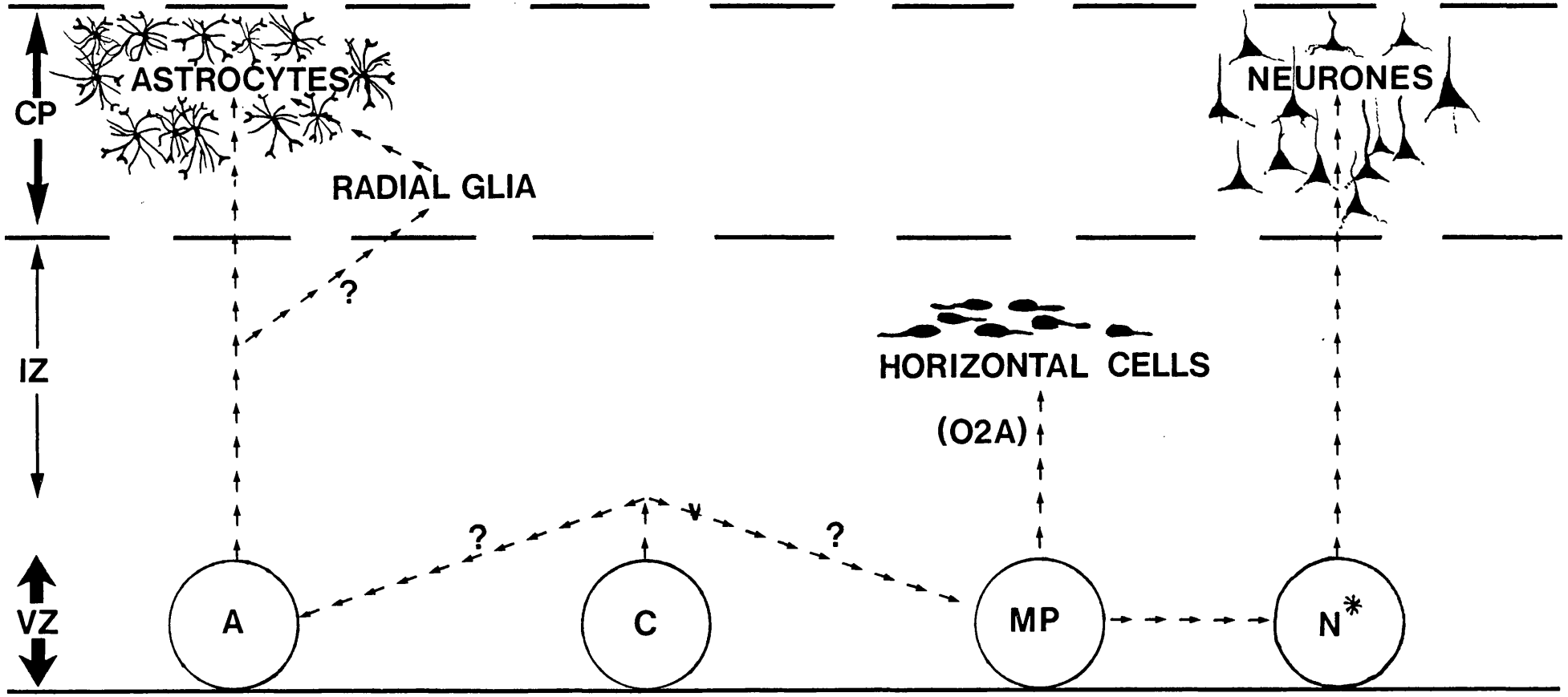
Figure 1.12. A Model of Cell Lineage in the Developing Rodent Neocortex.

The precursor cells are located in the ventricular zone (VZ) and migrate superficially through the intermediate zone (IZ) to the cortical plate (CP). The model principally has two distinct lineages.

(i) Cortical astrocytes are derived from an astrocytic precursor (A). However the exact route from A to cortical astrocytes is not clear as cell A may be a radial glial cell which subsequently transforms into an astrocyte.

(ii) The second lineage derives from the multipotential cell (MP) which can generate neurones and white matter glia, the latter of which have been termed horizontal cells. The horizontal cells are believed to be the cortical equivalent of the O2A cell, a bipotential cell that has been extensively studied in the optic nerve. Neurones are generated from MP *via* a committed neuronal precursor (N^*). Thus if MP is labelled by 'BAG' then a clone containing neurones and horizontal cells will be produced. However only neurones will be produced by infection of N^*.

It is likely, although not proven to date, that both the lineages described above were derived from a common progenitor cell (C) at some earlier point in development.


CP
ASTROCYTES
RADIAL GLIA
NEURONES
IZ
?
HORIZONTAL CELLS
(O2A)
?
?
VZ
A
C
MP
N*

hypothesis. This has been done by considering cell lineage relationships, specifically the spatial distribution of clones found within rat Sm1. Cell lineage was examined with the 'BAG' retroviral vector.

The initial studies (Chapter Two) centred upon producing and characterizing viral stocks. All assays involved the histochemical detection of 'BAG' encoded ß-galactosidase using X-Gal. Selected virus stocks were then used to infect the VZ of embryonic rats *in utero* (Chapter Three). In the mature animal 'BAG' labelled cells were detected histochemically and the topological distribution of clones examined in relation to Sm1 barrel/columns. The implications of the observed distribution are discussed in relation to proposed models of neocortical development. Finally the data in Chapter Four relates to an attempted cell lineage study in the chick forebrain.

Chapter Two : Retroviral Vector Production and Concentration

2.1 Introduction

In order to infect rat neocortical progenitor cells in the VZ and progenitor cells within the chick neural tube stocks of 'BAG' retrovirus, with both ecotropic and amphotropic were prepared (see below) specificity. Preliminary studies in the rat suggested that the titre of virus made by the ecotropic producer line was insufficient to infect VZ cells *in utero* (J.Price, unpublished observations). Hence all viruses were concentrated to increase the titre prior to injection. The number of virions in each concentrated viral sample was assayed in order to provide an estimate of the number of virions introduced near the target. Similarly, the effect of polybrene on viral infectivity was examined. The latter was important as, if possible, polybrene should have not been injected with the virus. The viral samples used in my studies were assayed for helper virus in order to show that horizontal spread of the 'BAG' virus was unlikely.

The ecotropic virus was used to infect VZ cells in the rat embryo. Several aspects of the experimental design and results are presented in Chapter Three.

In order to infect avian cells and examine cell lineage within the avian forebrain virions were created with amphotropic specificity. The amphotropic virion contained an *env* protein in its lipid envelope that could bind to the cellular receptor(s) present on cells from a wide variety of species e.g. human, monkey, rodent and avian cells (e.g. Sorge *et al.*,1984; Delouis *et al.*,1990). Amphotropic viruses were successfully produced and concentrated. They were applied to the study of clonal migration in the avain forebrain and the results of the experiments are presented in Chapter Four.

2.2 Materials and Methods

2.2.1 Cell Culture and Cell Lines

All cell culture techniques were performed inside biohazard laminar flow hoods. All cells were propagated at 37°C in a humidified atmosphere of 95%(v/v) air, 5%(v/v) CO_2, in monolayers on stationary dishes; either 10cm (Becton Dickinson Labware,USA) or 3.5cm (Nunc,Denmark) diameter. Cell lines were regularly screened for *Mycoplasma* sp. (see 2.2.5).

All tissue culture medium was supplemented with 2mM L-glutamine, 0.3mg/ml penicillin and 0.5mg/ml streptomycin. Cells were routinely dissociated using a trypsin (0.125% w/v)-EDTA (0.01% w/v) solution. All tissue culture solutions and medium were stored at 4°C until required, but were prewarmed to 37°C before addition to cultured cells. Serum was heat inactivated at 56°C for 30 min prior to aliquoting. Serum aliquots were stored frozen at -20°C.

The nomenclature applied to the producer cells and the virus produced by them is shown in Table 2.1. All producer cells, except LZ10 (Galileo *et al.*,1990; see Table 2.1), contained the 'BAG' genome.

The S2, psi-2 (Mann *et al.*,1983), CRIP (Danos and Mulligan,1988) and NIH 3T3 (Jainchill *et al.*,1969) cells were cultured in DMEM (made from

TABLE 2.1.

The Nomenclature of the Packaging Lines and Retroviral Vectors Used in this Study.

Packaging Cell Lines	Name of Producer Cells	Name of Virus
Psi-2	S2	BAG-psi^{+}
CRIP	SL88	BAG-C*
PA317	JPE87	BAG-P*
-	LZ10	vLZ10$^{\#}$

+ denotes an ecotropic virus, * denotes amphotropic virus and # denotes avian virus

powdered medium no.074-2100; Gibco,UK) with supplements of 0.11mg/ml sodium pyruvate, 3.7mg/ml sodium hydrogen carbonate and 10% (v/v) NCS (Sera-Lab,UK).

The PA317 (Miller and Buttimore,1986) packaging line was grown in MEM-FXV medium (made from powdered medium no.072-1500; Gibco,UK) with supplements of 2.2mg/ml sodium hydrogen carbonate, 10% (v/v) foetal calf serum (FCS) (Sera Lab,UK), non-essential amino acids (Gibco,UK), 0.45% (w/v) D-glucose (Sigma,UK) and HAT (from a 50x supplement supplied by Gibco,UK) selection. The final concentrations of HAT components in the medium were 10^{-1}mM hypoxanthine, $5x10^{-4}$mM aminopterin and $5x10^{-2}$mM thymidine.

The LZ10 producer cells and QT6 (Moscovici *et al.*,1977) cells were grown in Medium 199 (made from powdered medium no.071-01100; Gibco,UK) supplemented with 5.5mg/ml sodium hydrogen carbonate, 10% (v/v) tryptose phosphate broth (Gibco,UK), 5% (v/v) FCS (Sera-Lab,UK), 1% (v/v) chicken serum (Gibco,UK) and 1% (v/v) dimethylsulphoxide (DMSO) (BDH,UK).

Only LZ10 producer cells were grown in the presence of G418 ('Geneticin' disulphate salt; Sigma,UK; lot no.107F-3538 containing 483μg Geneticin per mg) at 400μg/ml in the medium. This was removed whenever medium was conditioned for virus production. A stock of 100mg/ml G418 was made by dissolving the solid in DMEM, filter sterilizing and titrating the pH to 7.4.

The XTC, XL-177 or XL *Xenopus* cell lines were cultured at a room temperature of 25°C. For references relating to the source of the cells and precise culture conditions see Smith (1987). All of the *Xenopus* culture work was performed using the tissue culture facilities kindly provided by Dr.J.C.Smith.

All attempts at viral infections *in vitro* were performed in the presence of 10μg/ml polybrene (Hexadimethrine bromide; Sigma,UK). This was made as an aqueous stock at 1mg/ml and sterilized by filtration.

2.2.1.1 Preparation of Chick Embryo Fibroblasts

The skin was removed from the heads of E7-8 chick embryos and was chopped up in Ca^{2+}/Mg^{2+} free saline (the chicken embryos were procured as outlined in 4.2.1). This tissue was then placed in a Sterilin tube and incubated at 37°C in 0.1% (w/v) collagenase (Sigma,UK; type 1A from *C.histolyticum*) for 15 min and then in 0.1% (w/v) trypsin (Sigma,UK; from bovine pancreas) for 10 min. The enzyme action was stopped by the addition of medium containing serum (see below). After allowing to settle the supernatant was removed and the cells were washed again in medium containing serum. The cells were triturated in 1-2mls of complete medium (see below) and filtered through VS monodur gauze. Cells were plated at a density of $2x10^5$ per 3.5cm dish and grown in DMEM (see 2.2.1) containing 10% (v/v) horse serum (HS) (Gibco,UK) and 2% (v/v) chick embryo extract (CEE) (see below). The 3.5cm dishes were coated with 10μg/ml poly(D-lysine) (PDL) (Sigma,UK; molecular weight range 70-150kDa) for a few hrs prior to plating. When confluency was reached the

cells were passaged just like a cell line, but all dishes were pretreated with PDL as described above.

CEE was made by pushing whole embryos aged E10 or E11 through a syringe. This suspension was then stirred at room temperature with DMEM in the ratio of 1:1 for 1 hr and then centrifuged at 10,000rpm for 30 min in a Sorvall Superspeed RC2-B centrifuge at 4°C. The supernatant was aliquoted and stored at -70°C.

2.2.1.2 Cryopreservation of Cell Lines

Cell lines were frozen in cryotubes (Nunc,Denmark) in the medium they were grown in supplemented with 10% (v/v) DMSO (BDH,UK) as a cryoprotectant (Mazur,1984). Cells were dissociated from a confluent plate, suspended in cryoprotectant medium and aliquoted as 1ml samples in cryotubes. The cryotubes were cooled to -20°C, transferred to the -70°C freezer overnight, and then to liquid nitrogen.

2.2.2 Generation of Amphotropic 'BAG' Producing Cells

PA317 and CRIP cell lines were infected with the ecotropic BAG-psi virus by adding BAG-psi virus, to adherent PA317 or CRIP cells growing *in vitro*, for 24 hrs in the presence of 10μg/ml polybrene. The multiplicity of infection was one. Cells were counted with a haematocytometer and the virus was from a previously titred stock (see below for titration details). Two days later 'BAG' infected cells were selected for by the addition of 1mg/ml G418 to the medium. The names given to the infected producer cells and the retroviral vectors they produced are shown in Table 2.1.

2.2.2.1 Cloning of 'BAG' Infected Cells

'BAG' infected cells were cloned by serial dilutions of a trypsinized suspension of cells, plated in 96 well plates (Nunc,Denmark). Selected clones were cultivated on successively larger tissue culture plates. Both the SL88 and JPE87 producer cells were cloned in this fashion and several independent clones were isolated for each producer: three for JPE87 and four for SL88. Different clones were denoted by a Greek letter after the cell name e.g. SL88α.

2.2.3 Production of Retroviral Stocks

Two methods have been used to produce concentrated stocks of the 'BAG' retrovirus (Cepko,1989a; Morgenstern and Land,1990a). Throughout this study one method was used (Price *et al.*,1987) which required the minimum of equipment and was accomplished by an overnight centrifugation.

Producer cells that were 70% confluent in 10cm diameter dishes (approximately 2.8×10^6 cells per dish) were fed 5ml, rather than the normal 10ml, of medium. Harvested medium was usually frozen and stored at -20°C at this point; in fact this step often resulted in precipitation of unwanted clumps which were subsequently removed. Upon thawing this conditioned medium was filtered through a 0.45μm Nalgene

filter (Nalge Company,USA). A small volume of this unconcentrated medium was frozen for subsequent viral titration. A full polyallomer centrifuge tube (Beckmann,USA) (normally containing 40ml of unconcentrated medium) was spun for 16 hrs at 14,000rpm in a Beckmann SW28 rotor, using a Beckmann L8-70 ultracentrifuge, at 4°C. After carefully decanting the medium the pellet at the bottom of the tube was *gently* resuspended in one hundredth of the original volume with FCS (usually 400µl), aliquoted into cryotubes and stored at -70°C. Amphotropic viruses (BAG-C, BAG-P and vLZ10) were also concentrated by this protocol but in order to increase titre the pellet was resuspended in 100µl of FCS, which was also divided into aliquots and frozen.

Virus stocks were never frozen on dry-ice. This procedure lowered the pH and inactivated the virus.

After reading a recent publication (Knight,1990), in order to remove putative toxic factors, all Nalgene filters were washed first with a few mls of sterile warm water and then medium prior to use.

2.2.3.1 Titration of Viral Stocks

2.2.3.2 Titration of Ecotropic Virus

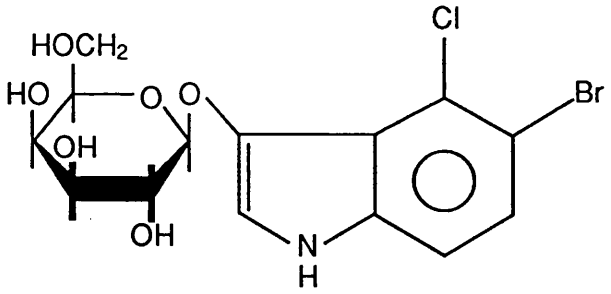
All ecotropic virus titrations were performed on the mouse NIH 3T3 cell line. The titration, outlined below, was an assay for active viral particles. NIH 3T3 cells were plated at a density of $2x10^5$ per 3.5cm plate. The following morning the medium was removed and 1ml of fresh medium was added to each plate. The virus was added to the medium together with 10µg/ml of polybrene. A range of volumes of virus was added. For the higher titre BAG-psi virus these were 0.1µl, 1µl, 2µl per plate from the concentrated stock. The volumes was increased to 10µl and 50µl per plate for unconcentrated supernatant. For the amphotropic viruses these volumes were increased to 1µl, 2µl of concentrated stock and 100µl of unconcentrated. These volumes were added to duplicate plates. A positive control of previously titred virus was included in each experiment, together with a plate of cells that received no virus as a negative control.

The cells were then returned to the incubator and occasionally were gently shaken. After 3 hrs, 1ml of fresh medium was added. The following day the 2mls of medium was removed and replaced with fresh medium. The cells were then allowed to grow to confluency, which normally took between three and four days. They were then stained histochemically for ß-galactosidase (see figure 2.1 for a description of the reaction). Cells were fixed in 1ml of 0.5% (v/v) glutaraldehyde (from a 25% solution; BDH,UK) in PBS for 5 min. The plates were then rinsed for 3x10 min with an excess of PBS containing 2mM $MgCl_2$. This washing step was found to be very important for subsequent X-Gal staining, as some step of the X-Gal reaction was inhibited by glutaraldehyde. After the final rinse, the plates were incubated overnight in a solution of 1mg/ml X-Gal, 30mM potassium ferrocyanide, 30mM potassium ferricyanide, 0.01% (w/v) sodium deoxycholate, 0.02% (v/v) NP40 and 2mM $MgCl_2$ in PBS pH 7.4.

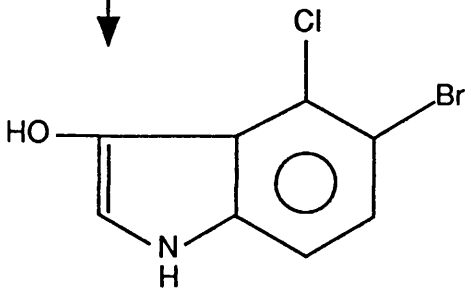
Figure 2.1. The Indigogenic Method of ß-Galactosidase Detection.

This histochemical reaction is widely used in a variety of applications and renowned for its sensitivity. The reaction involves the cleavage of the ß-D-galactoside bond of 5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (X-Gal) (I) liberating a substituted molecule (II). This molecule then undergoes an oxidative dimerization to generate a blue coloured, alcohol insoluble, indigo molecule (III). The oxidation is enhanced by the presence of an oxidizing agent such as Fe^{2+} (Holt and Withers,1952) or NBT (McGadey,1970). The Fe^{3+} ion is needed to stop the reaction from generating a further reduced and colourless molecule (Dannenberg and Suga,1981). The indigo molecules are of the *trans* configuration and can undergo hydrogen bonding (Holt and Sadler,1958) and form microcrystalline aggregates. The indigo produced from the reaction is electron dense and indigo aggregates can be seen with the electron microscope (Bonnerot *et al.*,1987; Bunge *et al.*,1989). This X-Gal histochemical technique is not a vital staining method (MacGregor *et al.*,1987); only one such method for ß-galactosidase exists (Nolan *et al.*,1988; Kerr *et al.*,1989). Counterstaining is compatible with the X-Gal technique (Thomason and Booth,1990; Cepko,1989a; Price and Thurlow,1988).

(I)

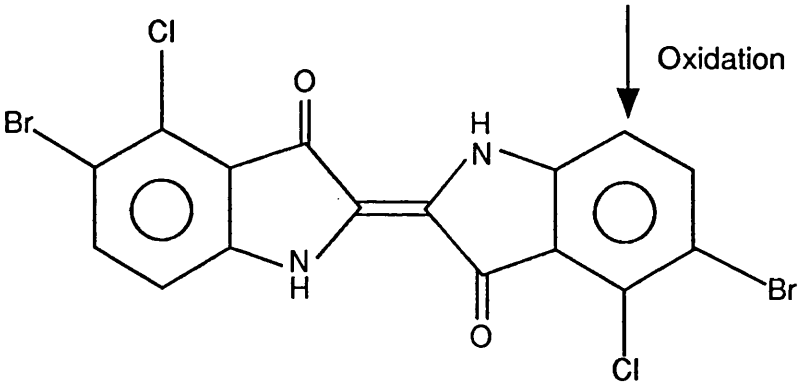


(II)



Oxidation

(III)



The overnight incubation was performed in a tissue culture incubator at 37°C. The X-Gal was diluted from a stock of 40mg/ml in dimethylformamide (DMF) (this X-Gal stock was stored in the dark at 4°C). The X-Gal stock was always aliquoted with a glass pipette. X-Gal sometimes crystallized after addition to the staining solution. This was reduced by pre-warming the solution that contained ferrocyanide and ferricyanide prior to the addition of X-Gal. X-Gal was obtained from a variety of sources (Sigma,UK; Gold Biotechnology,USA; Novabiochem,UK).

The number of X-Gal positive clones found in 5x 0.25cm^2 of each dish were counted using an inverted phase contrast microscope at a low magnification (x10). At low densities of infection clones were defined as discrete clusters of stained cells in dishes. Clones could be easily distinguished from one another up to a point where 25 were found in 0.25cm^2.

The X-Gal histochemical assay provided a convenient means of monitoring cross contamination of cell lines as no staining was ever seen in any of the vector negative lines; any cells that were X-Gal positive contained the 'BAG' genome.

2.2.3.3 Titration of Amphotropic Virus

Amphotropic viruses (except vLZ10) were also titred on NIH 3T3 cells. In order to assay if amphotropic viruses infected avian cells the titration was also performed on chick embryo fibroblasts (see 2.2.1.1) and the QT6 cell line (Moscovici *et al.*,1977). The titration of the amphotropic viruses were performed in a manner directly analogous to that stated above.

2.2.4 Screening for Helper Virus

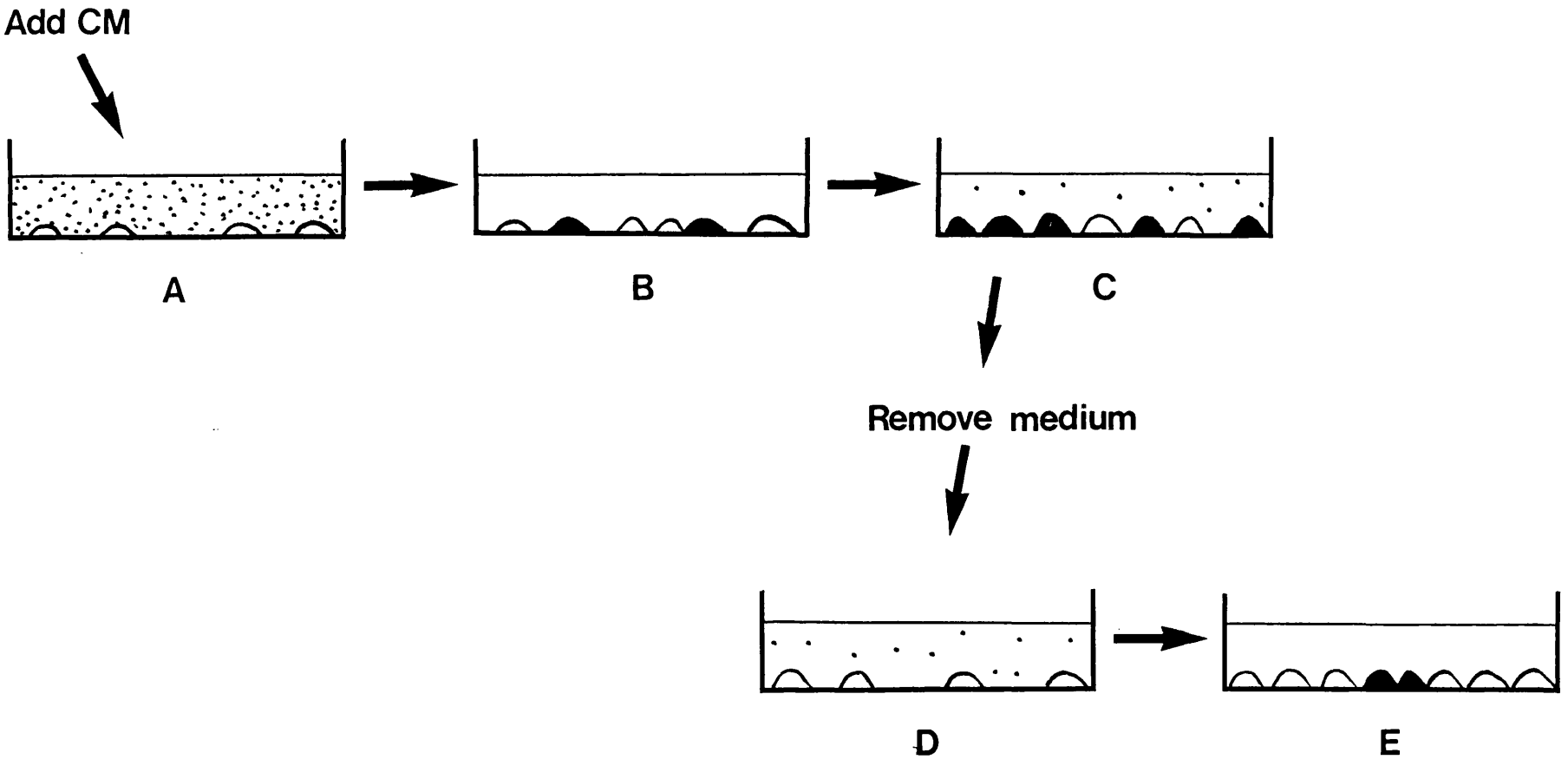
An assay was used which involved infecting NIH 3T3 cells and then examining to see if these infected cells became producers (figure 2.2) (Cepko,1989a). It was *crucial* to ensure that at all steps in the assay where medium is transferred, no cells were transferred. If this was not performed then a false positive was obtained. Removal of cells was accomplished by filtering medium through a 0.45μm Nalgene filter (Nalge Company,USA).

NIH 3T3 cells at a density of 2x105 per 3.5cm plate were infected in the presence of 10μg/ml polybrene, with filtered medium from producer cells. The following day they were fed with fresh medium which was then kept on the cells for two or three days. This medium was then removed and filtered. The NIH 3T3 cells were then stained histochemically with X-Gal, as described in 2.2.3.1. This step ensured that the initial infection was successful. The filtered medium collected from these cells was then added to a new culture of NIH 3T3 cells (at a density of 2x105 per 3.5cm plate) in the presence of 10μg/ml polybrene. After 24 hrs this medium was removed and the cells were allowed to grow to confluency. Upon reaching confluency they were fixed and histochemically stained with X-Gal (as in 2.2.3.1) together with a positive control of producer

Figure 2.2. The Experimental Protocol Used to Assay for Helper Virus

The figure illustrates the outcome of a positive result i.e. helper virus present in stocks. 'BAG' infected cells are coloured black.

A. Filtered conditioned medium (CM) is added to subconfluent NIH 3T3 cells.
B. These cells become infected.
C. If any of these cells start to produce 'BAG' virus it will be secreted in the medium.
D. This medium is added to new NIH 3T3 cells.
E. If helper virus is present then these NIH 3T3 cells will be infected and can be detected histologically with X-Gal.


Add CM
A
B
C
Remove medium
D
E

cells and a negative control of NIH 3T3s. Helper virus was considered to have been present if any X-Gal stained cells were found in the second plate of NIH 3T3s.

2.2.5 Mycoplasma Screening of Cell Lines

Mycoplasma screening was performed using Hoechst 33342 (Aldrich,UK) (Russell *et al.*,1975). This is a DNA fluorochrome which stains the nuclear DNA at AT rich regions (Arndt-Jovin and Jovin,1977). Sterile 1cm diameter circular glass coverslips (BDH,UK) were placed in a culture dish of newly dissociated cells. The cells were allowed to settle and grow for several days. The coverslip was then removed and fixed in 4% (w/v) paraformaldehyde, and after a rinsing in PBS were stained for 10 min with a 0.02mM aqueous solution of Hoechst 33342. *Mycoplasma* positive cultures contained staining in the cytoplasm. All material for immunofluoresecent examination was mounted with Citifluor (Citifluor Ltd.,UK). Coverslips were sealed with colourless nail polish (Rimmel,UK) and examined under a Zeiss Photo-Microscope III. Any cultures judged infected after examination were discarded.

2.3 Results

2.3.1 The Viral Producer Cells and X-Gal Staining

Tissue culture cells not infected by any of the retroviruses in Table 2.1 never stained using the X-Gal histochemical protocol outlined in 2.2.3.2. Any producer cells that were stained with X-Gal had indigo uniformly distributed uniformly throughout the cytoplasm.

The percentage of SL88, JPE87 and S2 cells that stained with X-Gal was approximately 98%. This value remained constant throughout subculture, even though the none of these cells were not kept in constant G418 selection. However the LZ10 producer line stained heterogeneously, with only approximately 60% of the cells were positive (although the cells that were stained were strongly positive). G418 selection did not increase this figure.

2.3.2 Retroviral Concentration and Titres

Freezing virus conditioned medium did not alter viral titres. This proved to be very useful as it was possible to collect conditioned medium and freeze it, and then concentrate virus some time later. After conditioned media was centrifuged as described in 2.2.3, no virus whatsoever was detected in the supernatant for any of the viruses used in this study.

2.3.2.1 Retroviral Titres From the Ecotropic Producer Cells

When titred on NIH 3T3 cells the unconcentrated supernatant from the S2 producer line contained $2x10^5$cfu/ml. Upon concentration this value was increased to 10^6cfu/ml. These figures represent a five fold increase in titre and a 5% recovery of virus.

2.3.2.2 Retroviral Titres From the Amphotropic and Avian Virus Producer Cells

When titred on NIH 3T3 cells, the unconcentrated supernatant produced by all but one (see below) clone from both the JPE87 and SL88 cells contained $3x10^3$cfu/ml. After concentration this value was increased to 10^5cfu/ml with an 8% recovery of virus.

As the SL88 cell line had the least complex medium requirements it was used for most of the subsequent studies on avian embryos. One SL88 clone (SL88ϵ) produced very little virus. The vast majority of SL88ϵ cells stained positive with X-Gal. Presumably the lack of viral production was due to contamination by non-viral producer cells.

When titred on CEF, the unconcentrated BAG-C virus gave a titre that was approximately 57% of that seen with this virus added to NIH 3T3 cells. However no titre could be obtained for the concentrated SL88 supernatant on chick embryo fibroblasts, partly due to problems in cultivating the fibroblasts.

In view of these disappointing results an attempt was made to produce stocks of the LZ10 retroviral vector (Gray *et al.*,1988). This virus did not infect NIH 3T3 cells and was titred on the QT6 cell line or chick embryo fibroblasts. However no significant viral production from the LZ10 cells could be demonstrated. When unconcentrated supernatant was added to QT6 or chick embryo fibroblasts very few virions were present (QT6 ,1cfu/ml; chick fibroblasts, 5cfu/ml).

On the advice of P.Savatier (ICRF Developmental Biology Unit,UK) medium was conditioned by LZ10 cells for only 4 hrs. This was an attempt to evade an extreme acid pH value which can be found in some *in vitro* avian cultures. The acid pH values can inactivate retroviruses. However this precaution did not increase the viral titre.

Attempts at cloning individual cells from the heterogeneous LZ10 population, by serial dilution (see 2.2.2.1), were uniformly unsucccessful as the cells died when plated at low densities.

2.3.2.3 Modification of the Virus Concentration Method

In a preliminary experiment virus containing supernatant, prepared as described in 2.2.3, was centrifuged for only 2 hrs at 14,000 rpm. Pre-centrifugated and post-centrifugated supernatant and the resuspended pellet, all from BAG-C producer cells, were then titred on NIH 3T3 cells. Post-centrifugation, the supernatant sample was removed from half way down the centrifuge tube. Samples taken at this point from the centrifuge tube contained $3.8x10^2$cfu/ml. Hence not all of the virus was pelleted after 2 hrs centrifugation as 13% of the pre-concentrated titre still remained in the supernatant. However normal viral titres (10^5cfu/ml) were obtained from the resuspended pellet.

2.3.3 The Effect of Polybrene on 'BAG' Infectivity in Vitro

In one experiment the quantitative effect of polybrene upon 'BAG' infection of NIH 3T3 cells *in vitro* was examined. The number of

infective events was assayed by viral titration, as outlined in 2.2.3.2. Polybrene was mixed with the cells on addition of virus; no attempt to pretreat cells or virus was attempted. Cultures of NIH 3T3 cells received an addition of $3x10^5$ or $6x10^5$ cfu BAG-psi virions, with or without 10μg/ml polybrene. In the absence of 10μg/ml polybrene only 0.8% (plates received 3x105 virions) and 2.3% (plates received 6x105 virions) of cfu was observed compared to the titre obtained when the same titre of virus was added to cells with 10μg/ml polybrene. This effect with 'BAG' was strikingly visible macroscopically (figure 2.3). In figure 2.3 both plates A and B received the same titre of virus ($3x10^5$ BAG-psi virions), but B additionally received 10μg/ml polybrene. At 10μg/ml polybrene did not have any apparent adverse effects on the NIH 3T3 cells but it was cytotoxic at and above 50μg/ml.

2.3.4 Assay for Helper Virus in Viral Stocks

Using the assay described in 2.2.4 no helper virus was detected in any of the 'BAG' viral stocks, either concentrated or unconcentrated.

2.3.5 Infection of Xenopus Cell Lines With Amphotropic Retrovirus

BAG-C virus was added to the *Xenopus* cell lines XTC, XL-177 and XL, all growing at room temperature. The dishes contained approximately $2.4x10^6$ cells. Approximately $5x10^5$cfu of BAG-C virions were added to these cells in the presence of 10μg/ml polybrene. The medium was removed after 24 hrs and the cells were fed fresh medium. Four days post infection the cells were stained with X-Gal as outlined in 2.2.3.2. No staining was found in the uninfected control and the infected cell lines.

2.4 Discussion

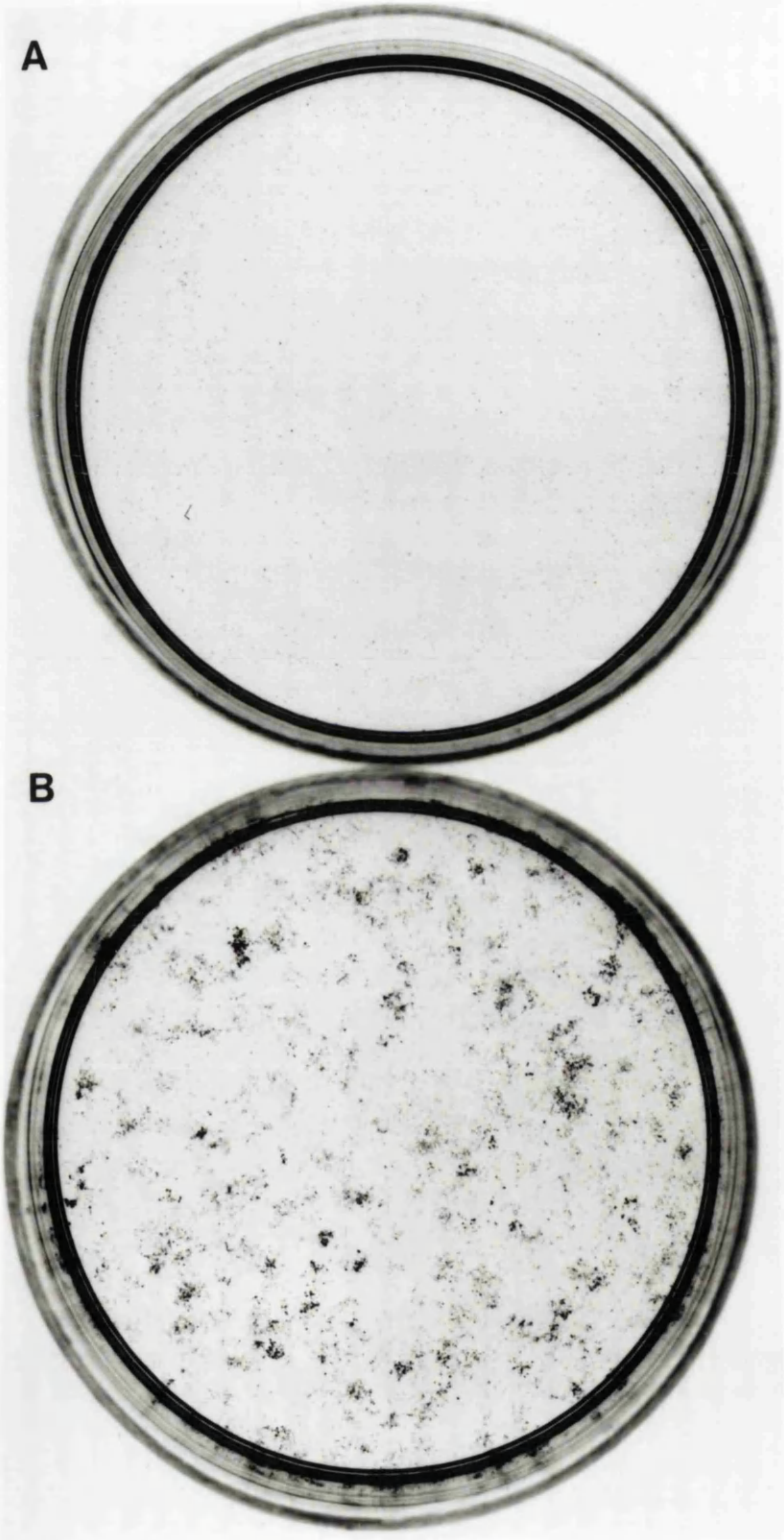
2.4.1 Viral Titres Obtained and Their Usefulness

The viral titres obtained from the ecotropic producing cell line were close to the values obtained for cells infected by MoMLV (Muenchau *et al.*,1990). Furthermore the titre was similar to those values obtained for other retroviral genomes packaged by the psi-2 line (Williams *et al.*,1986; Dumenco *et al.*,1989). The important fact to note however is that the ecotropic titre was theoretically sufficient for the infection of VZ cells in the rodent as similar titre values were reported in previously successful studies (Price and Thurlow,1988; Luskin *et al.*,1988).

The titres obtained from the SL88 and JPE87 producer lines were slightly lower than those reported with other retroviruses packaged by the CRIP (Danos and Mulligan,1988) and PA317 (Eglitis *et al.*,1988; Zwiebel *et al.*,1990) packaging lines. Perhaps this was due to the length of the 'BAG' genome, which is one of the larger genomes packaged by the amphotropic lines. In general packaging efficiency is known to decrease as genomic RNA length increases (Gelinas and Temin,1982; Morgenstern and Land,1990a). Unfortunately it was not immediately clear if the titres obtained were sufficient to be used practically in infect cells in the

Figure 2.3. The Effect of 10μg/ml Polybrene on Infection of NIH 3T3 Cells in Vitro.

The NIH 3T3 cells in A were incubated in 'BAG' virus whereas the cells in B received virus in the presence of 10μg/ml polybrene. Clones of X-Gal stained cells are coloured dark grey. The difference between the two dishes is clearly apparent, with most clones found in dish B. The culture dishes are 35mm in diameter.


A
B

early chick neural tube. For example the target could be very precisely located *in ovo* and a large volume of virus introduced.

Independent clones from the SL88 and JPE87 producer lines were isolated but, with one exception (SL88ϵ, discussed in 2.3.2.2), they all produced the same number of viral particles. Presumably the MoMLV LTR was sufficiently strong to over-ride any effects dependent upon integration position, although other studies with different retroviral vectors have reported some clonal variation (Collins *et al.*,1990).

However there was a two hundred fold difference between the unconcentrated titre of 'BAG' virus obtained from the ecotropic and amphotropic packaging lines. This difference was not due to a mosaicism in the population as both ecotropic and amphotropic producer cells stained equally (both in percentage numbers and intensity) with X-Gal. Presumably the differences in titre were due to the different abilities of the ecotropic and amphotropic packaging lines to package the 'BAG' genome into a virion.

The percentage recovery of virus after the centrifugation was very low. On average only one out of twenty virions initially loaded into the centrifuge tube was recovered. Decreasing the time of centrifugation resulted in the same titre as longer centrifugations. These observations suggest that the majority of virions were inactivated by the centrifugation concentration process. Perhaps there was a limit to the number of virions that could be recovered from the centrifugation process, although if this was the case then all viral titres after concentration should be equal. Another equally feasible interpretation was that virus particles clumped when centrifuged. Hence when the resuspension was titred, a cluster of twenty virions were detected as one cfu. Whichever mechanism was responsible for this loss of titre is currently unknown.

Unfortunately the BAG-C amphotropic virus did not infect three *Xenopus* cell lines tested (XTC, XL-177 and XL). As the MoMLV is constitutively active in most cells the lack of staining was due to a lack of binding of virions to the *Xenopus* cells and not due to a lack of ß-galactosidase expression.

2.4.2 Polybrene and Retroviral Infectivity

Polybrene was found to be an essential factor for 'BAG' infectivity. Very few clones of 'BAG' infected NIH 3T3 cells were observed *in vitro* without polybrene treatment but at a concentration of 50μg/ml or higher polybrene had a cytotoxic effect on cells. In order to ensure that a sufficient number of cells could be infected, 10μg/ml polybrene was used in all *in vivo* or *in vitro* experiments.

The mechanism(s) of this enhancement are not conclusively clear. Toyoshima and Vogt (1969) speculated that as a polycation, polybrene neutralized the repulsive negative charge present on the surface of cells and hence enhanced adsorption of virus to cells. Polybrene has been shown to enhance the infectivity of a number of viruses, as have a

number of different polycations (Toyoshima and Vogt,1969; Cornetta and Anderson,1989). Polybrene is extensively used however because of its low toxicity (Manning *et al.*,1971). Although never omitted from *in vivo* infections it was not essential for successful infection of primary rat cerebral cortex cultures *in vitro* (J.Read, personal communication). Possibly negatively charged extracellular matrix in these primary cultures may substitute for the polybrene.

The findings obtained by this experiment have been previously noted with retroviruses although with a retroviral vector such as 'BAG' that can be detected histologically the effects are perhaps easiest to appreciate.

Chapter Three : Studies on Barrel/Column Development in the Rat

3.1 Introduction

As outlined in Chapter One, retroviral vectors, such as 'BAG', are ideal tools with which to indelibly and innocuously label mitotic cells and their progeny. In this particular aspect of study an attempt was made to see if the neocortical VZ was positionally specified in a manner suggested by the radial unit hypothesis. Specifically this was addressed by a lineage study using the 'BAG' retroviral vector (Price *et al.*,1987).

By the time of birth essentially all neocortical neurones have been generated by the VZ. Hence any attempts to examine lineage relationships using 'BAG' must involve an infection of VZ cells *in utero*. Either the embryo needs to be injected in an *exo utero* fashion (e.g. Turner *et al.*,1990; Cepko,1989a) or, more preferably, injected with the minimum amount of surgery on the uterus (see below; Price and Thurlow,1988). Furthermore because retroviruses only penetrate tissue poorly, for a productive infection 'BAG' must be injected close to dividing cells. Fortuitously the VZ cells surround the lateral ventricle, a relatively large fluid filled vacuole into which virus can be introduced. Indeed all VZ cells border the lateral ventricle, even during mitotic division (Seymour and Berry,1975). Thus VZ cells can be infected by introducing virus stocks into the lateral ventricle. Provided no restrictions to diffusion occur then the virus will move away from the injection site and infect cells irrespective of their position or physical size in the VZ. As the lateral ventricles are fluid filled they can be visualized by illumination of the uterus with a light source, even though the ventricles are positioned behind the uterine wall. Thus the target for injection can be visualized very simply. A known volume and titre of 'BAG' can then be released within the lateral ventricle via a small syringe needle. This protocol has been followed by several laboratories (Price and Thurlow,1988; Luskin *et al.*,1988; Walsh and Cepko,1988). This was the technique followed in the series of experiments reported below.

A concentrated viral stock of BAG-psi retroviral vector (prepared as outlined in Chapter Two) was the only virus used. As the BAG-psi virus has a short half-life (Sanes *et al.*,1986; Cepko,1989a; Austin and Cepko,1990; Rocancourt *et al.*,1990) it will only be able to infect cells shortly after injection near the target. In order to examine how far the virus spread within the ventricle and to examine any malformations caused by the injection procedure, the injections were unilaterally directed. Once infected with 'BAG', cells will be detectable by their expression of ß-galactosidase, but will otherwise be undisturbed.

As outlined in Chapter One the barrel/columns provide a unique example of cortical columns that can be detected by simple histological techniques. They are present in mature Sm1 of a number of rodents including the rat and classically they have been studied in the tangential plane i.e. a plane of section parallel with the neocortical layers. Several questions surrounding the development of the barrel/columns relate to the lineage relationships of the neurones and

glia comprising them. For example is one barrel/column derived from one VZ cell only? Is one barrel/column derived from a specific cluster of VZ cells as suggested in the radial unit hypothesis? The former question has been addressed using mouse aggregation chimeras; clearly individual barrel/columns are derived from more than one VZ progenitor (Goldowitz,1987). The latter question can only be addressed by a study of cell lineage, the labelling of individual cells and their progeny coupled with an examination of the distribution of individual clones within Sm1.

Cell lineage in the developing neocortex has previously been investigated by several groups using retroviral vectors, including 'BAG', as lineage labels (Walsh and Cepko,1988; Price and Thurlow,1988; Luskin *et al.*,1988; Austin and Cepko,1990; see 1.13). All of these studies demonstrated that lateral clonal spread was greater than that seen in the retina (Turner and Cepko,1987) and optic tectum (Gray *et al.*,1988) where strict radial arrays of clones were observed. Hence the retroviral studies, and that of Goldowitz (1987), suggested that minicolumns in the neocortex may not originate from one VZ progenitor. Hence one aspect of the radial unit hypothesis, that one proliferative unit generates one ontogenetic column (and subsequently a minicolumn), was not supported by several experiments. However none of these studies addressed the remaining (and tenable) aspect of the radial unit hypothesis; that some of the larger physiological columns such as ocular dominance or barrel/columns originated from a prespecified VZ.

In this Chapter an examination of how individual clones of cells were distributed in relation to the barrel/columns was performed. BAG-psi virus was introduced within the lateral ventricle of staged rat embryos, *in utero*, as described above. The size of the target and the tissue penetration required to introduce virus near to it, can be appreciated from a coronal section of the embryonic telencephalon (figure 1.2). The embryos were then allowed to complete development and were sacrificed at two weeks postnatal age. All the cortices were sectioned in a plane tangential to the layers of Sm1. The series of sections obtained were alternately stained for ß-galactosidase using X-Gal and cytochrome c oxidase (an enzyme uniquely concentrated within barrel/columns). The positions and characteristic staining patterns of stained cells were noted. Provided the sections contained a suitable number of X-Gal stained cells then all sections containing stained cells and barrel/columns were drawn by hand with a *camera lucida* facility. The position of large blood vessels in each section was also noted.

Using computer aided, three-dimensional reconstruction, the entire series of tangential sections were reconstructed into three dimensions. This was performed to enable an examination of how individual clones spread in three dimensions throughout Sm1. Three-dimensional alignment was achieved by using the radial alignment of large cerebral blood vessels. Clonal relationships of infected cells were inferred from their two-dimensional spatial relationships to one another. This was necessary

as the position and number of infective events in the VZ and the size of individual clones were not known *a priori*. However it was assumed that the progeny of one VZ cell remained relatively closely apposed to one another. A previous study (Price and Thurlow,1988) suggested that the vast majority of clones were found laterally within 300μm of each other. This dimension was used in this study to classify clonal events (see 3.2.8) and could still be compatible with the radial unit hypothesis. For example, as reported below, some barrel/columns were large enough to accomodate a clonal spread of 300μm.

Additionally by inferring clonal events and examining the staining characteristics of the 'BAG' labelled cells the phenotype of progeny derived from a single progenitor were examined. The results obtained from the three-dimensional reconstruction suggested that separate cell type specific progenitors coexisted in the VZ, data in accord with the proposed lineage tree outlined in figure 1.12.

Furthermore the descendants of individual neuronal progenitors, i.e. cells that produced only neurones, were spread in a manner incompatible with the radial unit hypothesis. Such findings have not been previously reported and the implications of them are discussed and a model of neocortical development proposed.

3.2 Materials and Methods

3.2.1 Procurement of Embryonic Rats

Pregnant Wistar rats were used in this study. They were mated by caging virgin females with males for a single night. The following day, when vaginal plugs were detected, was considered embryonic day zero (E0). The pups were born after 21/22 days, the day of birth being postnatal day zero (P0). The next day of postnatal life was termed P1. The rats received standard food and water *ad libitum* and were kept at a constant temperature of 20°C, with a light/dark cycle of 12/12 hrs. In this study rats were injected with virus on E14,E15 or E16.

A brief outline of the experimental protocol followed in order to test the radial unit hypothesis is shown in figure 3.1.

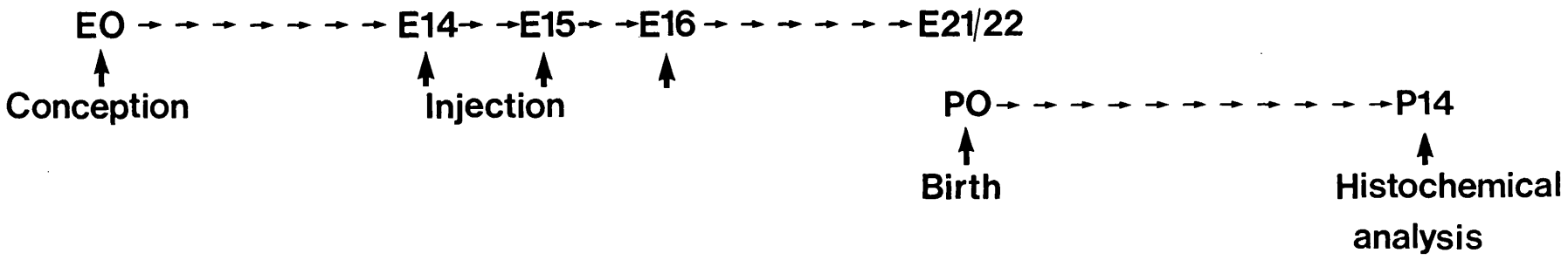
3.2.2 Injection of BAG-psi Virus into the Embryonic Lateral Ventricle

Rat surgery was performed as according to Price and Thurlow (1988) with only one modification. At the early stages of this project it was discovered that another general anaesthetic could be used that had less detrimental effects and resulted in a much higher maternal survival rate. This was a 1:1 mixture of Hypnovel (Roche Products,UK) and Hypnorm (Janssen Animal Health,UK) (further diluted 1:1 with sterile water immediately prior injection). This was injected intraperitoneally at a dose of 0.68ml per 250g. This dose allowed 1 hr of general anaesthesia.

After shaving animal fur, mid-ventral laparotomy was performed to expose the uterus. The target for injection, the embryonic left cerebral vesicle, was located by transillumination of the exposed uterine wall.

Figure 3.1. An Outline of the Experimental Protocol Followed in an Attempt to Study Clonal Migration in the Developing CNS.

Progenitor cells in the neocortical VZ were labelled with the 'BAG' retroviral vector. Specifically rat embryos *in utero* received a single injection of BAG-psi virus at either E14, E15 or E16. After normal birth the cerebral cortices were histochemically analyzed on P14. Selected sections were analyzed by three-dimensional reconstruction.


E0
E14
E15
E16
E21/22
Conception
Injection
P0
P14
Birth
Histochemical
analysis

'Cold-light' from a flexible fibre optic light-guide (Schott KL 1500-T; Schott Glaswerke,FRG) was used for this purpose.

Concentrated ecotropic BAG-psi virus produced by the S2 cell line (see Table 2.1) was thawed from -70°C storage immediately prior to use. Polybrene was added to a final concentration of 10μg/ml. Black Pelikan Fount India Ink (Pelikan AG,FRG) was added at 1% v/v to make the innoculum visible. A volume of 1μl of this virus suspension was pressure injected through the uterine wall into the left cerebral vesicle using a 50μl Hamilton syringe (Hamilton,USA) equipped with a Luer lock fitted with a 30G hypodermic needle ('Microlance',Becton Dickinson,UK). In most experiments the viral stock was diluted 1 to 5 with sterile PBS. This meant that the 1μl injected had a theoretical titre of 10^3cfu. If possible all embryos in both uterine horns were injected. A small minority of embryos could not be injected but were allowed to survive. The peritoneal wall and skin were both sutured using 5/0 prolene monofilament suture thread (Ethicon Ltd.,UK). Recovery from anaesthetic was usually rapid and injected embryos were delivered normally on the predicted day. The surgery outlined above was performed by two people and the assistance of Miss Joanne Read and Miss Linda Thurlow was greatly appreciated.

3.2.3 The Histochemical Detection of 'BAG' Labelled Cells and Barrel/Columns

3.2.3.1 Tissue Fixation

On P14 all rats from an injected litter were anaesthetized with an overdose (100μl or 6mg) of pentobarbitone sodium ("Sagatal", May and Baker,UK). When they showed no response to a toe pinch they were perfused intracardially with a fixative solution of 2% (w/v) paraformaldehyde in 0.1M PIPES containing 2mM $MgCl_2$ and 1.25mM EGTA, pH 6.9. The brains were removed and the cortices cortices examined for any signs of damage resulting either from surgery or fixation. Any damaged cortices were discarded. Cortices were kept in the fixative solution at 4°C for a further two or three days.

3.2.3.2 Cryoprotection and Embedding of Tissues for Cryostat Sectioning

Cortices were cryoprotected by overnight immersion at 4°C in 30% (w/v) sucrose containing 2mM $MgCl_2$. Cortices were then carefully separated from each other and individually embedded in O.C.T. compound (Tissue-Tek, Miles Diagnostics,USA) within a cup-shaped holder made from two layers of aluminium foil. The animal code was inscribed on the bottom of this holder at this point. Cortices were embedded in such a fashion that Sm1 was parallel to the upper surface of the block (topography dictated by Woolsey and Van der Loos,1970; Welker and Woolsey,1974; Strominger and Woolsey,1984). The block was frozen on a level surface of broken dry ice. As frozen O.C.T. is opaque, just prior to freezing a few drops of ink were placed on top of the block in a unique manner, so as to orient the tissue. All blocks were then stored at -70°C until required for

sectioning. Both cortices of an individual animal were processed in this manner even though only the left was injected with virus.

3.2.3.3 Subbing of Microscope Slides

All slides were subbed to provide an adhesive surface for section mounting. Super Premium, twin-frosted microscope slides (BDH,UK) were dipped in an aqueous solution of 1% (w/v) gelatin (Sigma,UK, from porcine skin) at 60°C containing a trace of chromic potassium sulphate (chrome alum; BDH,UK). The slides were then air dried overnight in an upright position and stored at 4°C.

3.2.3.4 Cryostat Sectioning of Infected Cortices

25μm tissue sections were cut at -20°C in a Slee TE Cryostat with the Slee R3 microtome (Slee Medical Equipment Ltd.,UK), equipped with stainless steel knives and perspex anti-roll plate. Consecutive sections were thaw mounted onto subbed slides in the same topographic manner. All slides containing tissue sections were stored at -20°C until stained.

The complete series of sections through each cortex was collected, from the pial surface to the inner surface of the grey matter. Each series comprised approximately forty sections. The position of missing, inverted or sections transferred to other slides were scratched onto the surface of the slides.

The sections were grouped as follows. After approximately twelve sections were collected, every fourth subsequent section was mounted on a separate slide, until about seven or eight sections (20% of the total) had been collected on this slide. This separate slide was stained for cytochrome c oxidase (see 3.2.4.1) to detect the barrel/columns in the PMBSF. The remainder were processed by X-Gal histochemistry.

3.2.3.5 The Histochemical Detection of 'BAG' Infected Cells Using X-Gal

The staining protocol followed was basically that previously reported (Price and Thurlow,1988; Dannenberg and Suga,1981; Lojda,1970) with only a few modifications. Sections were post-fixed for 10 min in 0.5% (v/v) glutaraldehyde (from a 25% solution; BDH,UK) in PBS, pH 7.4 containing 2mM $MgCl_2$ and 1.25mM EGTA. After washing 2x10 min in PBS they were immersed in a detergent solution of 0.01% (w/v) sodium deoxycholate, 0.02% (v/v) NP40 and 2mM $MgCl_2$ in PBS pH 7.4, for 10 min. All of these procedures were performed at 4°C. The sections were then incubated at 37°C in the dark for three hrs in the same detergent solution supplemented with 1mg/ml of X-Gal, 30mM potassium ferrocyanide and 30mM potassium ferricyanide. Sections were dehydrated, cleared and mounted as in 3.2.5. The X-Gal staining solution was stored in the dark at room temperature when not in use and was remade every week. The ferrocyanide and ferricyanide were used at higher concentrations than reported by Price and Thurlow (1988) as this resulted in less diffusion of the indigo, presumably due to a more rapid dimerization.

3.2.3.6 The Histochemical Detection of 'BAG' Infected Cells Using Nitro Blue Tetrazolium (NBT)/X-Gal

The NBT/X-Gal reaction involves the substitution of the ferrocyanide and ferricyanide solutions with NBT (McGadey,1970). In a coupled redox reaction with indigo, the NBT is rapidly reduced to an alcohol insoluble, electron dense blue diformazan (see mechanism reported by McGadey,1970).

Sections were processed exactly as described above for X-Gal histochemistry. They were then reacted for 15 min in detergent solution (see 3.2.3.5 for composition) containing 0.5mg/ml NBT (Sigma,UK) and 0.25mg/ml X-Gal at 37°C. This X-Gal/NBT solution was made fresh every time prior to use. Sections were dehydrated, cleared and mounted as in 3.2.5.

3.2.3.7 ß-Galactosidase Staining Positive Control

All ß-galactosidase histochemistry was performed in the presence of a positive control slide. These slides were made by scraping unfixed 'BAG' virus producer cells (typically S2) from a tissue culture dish, embedding in O.C.T. and cryostat sectioning (3.2.3.4) at 25μm. After fixation and X-Gal staining (see 3.2.3.5) these sections always stained macroscopically blue within a few hrs. However these sections stained blue within minutes if the X-Gal/NBT method (3.2.3.6) was used.

3.2.4 The Histochemical Detection of Barrel/Columns

3.2.4.1 Detection of Barrel/Columns By Cytochrome c Oxidase Histochemistry

Cytochrome c oxidase (E.C. 1.9.3.1) histochemistry was performed to detect the barrel/columns (Wong-Riley and Welt,1980; Land and Simons,1985; Wong-Riley,1989) by a previously reported method (Wong-Riley,1979). Sections were incubated for four hrs in a solution which contained 0.5mg/ml 3,3'-diaminobenzidine (DAB) (Sigma,UK), 0.3mg/ml cytochrome c (Sigma,UK, from horse heart, type II-S) in 0.1M phosphate buffer, pH 7.4. This solution was made fresh every time prior to use. In this reaction the DAB is oxidatively polymerized to a dark brown, alcohol insoluble, indamine polymer. Sections were dehydrated, cleared and mounted as in 3.2.5. In preliminary experiments the removal of endogenous peroxide by the addition of 200μg/ml of catalase (Sigma,UK; from bovine liver) had no effect on the final histochemical result and was not subsequently included in the reaction. All DAB containing solutions were oxidized by an excess of Chloros prior to disposal.

3.2.4.2 Detection of Barrel/Columns By Succinate Dehydrogenase Histochemistry

Succinate dehydrogenase (E.C. 1.3.99.1) histochemistry was performed to detect the barrel/columns according to a previously reported method (Killackey and Belford,1979). The animals were perfused intracardially with an aqueous solution of 10% (v/v) glycerol (BDH,UK) at 4°C. The

cortices were then embedded in O.C.T. as described in 3.2.3.2 and cut in a cryostat as in 3.2.3.4. Sections were then stained in a solution of 4.5mg/ml NBT, 0.05M sodium succinate in 0.1M phosphate buffer, pH 7.4 for 45 min at 37°C. Sections were dehydrated, cleared and mounted as in 3.2.5.

3.2.5 Mounting of Sections

All sections were dehydrated sequentially through aqueous solutions of alcohols (50,70 and 100% (v/v); 2x5 min each), cleared in Histo-Clear (National Diagnostics,USA) (2x5 min), mounted in D.P.X. (BDH,UK) and allowed to dry. The sections were then examined microscopically as soon as possible.

3.2.6 Microscopical Analysis of Stained Tissue

Sections were examined under a Leitz Diaplan microscope (Leitz,UK) equipped with differential interference optics, *camera lucida* facility and a phototube. Inventory of the co-ordinates and staining patterns of X-Gal stained cells were taken using a Vernier scale. Photographs were taken with a Olympus OM-2 camera using Kodak Ektachrome 50 ASA colour slide film (Kodak,UK). The films were processed and prints developed by a commercial processor (Bushy Colour Labs.,UK).

Camera lucida of rat cortices were drawn for each section using the x2.5 objective. The position and staining characteristics of cells and the outlines of the barrel/columns were drawn. Occasionally other components of the rattunculus were visible after cytochrome c oxidase staining, and were drawn on the *camera lucida*. Furthermore the position of all large blood vessels were drawn.

3.2.7 Three-Dimensional Reconstruction of 'BAG' Infected Tissue

The tangential sections were radially aligned by using the natural radial alignment of blood vessels in the cerebral cortices. The first stage in the three-dimensional reconstruction involved finding large blood vessels in Sml that ran through all of the serial sections. This was accomplished by sequentially placing the *camera lucida* tracings on a light box and finding two vessels (or three if possible) common to all sections. At least one of the vessels chosen for this purpose was from within the barrelfield.

Three-dimensional reconstructions were performed using a computerized system: SSPROF and SSRCON software (Shepherd *et al.*,1984) run under a RT-11 (XM) operating system (Digital Equipment,USA) on a PDP11 computer. Data was entered into the SSPROF program from the *camera lucidas* by tracing with a mouse on a digitizing tablet. The following details for each section were entered: the positions of the selected blood vessels, the boundaries of the barrel/columns and the position and phenotype of stained cells. All of these data were entered into a separate category in the SSPROF program. A hardcopy of the data was obtained using a Hewlett Packard 7221A plotter equipped with coloured pens. For clarity

the barrel/columns and different cell types were displayed in different colours and symbols.

The anterioposterior and mediolateral axes were assigned to the data from the known topography of the PMBSF (Woolsey and Van der Loos,1970; Welker and Woolsey,1974) and from the manner in which the cortex was embedded and sectioned. The assignment of the mediolateral axis was aided by the 'notch' (Woolsey and Van der Loos,1970), an indentation of barrel-free cortex between the PMBSF and the ALBSF. At the juction of the two is a precise mediolateral axis (Woolsey and Van der Loos,1970; Welker and Woolsey,1974).

3.2.8 How Clones of 'BAG' Infected Cells Were Defined

Clones were assigned to the tissue after completion of the three-dimensional reconstruction. Price and Thurlow (1988) suggested that, in the rat cerebral cortices after infection at E16 and examination at P14, the majority of 'BAG' labelled cells constituting an individual clone were clustered within 300μm of each other. Since most of my experiments closely followed their experimental criteria, the same definition was adopted. Specifically this clonal definition was applied to E14, E15 and E16 reconstructions by measuring radially from the centre of a cell in all dimensions (figure 3.2). Thus each 'BAG' labelled cell was surrounded by a circular clonal domain with a radius of 300μm and area of 0.28mm^2. If any other cell fell within this 300μm clonal domain, irrespective of the depth of the cell within the cortex, then the two were classed as clonal. The procedure was then repeated for the clonal domain of the two cell clone, and so on. The 300μm clonal domain was applied to the entire reconstruction even though towards the edges there will have been little radial alignment of the cortex. Clones were further classified on the basis of the staining characteristics of cells comprising them e.g. neuronal clones contained only neurones.

3.2.9 Analysis of The Three-Dimensional Orientation of Individual Clones

An examination of how clones of neurones were spread in three dimensions was performed (see 3.3.7). The data obtained were displayed in the form of a vector diagram. The vector diagrams were constructed by first drawing the position of cells in two cell neuronal clones, to scale and with respect to the axes, on a square transparency. The cells were joined together by a line and the position of the deeper cell, i.e. the cell found in the deepest section, was noted. The entire data were then superimposed on top of each other with the deepest cell of each clone positioned at the origin. A diagram of the composite data was then drawn.

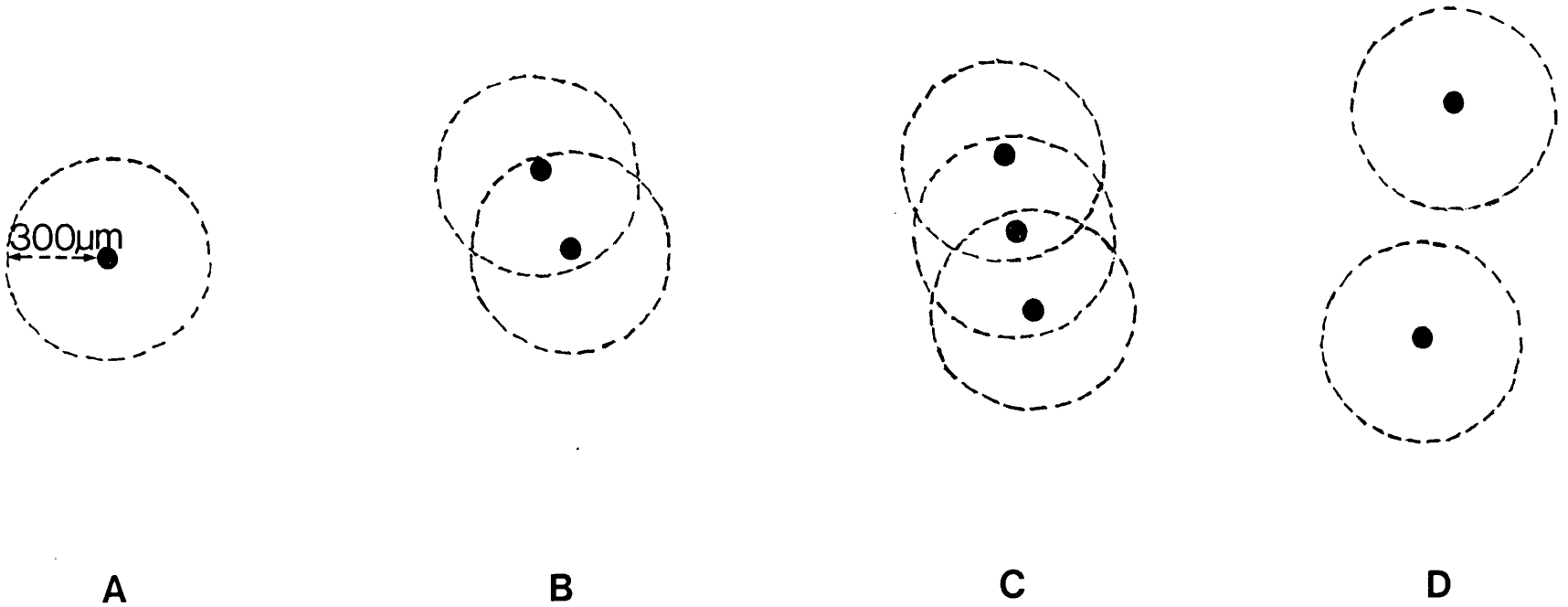
3.3 Results

3.3.1 The X-Gal Histochemical Staining of Uninjected Animals

Some cells in the cerebral meninges stained lightly with X-Gal in control embryos. However no endogenous X-Gal staining was found in grey

Figure 3.2. The Definition of 'BAG' Labelled Clones in Three-Dimensional Reconstructions.

Each 'BAG' labelled cell was surrounded by a circular clonal domain with a radius of 300μm. This is shown by the dotted line in surrounding a single cell clone in A. If, after three-dimensional reconstruction, any other cell was found within this domain then the cells were classed as clonal. For example a two cell clone is shown in B. The enlarged clonal domain of a two cell clone was then examined to see if any other cells were found within it (as in C). Cells not encompassing each other in a clonal domain (as in D) were classed as single cell clones. This procedure was performed for all cells obtained from a reconstruction.


300µm
A
B
C
D

or white matter. Presumably because of the difference in pH and ion requirements for mammalian and bacterial enzymes (e.g. Shimohama *et al.*,1989), all of the staining reported below was due to 'BAG' encoded ß-galactosidase.

3.3.2 The Survival of 'BAG' Injected Embryos

The injection procedure was performed on a regular basis and usually the animals quickly recovered from the operation. The data in this study was derived from a small population of them (see below). However some pregnant animals died after the operation and this seemed to depend upon the age of embryo injected. For example during 1989 a total of 24 animals were operated upon. Whereas all the injected animals containing E16 (7/7) and E15 (2/2) embryos survived only 60% (9/15) of animals containing E14 embryos recovered from the operation.

All of the three-dimensional reconstructions presented below were obtained from surgery of nine animals which contained a total of 99 embryos; 47 of these embryos were born alive (E14 14/35 or 40%; E15 16/36 or 44%; E16 17/28 or 60%). All surviving postnatal rats at P14 appeared to be normal and had a mass between 20.8-33.2g, with a mean of 27.4g.

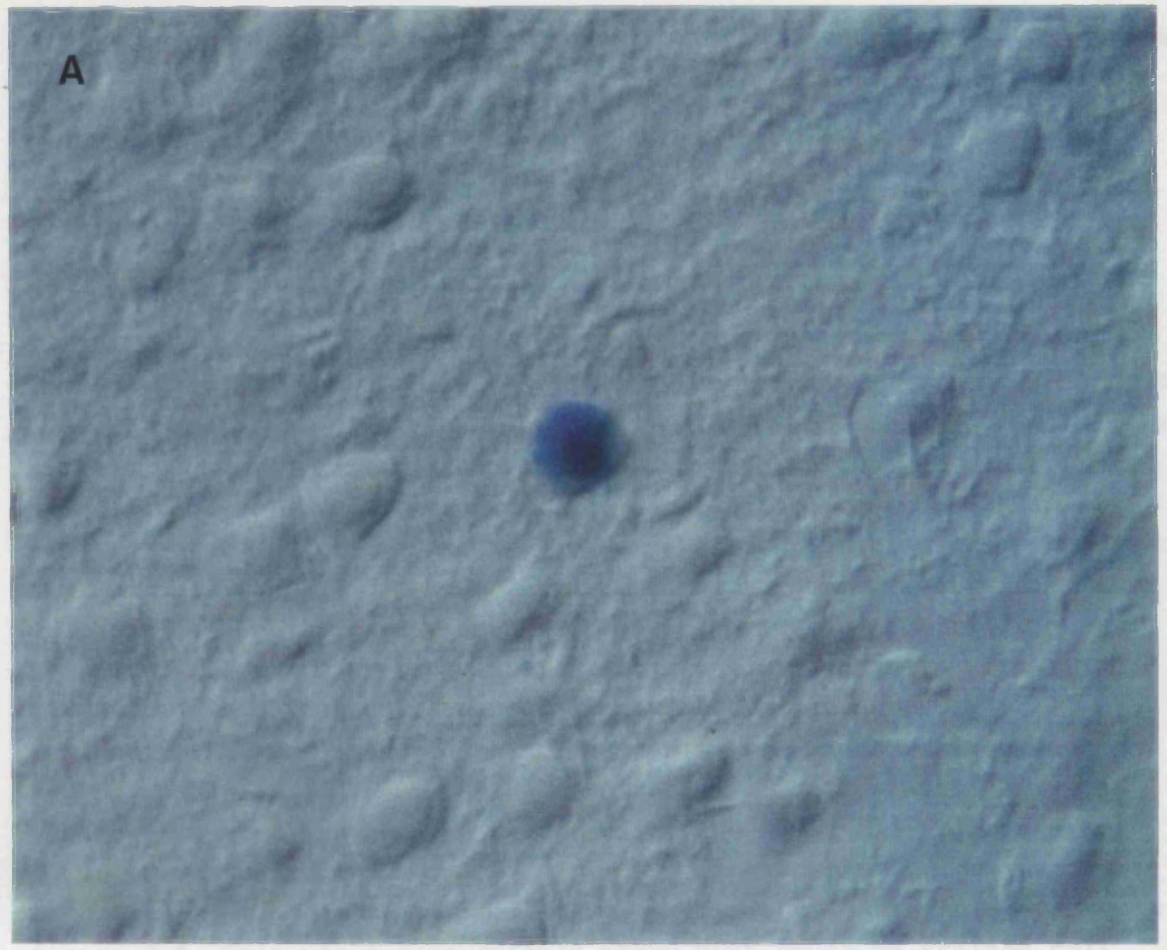
3.3.2.1 The Histological Appearance of 'BAG' Labelled Cells in Tangential Sections of Cortex

In postnatal 'BAG' infected cortices, there were three distinct types of ß-galactosidase staining as described previously (Price and Thurlow,1988). The staining patterns were very similar to those described in the previous study even though the plane of section in this study was tangential.

One of the staining patterns was grey matter restricted and appeared as a small circular spot of staining overlying the nucleus of a cell (figure 3.3). These nuclear stained cells sometimes had one very small, extranuclear spot of staining (arrowed in figure 3.3B) which Price and Thurlow (1988) demonstrated was found next to a prominent apical dendrite. Price and Thurlow (1988) suggested that these cells were neurones for several reasons. First cells with this nuclear staining often had a neuronal morphology. Second this staining was found at the appropriate laminae position for neurones generated subsequent to the time of infection. Furthermore cells stained in this manner grouped into clones that had a small clonal size expected for neuronal progenitors (see 3.3.10). Recently single-cell filling experiments have confirmed that cells with this nuclear staining have a characteristic neuronal morphology (J.Price, personal communication). Consequently, cells stained in this pattern were classified as neurones. If any cells stained in this pattern were found within a 300μm clonal domain (as described in 3.2.8) then they were classed as clonal. As both non-pyramidal and pyramidal neurones stained in this manner (Price and Thurlow,1988) they were not possible to distinguish between. Thus

Figure 3.3. X-Gal Stained Neurones after Staining Sections of 'BAG' Infected Cortices With X-Gal.

Note the nuclear staining of the cell surrounding an unstained cytoplasm in both A and B. An extranuclear 'spot' found in some neurones and is arrowed in B. Scale bar 20μm.


A


B

although non-pyramidal and pyramidal neurones are cogenerated in the VZ (Miller,1985), in this study it was not possible to address if they were generated from the same or different progenitors.

A second staining pattern (figure 3.4) was also restricted to the grey matter but very different from that reported above. The staining spread extracellularly and was detectable under a low power (x2.5) objective. At the centre of the staining was a small cell body that stained uniformly (e.g. figure 3.4B). The cell had a small perikarya and several processes which often extended to adjacent sections. Some of the stained processes formed expansions or end-feet which extended to the pial surface or to blood vessels. These cells were often found in small clusters of two or three cells suggesting after migration the progenitor divided *in situ*. All of these features (i.e. morphology, *in situ* division) are characteristics of cortical astrocytes (Peters *et al.*,1976; Price and Thurlow,1988), which is how such cells were classified in this study. Indeed recent immunohistochemistry using GFAP antibodies has demonstrated that these cells are indeed astrocytes (E.Grove and J.Price, personal communication). Cells stained in this manner were also assigned to clones using the 300μm clonal domain as outlined in 3.2.8.

The clear difference between these two types of staining is shown in figure 3.5. This was a rare example where both types of stained cell were found adjacent to one another in the same section. However a minority of 'BAG' labelled cells showed neither staining patterns and were classified as unknown. Again these 'unknown' cells were classed as clonal using the 300μm clonal domain as outlined in 3.2.8.

The third staining pattern (figure 3.6) was restricted to the white matter. The phenotype of the stained cells is not known with certainty. They were termed by Price and Thurlow (1988) as 'horizontal cells' as in coronal sections the cell body and process appeared to be oriented parallel to the cortical layers. In the tangential plane most of the cells had a large nucleus, a small perikarya and usually one process which was found in the plane of section. Both cell body and processes were stained and the cells tended to occur in discrete clusters in the white matter. Price and Thurlow (1988) speculated that, on the basis of morphology, the horizontal cells were glia, specifically the cortical equivalent of the bipotential O2A cell that has been extensively studied in the perinatal and adult optic nerve (Raff,1989; Miller *et al.*,1989; Noble *et al.*,1990; Small *et al.*,1987). Exactly which type of O2A cell these putative cortical O2A cells resemble i.e. $O2A^{adult}$ (Wolswijk and Noble,1989), $O2A^{perinatal}$, a mixture of both, or some novel as yet undescribed O2A cell (e.g. $O2A^{horizontal}$), is not known. However the apparent unipolar morphology of the horizontal cells in figure 3.6 resembles that described for the $O2A^{adult}$ cell *in vitro* (Wolswijk and Noble,1989). The horizontal cells were not extensively analyzed in this study, although Price and Thurlow,(1988) suggested that they co-segregated with some neuronal clones (discussed in 1.13). Hence some of

Figure 3.4. X-Gal Stained Astrocytes after Staining Sections of 'BAG' Infected Cortices With X-Gal.

A and B show the characteristic X-Gal staining of astrocytes. Two stained cells are shown in both A and B. The processes and entire cell body are stained with X-Gal. Note that there is extracellular staining. Scale bar 20μm.

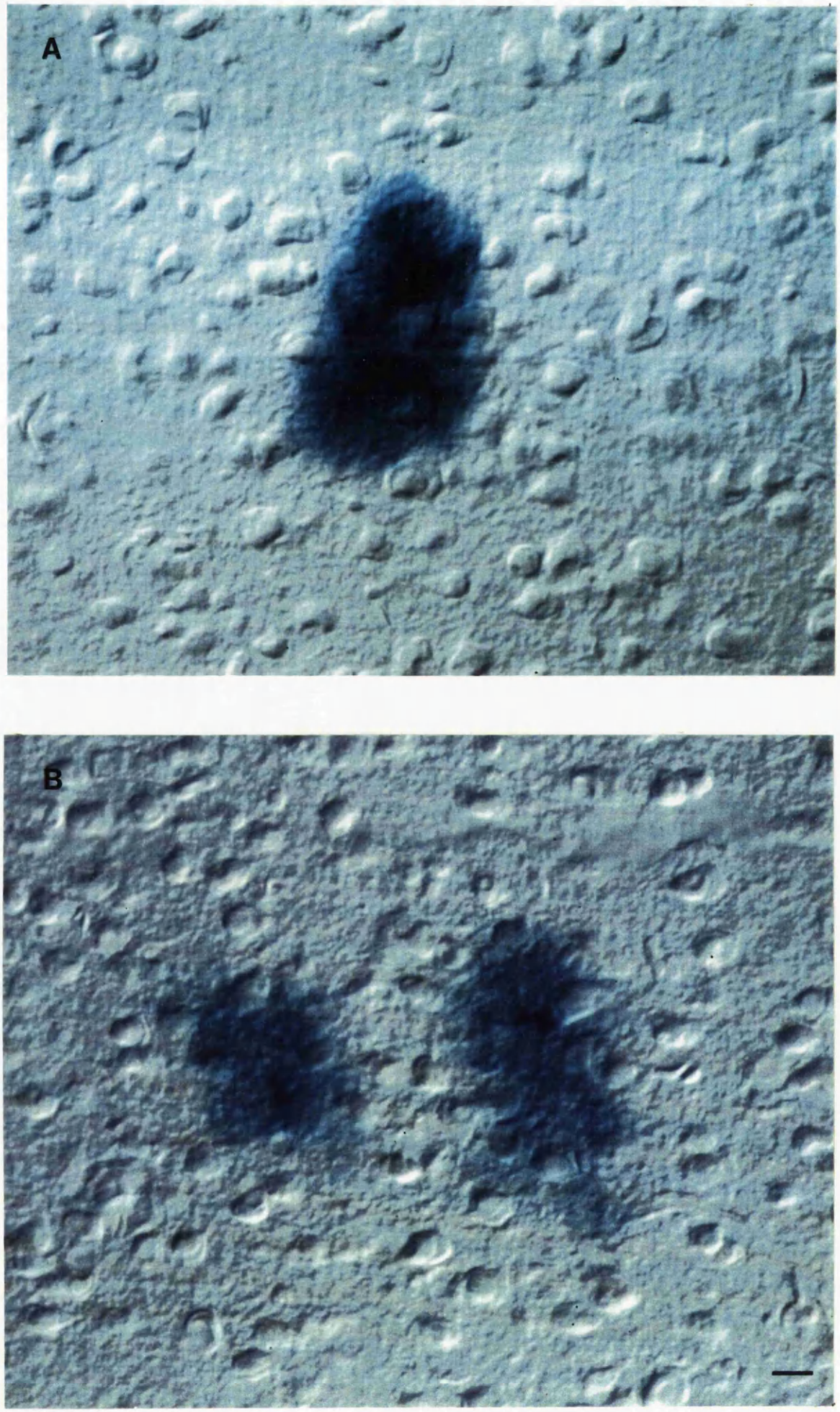

A
B

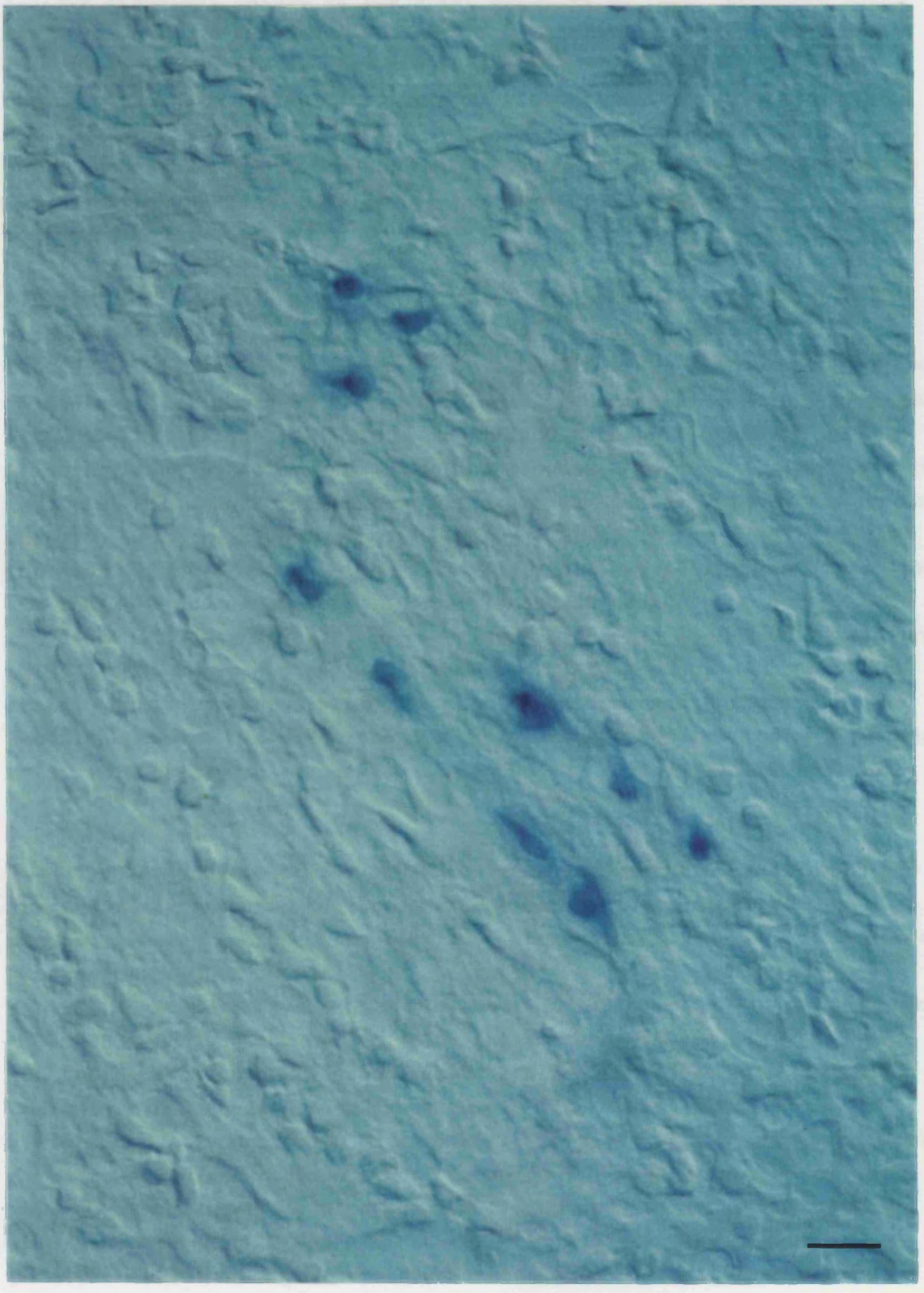
Figure 3.5. The Differences in the X-Gal Staining of 'BAG' Infected Neurones and Astrocytes.

This figure shows three neurones (denoted by 'N') and three astrocytes (denoted by 'A') found in the same section. The differences in the staining of the two cell types are clear. Scale bar 20μm.


N
N
N
A
A
A

Figure 3.6. The X-Gal Staining of 'BAG' Infected Horizontal Cells in the White Matter.

The cells are stained uniformly throughout and appear to be unipolar (see text for further description). Scale bar 20μm.



the neuronal clones in Sm1 discussed below may have had sibling cells in the underlying white matter.

3.3.2.2 Transient Nature of the X-Gal Staining of 'BAG' Labelled Cells

All X-Gal stained cells mounted in D.P.X. gradually faded such that, sometimes even over a few hrs, they disappeared (figure 3.7). This fading occurred more rapidly with some stained cells than others! However after storage of several weeks at room temperature all stained cells had faded. The phenomenon occurred much more slowly in thicker 200μm sections when mounted in D.P.X. (Dr.E.Grove, personal communication).

3.3.2.3 Histochemical Detection of 'BAG' Infected Cells By X-Gal/NBT Histochemistry

A minority of sections were stained by the X-Gal/NBT method and examples of staining are shown in figure 3.8. Cells were stained an intense blue although the staining was much more diffuse than that observed with X-Gal. Furthermore the staining reaction proceeded so quickly (complete by nearly 10 min) that overstaining was a serious problem. The staining in the grey matter was of two types, and by analogy to the X-Gal staining patterns reported above the more diffusely stained cells were astrocytes and the less diffusely stained were classed as neurones. Figure 3.8A shows a cell where most of the reaction product was localized within the cell (although was not exclusively nuclear). By analogy with the X-Gal staining patterns this cell was a possibly a neurone. However the reaction product was much more diffusely distributed in figure 3.8B. The cell was possibly an astrocyte, surrounded by a very diffuse halo of staining.

3.3.3 The Appearance of the Barrel/Columns in Tangential Sections

The PMBSF was detected by histochemical methods that detected two enzymes present in the mitochondria of all cells. Barrel/column hollows, because of heightened metabolic activity of mitochondria present in dendrites and perikarya (Wong-Riley and Welt,1980), stain stronger than the surrounding septum and neighbouring cortical tissue. Examples of tangential sections through the PMBSF stained for cytochrome c oxidase and succinate dehydrogenase are shown in figures 1.5 and 3.9 respectively. Cytochrome c oxidase positive cells were stained uniformly dark brown. This reaction product faded very slightly over a few weeks when mounted in D.P.X., although the fading was not as severe as that reported above with X-Gal stained cells. Succinate dehydrogenase positive cells had a punctate blue staining in the cytoplasm and red nuclei.

Both figures 1.5 and 3.9 show several characteristic features of the PMBSF. Individual barrel/column hollows in the PMBSF, although stained equally in intensity, did not have the same size nor the same cross sectional shape. The smaller barrel/column hollows tended to be more

Figure 3.7. The Transient Nature of the X-Gal Staining.

Figures 3.7A,C and E represent three stained neurones located within a few minutes after mounting. Figures 3.7B,D and F represent the same cells, located three hours later. The cell in figure 3.7A and B faded very slightly over three hours. The cell in figure 3.7C and D faded even more whereas the final cell (in figure 3.7E and F) practically disappeared over three hours. Scale bar in A and B is 40μm and for C, D, E and F 20μm.

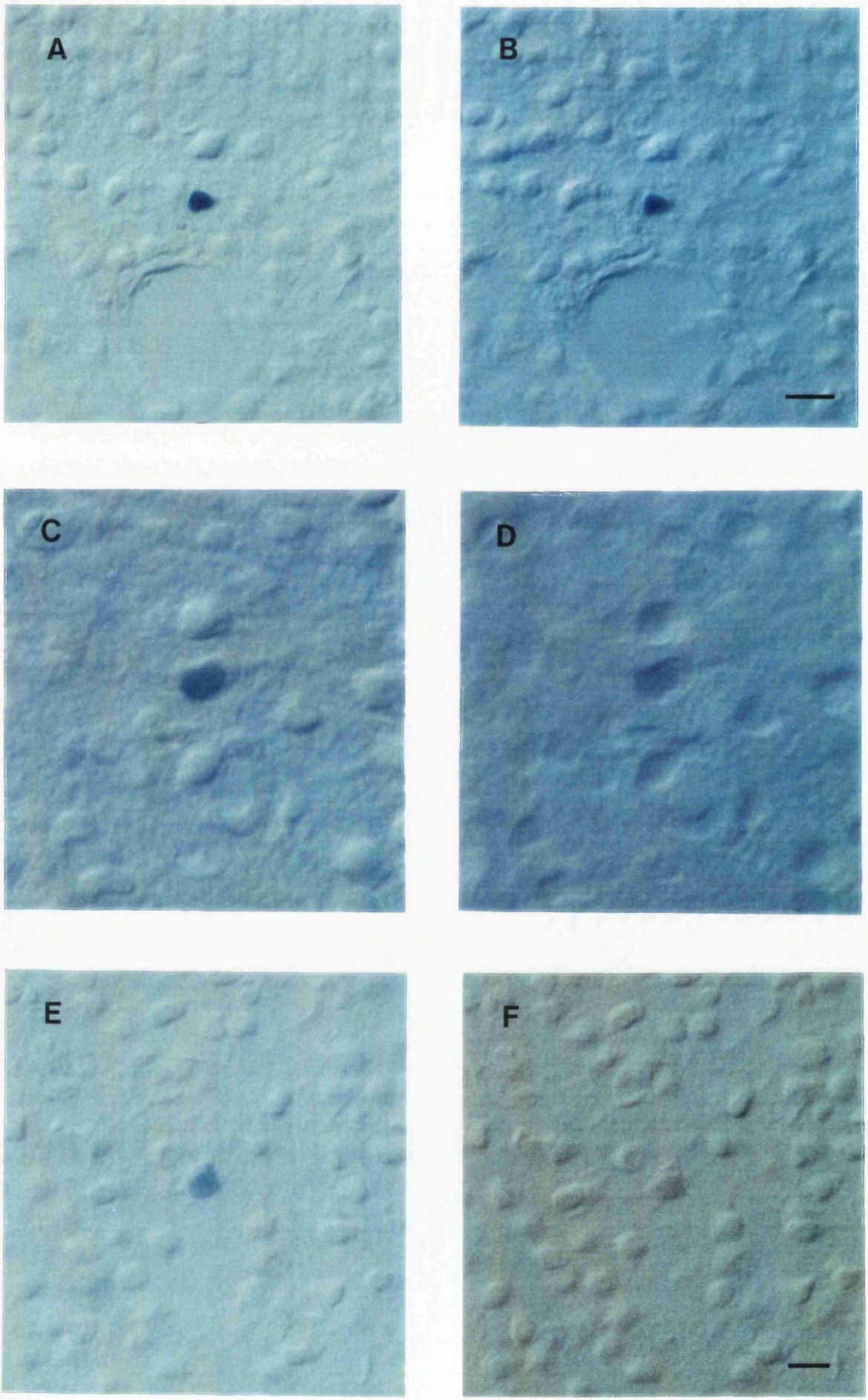
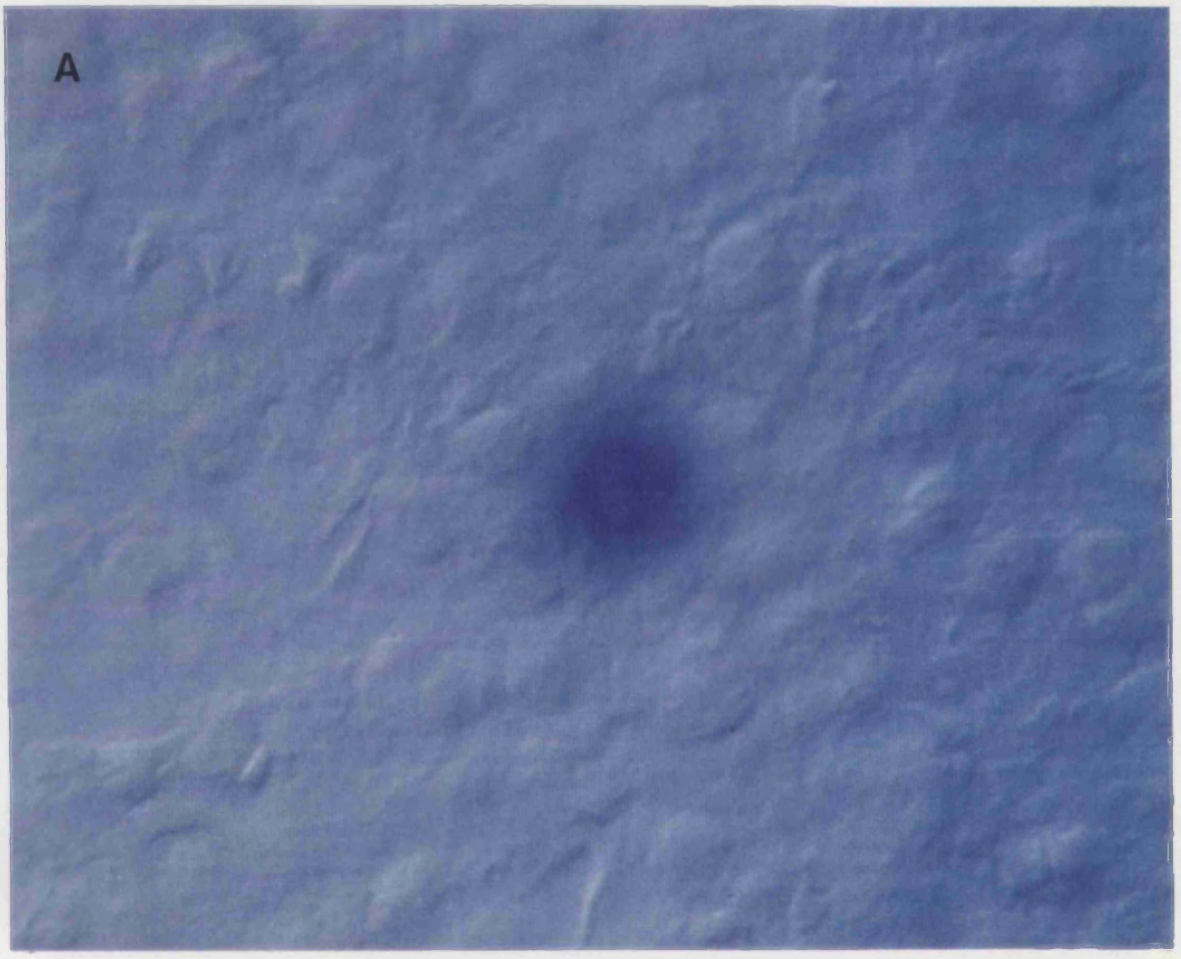

A
B
C
D
E
F

Figure 3.8. The X-Gal/NBT Staining of 'BAG' Labelled Neurones and Astrocytes.

A shows the staining of a putative neurone where the reaction product is more discretely localized compared to that obtained with a putative astrocyte in B. Scale bar 20μm.


A

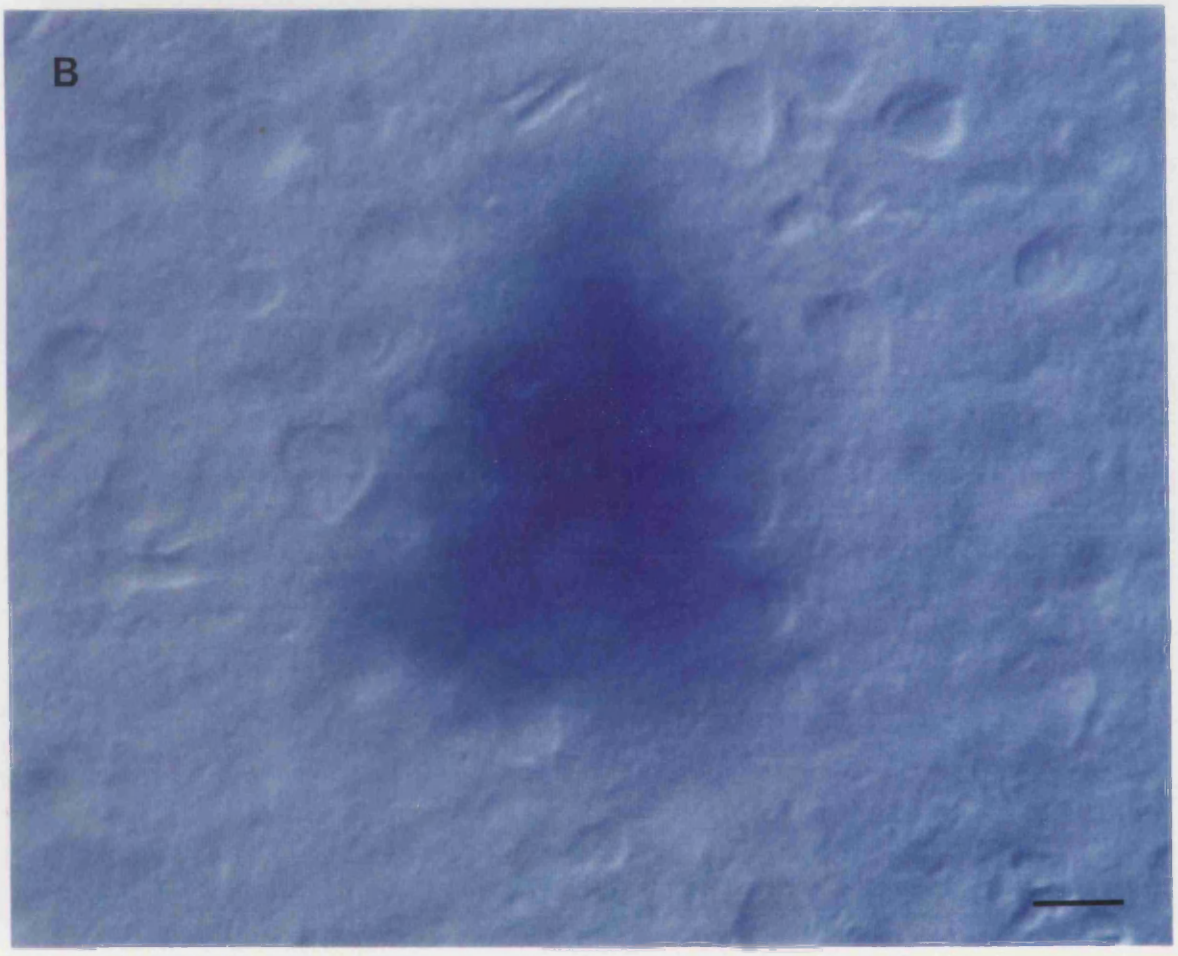
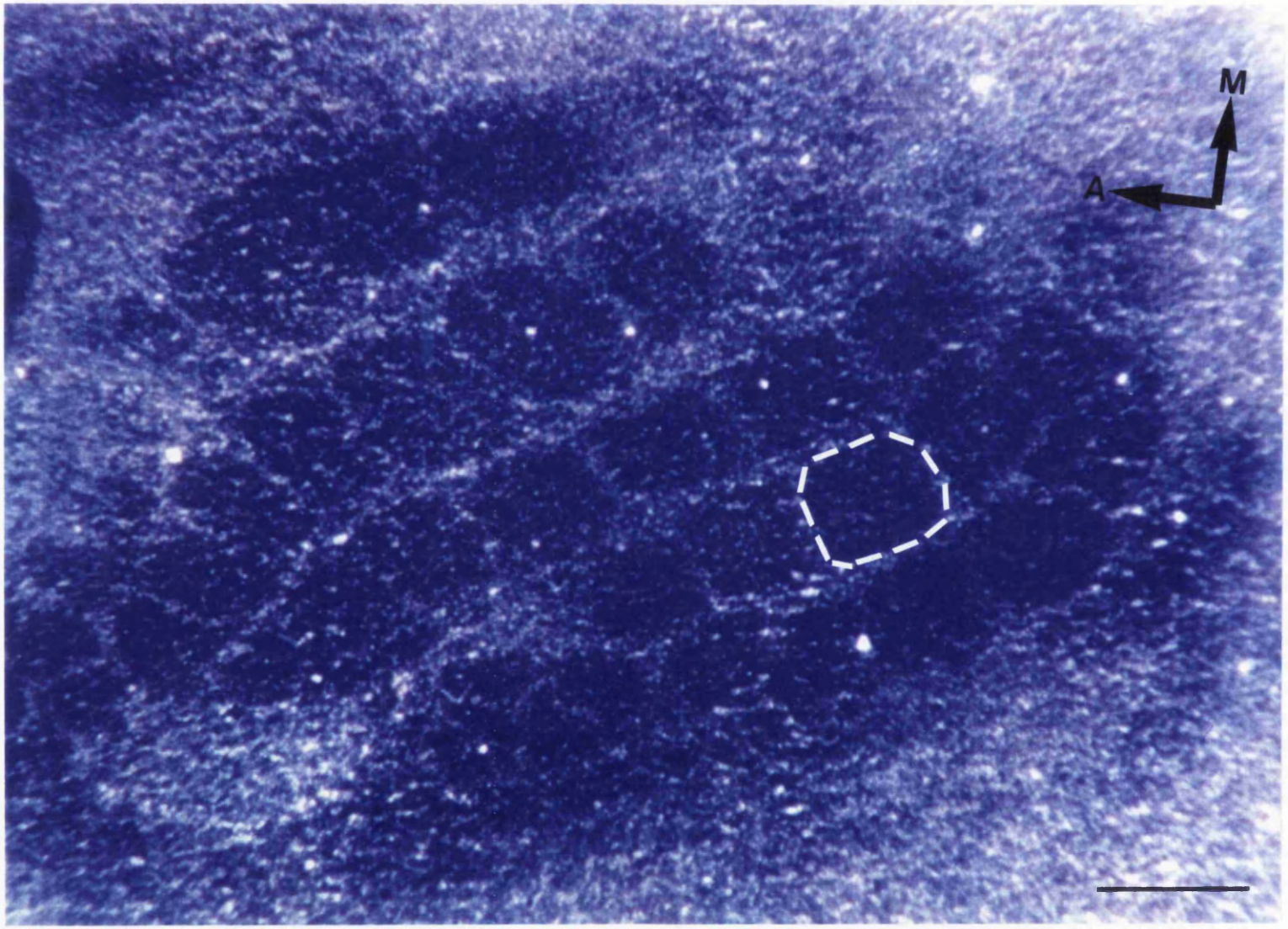

B

Figure 3.9. The Appearance of Barrel/Columns in a Tangential Section of Rat Primary Somatosensory Cortex Stained for Succinate Dehydrogenase Activity.

This section was stained histochemically for succinate dehydrogenase. Individual barrel/column hollows are strongly stained whereas the surrounding septal region is stained less so. The border between the septum and hollow for one barrel/column is outlined in white. Note that the barrel/columns do not have the same size nor shape although they are stained at equal intensity. Scale bar 300μm.


M
A

circular whereas the larger tended to be rectangular shaped with sides of up to 400μm long. The larger barrel/columns were located posterior and medially in the PMBSF. The septum separating individual hollows within a row was relatively constant in size but smaller than the septum between rows. However the septal distance tended to vary in size between individual rows. The border between the hollows and septa (an example is outlined in figure 3.9) were very precise and no diffuse gradient of staining was seen. The cells in the barrel/column hollow and septum have many different physiological and anatomical characteristics (discussed in 1.5.1). Hence the border between the two was viewed as a boundary which no clones would cross if the radial unit hypothesis applied to cortical development.

The barrel/column morphology was best appreciated using a x2.5 objective and difficult to appreciate at any higher magnification. However examination at higher magnification revealed that although the septa appeared uniformly stained, staining within individual barrel/column hollows was not. Typically near the hollow/septal boundary there were 'patches' (Land and Simons,1985) of increased staining.

In preliminary experiments attempts were made to flatten cortices so that all of the barrel/columns were visible in the same plane of section as one another (Welker and Woolsey,1974). However although the tissue stained, barrel/columns could not be consistently detected using this technique. Second the succinate dehydrogenase staining of barrel/columns was not pursued further for two reasons. The succinate dehydrogenase enzyme was aldehyde fixation sensitive and, as tissue used for staining was unfixed, cryostat sectioning was difficult. Hence subsequently all barrel/columns were detected by cytochrome c oxidase histochemistry.

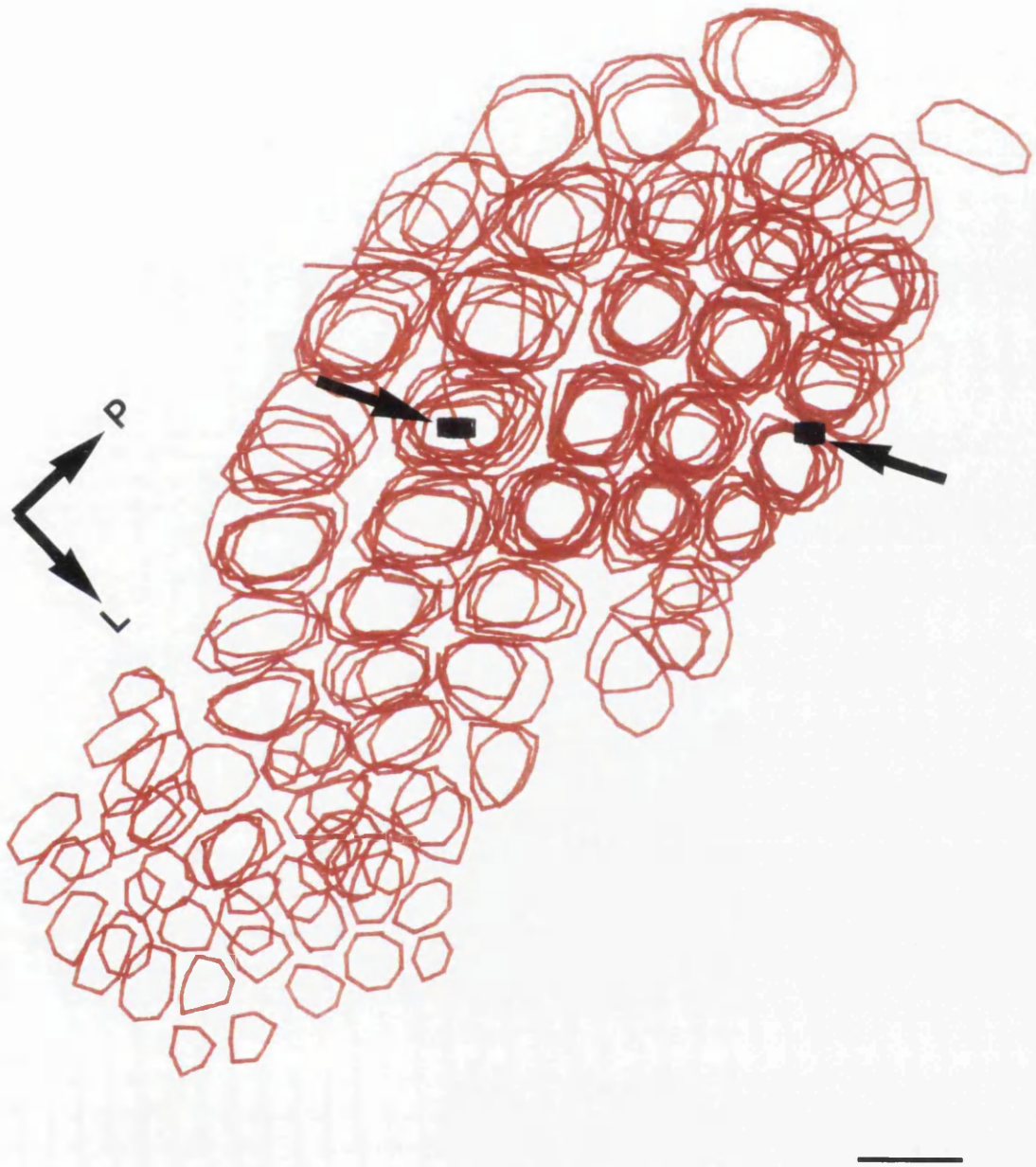
There was a non-random distribution of blood vessels in relation to individual barrel/columns. Blood vessels, which appear as white circles in the figures 1.5 and 3.9 had a tendency to be found in the septal regions surrounding the barrel/columns and only very rarely were they found within the barrel/column hollows (e.g. figure 1.5).

3.3.4 The Radial Alignment of Tangential Sections Using Blood Vessels

To test the feasibility of using blood vessels to align sections and the suitability of the SSPROF/SSRCON package, an entire series of tangential sections of cortex were stained for barrel/columns. Barrel/columns were found in twelve sections and after drawing with *camera lucida*, three blood vessels were common to all sections. The reconstruction obtained from this data is shown in figure 3.10. Several of the barrel/columns, shown in red, were radially aligned with overlapping circles outlining the hollow/septa border. The barrel/column boundaries were not exactly aligned as barrel/columns are indeed 'barrel' shaped i.e. wider at mid height than at the ends (Woolsey and Van der Loos,1970). Also shown by black squares (marked by arrows) are the positions of two blood vessels which were used to align the sections. The axes in figure 3.10 were assigned by comparison of the characteristic barrelfield anatomy with

Figure 3.10. The Three-Dimensional Reconstruction of Barrel/Columns in Rat Sm1 Using Blood Vessels.

The figure is a three-dimensional reconstruction of consecutive tangential sections stained exclusively for barrel/columns. The barrel/column boundary is shown in red and as this boundary was found in more than one section individual barrel/columns are outlined several times. The positions of the two large blood vessels are indicated by black squares. These two blood vessels were used to align the sections and they are denoted by arrows. Scale bar 300μm.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previous published data (Woolsey and Van der Loos,1970; Welker and Woolsey,1974).

Hence it appeared that radial blood vessels could be used as an accurate radial marker with SSPROF/SSRCON, at least in layer IV. Subsequent reconstructions were all performed on material where only a minority of sections were stained for barrel/columns.

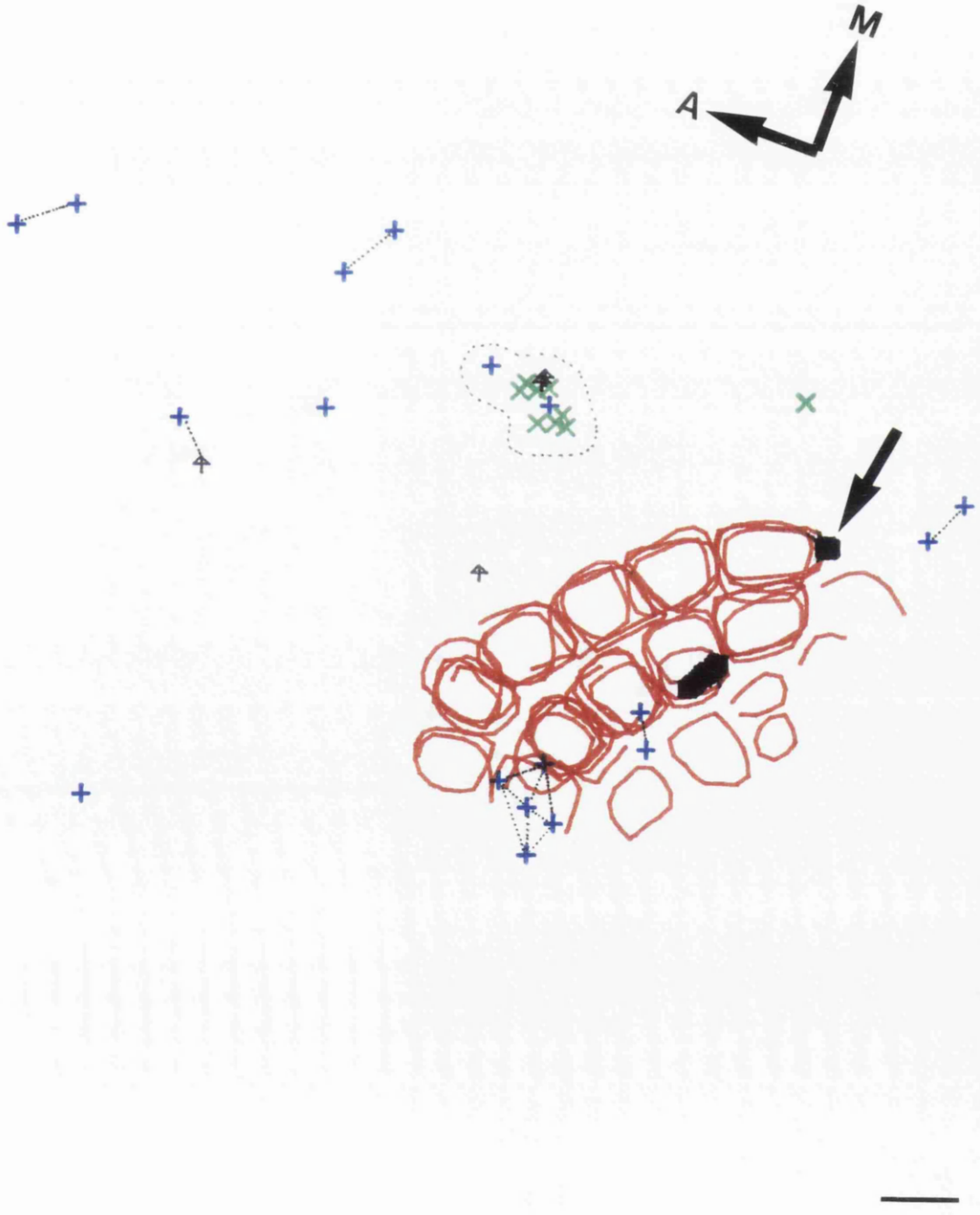
3.3.5 The Pictorial Presentation of Three-Dimensional Reconstructions

Figure 3.11 shows a printout of a three-dimensional reconstruction obtained from animal AG91R2. This figure was typical of many of the reconstructions generated where the majority of sections were stained for 'BAG' labelled cells and a minority were stained for barrel/columns. The boundary of individual barrel/columns, shown in red, was often found in several sections and such barrel/columns were concentrically outlined. The position of 'BAG' labelled cells are shown by symbols, with different coloured symbols representing different cell types (see figure legend for definitions). Clones of cells, defined using the 300μm clonal domain, are joined together by a dotted line (the one large clone is encircled). The reconstruction is only strictly radial near one blood vessel (arrowed in figure 3.11) and this is reflected by the fact that the barrel/columns were more skewed away from this point. Furthermore in some cases individual barrel/column boundaries were not possible to draw in entirety. Typically in the reconstructions not all of the barrel/columns in the entire barrelfield or PMBSF were detected (e.g. compare figures 3.11 and 3.10). This was probably because not all of the sections were stained for barrel/columns and the tangential plane of section may not have been optimal. Unfortunately this incomplete staining created some problems in assigning the orientation of the axes. The axes were of importance in assigning the orientation of individual clones. To illustrate the problem, and how it was solved, consider figure 3.11. Only part of the PMBSF was detected and the orientation of the axes was not immediately clear. However, as reported in 3.3.3, the inter-barrel/column distance separating barrel/columns within rows was much shorter than the same distance between barrel/colums in different rows. This fact assisted in axes identification as it meant that there were only two ways in which the axes could possibly be directed in figure 3.11. The correct axes were assigned by consulting published texts (Welker and Woolsey,1974) and confirmed by reference to the topographic manner in which the cortex was sectioned. For example the larger more rectangular shaped barrel/columns are found posteriorly whereas the smaller rounder barrel/columns are found anteriorly near the junction with the ALBSF.

Reconstructions presented in the form shown in figure 3.11 were difficult to discuss and interpret. For example focusing the reader on specific clones was difficult. More importantly however the precise boundary was not clear for some barrel/columns and stained cells were often difficult to see in amongst the barrel/column staining.

Figure 3.11. The Three-Dimensional Reconstruction Obtained from Animal AG91R2.

The animal received a single injection of 'BAG' virus at E16. The positions of two large blood vessels used for radial alignment are shown by black squares. The cortex is only strictly aligned at one of these blood vessels (arrowed in the figure). The barrel/columns are outlined in red, neurones are denoted by a blue cross, astrocytes by a green cross and cells of unknown phenotype by small black arrows. Clones are joined together by a dotted line, or in one case the clone is encircled. Scale bar 300μm.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Furthermore clones outside of the PMBSF were of no direct relevance to addressing the question of distribution within barrel/columns. Indeed cells at the perimeter of the reconstruction may have been misinterpreted as clonal because of the non-radial alignment in these regions. Therefore all reconstructions were processed by selecting the following:

(i) All clones either partially or totally within the barrelfield.

(ii) Only one 'average' barrel/column boundary.

Figure 3.12 is an example of this simplification process applied to the reconstruction in figure 3.11. The figure shows clearly both barrel/column boundaries and clonal distribution with respect to them. Individual stained neurones are represented by a small black circle. Cells of an unknown phenotype were represented by a black triangle (there were none in figure 3.12 but one is shown in figure 3.14). Astrocytes were represented by a cross (such cells are shown in figure 3.16). Clones of cells were joined together by a dotted line. The boundaries for each barrel/column in figure 3.12 were selected by tracing a single representative outline of a boundary from the several obtained from the initial analysis in figure 3.11.

However this relatively arbitrary simplification procedure may have led to a misinterpretation of barrel/column boundaries. This was obviously of important when assessing clonal distribution in relation to these boundaries. This possibility is discussed and examined for each clone and the collective data summarized in 3.3.10.

3.3.6 The Number of 'BAG' Labelled Cells Within Infected Cortices

Serial sections from a total 36 cerebral cortices from 25 rats were stained. All of these rats were from litters discussed in 3.3.2 which received a single injection of BAG-psi retrovirus. However it was not possible *a priori* to identify which animals contained none, few or too many 'BAG' labelled cells in the cortex or even within Sm1. Indeed nine cortices contained no 'BAG' infected cells. Presumably in these cases the 'BAG' virus was not accurately injected within the target, was able to leak out after injection or the titre introduced was insufficient for infection. At the other extreme however four cortices contained too many 'BAG' labelled cells for clonal relationships to be inferred. Possibly in such cases little or no spread of the virus throughout the neural tube may have occurred. Such densely stained material typically contained four or more stained neurones per section and no attempt at three-dimensional reconstruction of such material was attempted. Furthermore in some cases cortices contained stained cells none of which were found in Sm1 (an example of this is shown in figure 3.15).

The remaining twenty-three cortices were reconstructed in three-dimensions (7 from E14; 4 from E15 and 12 from E16). However after

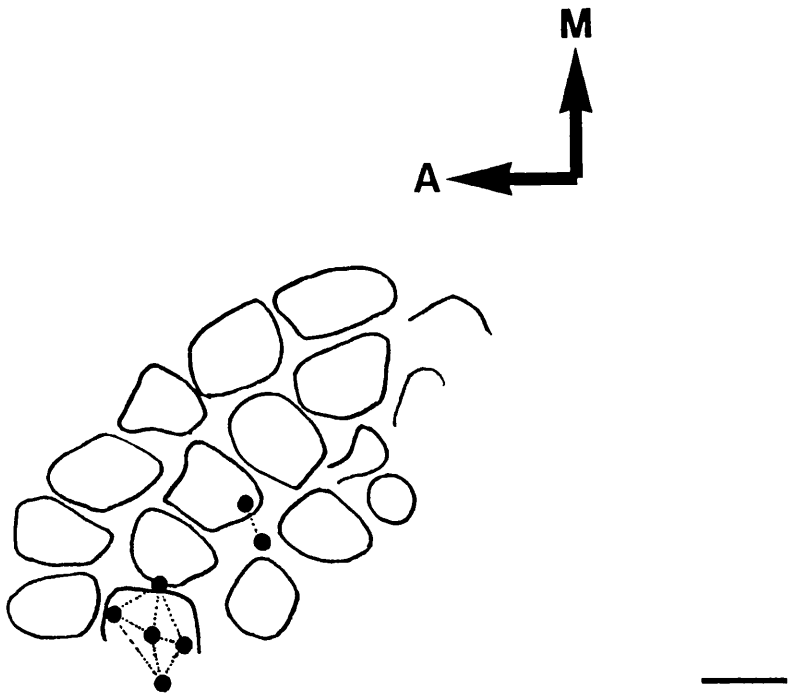


Figure 3.12. The Distribution of Clones Within the Sm1 of Animal AG91R2.

The reconstruction used in this figure was identical to that shown in figure 3.11. However the diagram has been processed for simplification (see text). Only a single boundary is shown for each barrel/column together with two clones found exclusively within the PMBSF. Cells comprising an individual clone are joined together by a dotted line. Scale bar 300μm.

three-dimensional reconstruction eight cortices were discounted from the analysis. In these cortices individual clones were difficult to distinguish between and in one case there was a very poor radial alignment of the barrel/columns. The clones in these eight cortices were often very large and probably represented clonal superimpositions. These exclusions left fifteen reconstructions suitable for further analysis (8 left and 7 right cortices; 5 from E14; 1 from E15 and 9 from E16). All the data presented below were derived from them.

3.3.7 The Three-Dimensional Orientation of Clones in Sm1

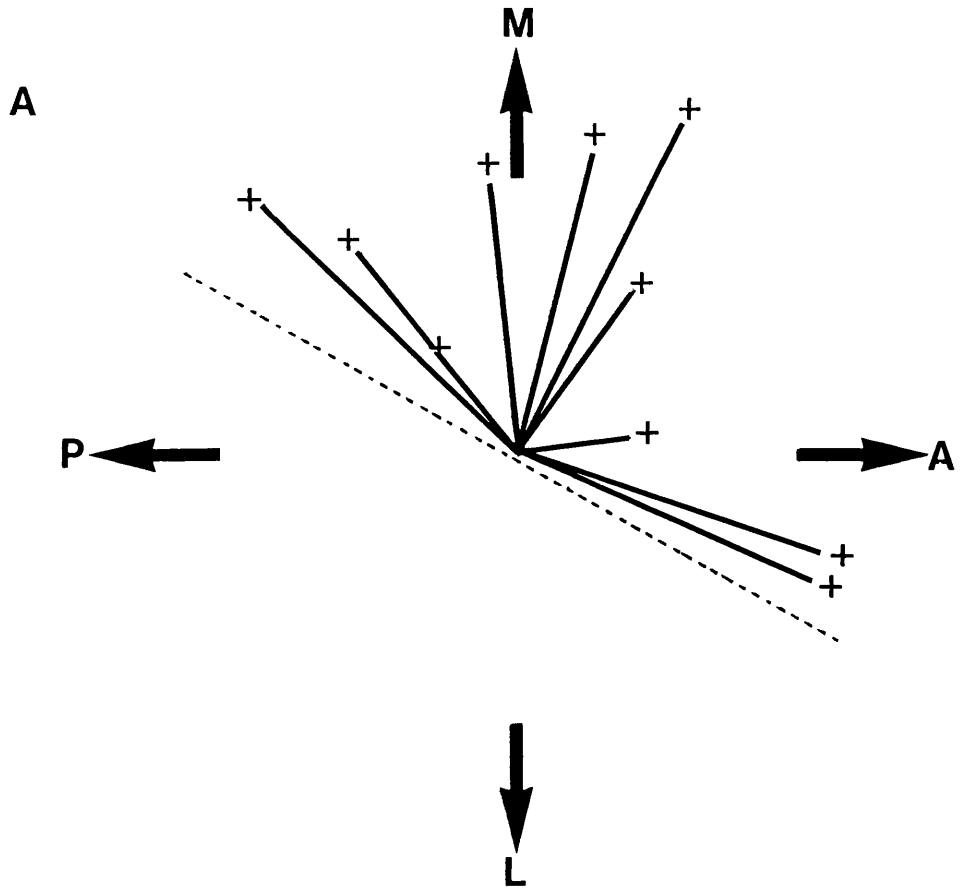
In this study clones were defined as stained cells restricted within 300μm of each other (see 3.2.8). This figure encompasses clonal spread up to, but not beyond, 300μm. Hence if cells within multicellular clones spread only 100μm from one another then such clones would still be classified as clonal. However if cells from an individual clone spread further than 300μm then they would be classified as separate clonal events. For reasons discussed in 3.4.8 this may be the case after 'BAG' infection of E14 embryos. Furthermore, and conversely, in situations where clones spread over 300μm the probability of superimposition of different clones would increase.

These points were addressed in two ways. First was to use animals which had only a small number of clones per cortex. In such material independent clonal events were well separated from one another. Indeed as mentioned above several cortices contained too many clones and were not included in the data analysis. Second the orientation of individual clones was examined (see below). If clones were found to be spread non-randomly in the cortex then the 300μm definition was probably a correct representation of clonal spread. There is a precedent for this; as mentioned in 1.3.2 there is an 'inside-out' gradient of neurogenesis in the rodent neocortex. As a single VZ cell can contribute cells to several lamina (Luskin *et al.*,1988; Price and Thurlow,1988), perhaps individual 'BAG' labelled clones may be distributed in a fashion dictated by this gradient.

To examine a possible non-random distribution of neuronal clones as defined by the 300μm clonal domain, clonal orientation was examined using a 'vector diagram' (figure 3.13). The vector diagrams were constructed by referring to the relative position of clones and the depth of constituent cells within a three-dimensional reconstruction. The deepest cell in a clone was positioned at the origin and a line was drawn to scale and with respect to the axes, to the position of the second more superficial clonal member (the construction of the vector diagrams is discussed further in 3.2.9). Only two cell clones are considered in figure 3.13. They were the most abundant multicellular clones (see 3.3.8.3) and, more importantly, larger clones were difficult to display in this fashion (see below). Furthermore the only data included in the figure are those where the two cells were located in separate sections. Figure 3.13 shows that the more superficial cell of a

Figure 3.13. Vector Diagrams Showing the Three-Dimensional Arrangement of Two-Cell Neuronal Clones Within the PMBSF.

For both A and B the deepest member of a two-cell clone was plotted at the origin and the position of the second clonal member was shown by drawing a line, to scale and with respect to the axis, to its position. A shows the three-dimensional distribution of neuronal clones at E16 and B at E14. Scale bar 100μm.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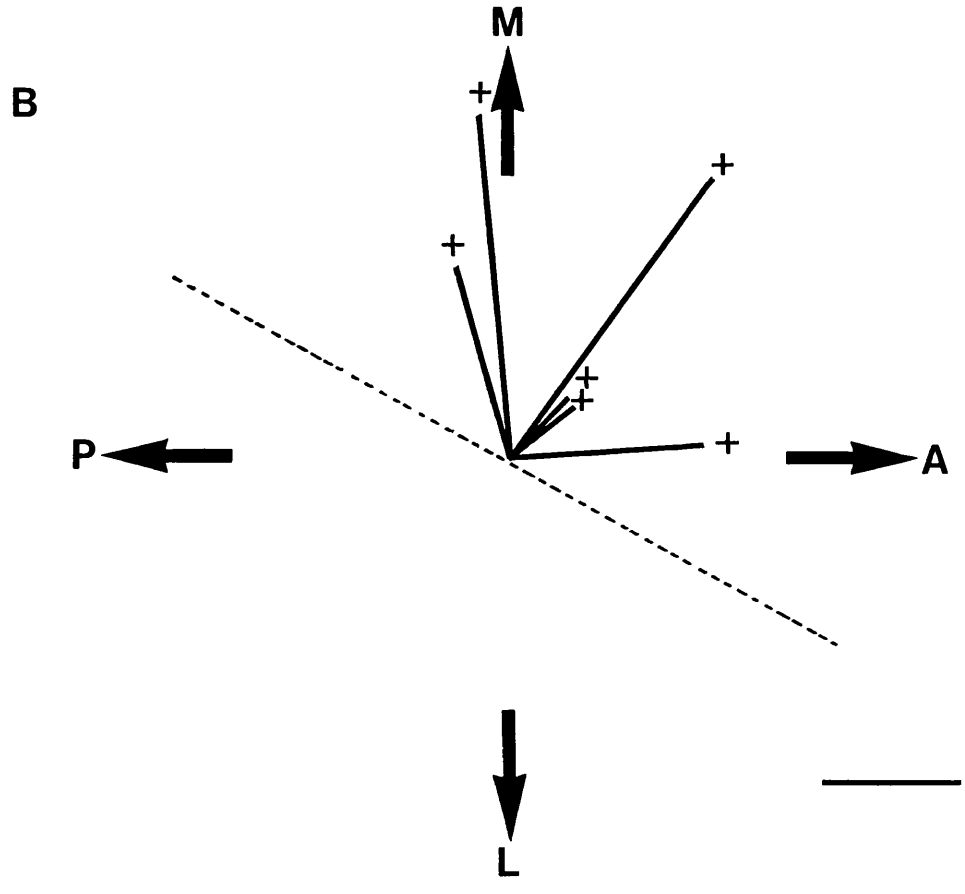
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pair tends to be found medial to the deeper cell, for both E16 and E14 data. To illustrate this a dashed line was drawn in both figures 3.13A and 3.13B at the same orientation dividing the diagrams into two. At both E16 and E14, clones did not spread below the dashed line i.e. superficial members of clones did not spread laterally of their deeper sibling. The dashed line was slightly skewed from the anterioposterior axis. In fact a line perpendicular to it would point slightly anterior of medial. Clones that were only separated from each other by a few tens of microns were equally dispersed within this domain as were clones which spread further apart. Furthermore this non-random distribution of clones reported above for the two cell clones was also true for larger ones. For example, in the five cell clone in figure 3.11 the deeper cells were found laterally whereas the superficial members were found medially.

Thus clones appeared to be distributed non-randomly suggesting that the 300μm clonal domain used in this study to classify clonal events was a true representation of clonal spread. The significance of these findings are discussed in 3.4.10.

3.3.8 The Characterization of 'BAG' Labelled Clones in Infected Neocortex

3.3.8.1 The Number of 'BAG' Labelled Clones in Infected Neocortex

Compendia of the different clonal types found after infection at E14, E15 and E16 are displayed in Tables 3.1, 3.2 and 3.3 respectively. Usually an individual cortex contained more than one type of clone, although some contained only neuronal clones e.g. animal AG09R1 in Table 3.3. The total numbers of clones in each cortex varied from a minimum of 1 to a maximum of 25. Ideally material containing the latter number of clones should not have been included in the data pool. Probably there is a chance of clonal superimposition in such material. However individual clones in the two cortices containing 25 clones were relatively well spaced apart. To justify their inclusion in the data pool both cortices have been presented and discussed below. Furthermore in 3.3.10 the effect of exclusion of cortices containing the larger number of clones upon the final data was examined.

3.3.8.2 The Phenotype of 'BAG' Labelled Clones

Table 3.4 shows the the classification of clones based upon phenotype. Despite minor differences, the proportion of clonal types at each age group remained relatively constant. The data is entirely consistent with the model of cell lineage in the neocortex outlined in 1.13. At all ages the majority of clones (on average 80%) consisted exclusively of neurones. These clones were analyzed further in relation to a number of respects e.g. average clonal size, distribution of clones within the PMBSF (see below). Clones consisting exclusively of astrocytes were second most in abundance and had a mean clonal size of 5.1 with maximum and minimum values of 1 and 27. Often several astrocytes in a clone were

TABLE 3.1.

Inventory of Neocortical Clones from Animals Injected at E14.

Key : N, neurone; A, astrocyte; U, unknown

Animal AG06L1

1N
2N
1N+1U
2N+1U
Total Clones = 4

Animal AG06R4

2N
Total Clones = 1

Animal AG06R3

6x1N
2N
5A+1N
2A
27A
Total Clones = 10

Animal AG06L7

7x1N
5x2N
2x3N
10N
3x1U
2x(1N+1U)
4N+1U
2A
1A+4N
16A+1N+1U
55A+1U
Total Clones = 25

Animal AG06L4

3x1N
10N
12A+5N
Total Clones = 5

TABLE 3.2.

Inventory of Neocortical Clones from Animals Injected at E15.

Key : N, neurone; A, astrocyte; U, unknown

Animal AF82L7

14x1N
6x2N
3N
2A
8A
4A
2A+1N
Total Clones = 25

TABLE 3.3.

Inventory of Neocortical Clones from Animals Injected at E16.

Key : N, neurone; A, astrocyte; U, unknown

Animal AG09L1
8x1N
4x2N
3x3N
1U
1A
Total Clones = 17

Animal AG09R1
2x1N
1x3N
Total Clones = 3

Animal AG09R4
2x1N
Total Clones = 2

Animal AG09L4
6x1N
2N
Total Clones = 7

Animal AG91L1
4x1N
2N
Total Clones = 6

Animal AG91R2
2x1N
4x2N
5N
1U
1N+1U
2A
2N+2U+7A
Total Clones = 11

Animal AG91R3
3x1N
1U
1A
2A
Total Clones = 6

Animal AG91R5
2x1N
Total Clones = 2

Animal AG91L5
17x1N
2N
1U
Total Clones = 19

TABLE 3.4.

The Phenotypic Classification of 'BAG' Labelled Clones Found After Injection of BAG-psi Virus at E14, E15 and E16.

(N, neurone clones; A, astrocyte clones; U, cells of unknown phenotype mixed clones are also indicated e.g. N/A neurone/astrocyte clones)

	N	A	N/A	U	N/U	A/U	N/A/U	Total
E14 TOTAL	29	3	3	3	5	1	1	45
	(64%)	(7%)	(7%)	(7%)	(11%)	(2%)	(2%)	(100%)
E15 TOTAL	21	3	1	-	-	-	-	25
	(84%)	(12%)	(4%)	-	-	-	-	(100%)
E16 TOTAL	62	4	-	4	1	-	1	72
	(86%)	(5%)		(5%)	(2%)		(2%)	(100%)
CUMULATIVE TOTAL	**112**	**10**	**4**	**7**	**6**	**1**	**2**	**142**
	(78.9%)	**(7.1%)**	**(2.8%)**	**(4.9%)**	**(4.2%)**	**(0.7%)**	**(1.4%)**	**(100%)**

found adjacent to one another in the same section and individual cells were difficult to distinguish. Hence the mean clonal size reported may be a slight underestimate. A small proportion of clones containing both neurones and astrocytes were found at E14 (7%) and E15 (4%) but not at E16. These could represent a superimposition of two separate clones, by say infection of adjacent cells. However a more attractive hypothesis, consistent with the decreasing proportion of these clones during development, is that the progenitors were bipotential. Perhaps these progenitors represent the common progenitor of the multipotential and astrocytic progenitors (C in figure 1.12). The remaining clones (cumulatively 14%) all contained cells with an unknown phenotype. These clones were classified separately to restrict data analysis to neuronal only clones. It is feasible that these clones may have migrated superficially by a different mechanism than that used by neuronal clones. However some clones containing non-neuronal cells were found within Sm1 and the distribution of them in relationship to barrel/column boundaries is reported below.

Previous studies involving retroviral vectors (Luskin *et al.*,1988; Price and Thurlow,1988) demonstrated that clones of astrocytes spread distances of the order of several millimetres and could not feasibly be constrained within individual physiological columns. However the more interesting question, unanswered from these previous studies, related to the distribution of clones of neurones. Were neuronal clones spread in a manner compatible with the radial unit hypothesis? In order to answer this question data analysis was restricted to clones of neurones found within Sm1, a region where the reconstructions were radially aligned. Furthermore as reported in 3.3.7 clones containing exclusively neurones were oriented non-randomly.

The data in Table 3.4 also reflects upon two problems associated with these experiments. Basically the clonal phenotypes were as expected from the lineage tree outlined in figure 1.12. This tree was suggested from a variety of both *in vivo* and *in vitro* experiments. Hence this tentatively suggests that both the clonal definition and radial alignment of the sections were essentially correct.

3.3.8.3 The Size of 'BAG' Labelled Neuronal Clones

As presented in Table 3.4 usually several clonal phenotypes were found in an individual cortex. However as discussed above a subset of data, only neuronal clones, were selected for analysis. In all of the reconstructions a total of 112 clones containing exclusively neurones were defined. The numbers and sizes of the clones obtained in each animal are shown in Table 3.5 together with the cumulative figures for E14, E15 and E16. Both odd and even numbered clones were found but because of cell death it was not possible to say if the clones were generated by symmetrical or asymmetric divisions.

At all three injection points the majority of neuronal clones were single cell clones, an observation in accord with a previous study

TABLE 3.5.

The Number and Size of Neuronal Clones Obtained After Three-Dimensional Reconstruction of Cortices Injected at E14, E15 and E16.

Animal No.	One	Two	Three	Five	Ten	Total
AG09L1	8	4	3	-	-	15
AG09R1	2	-	1	-	-	3
AG09R4	2	-	-	-	-	2
AG09L4	6	1	-	-	-	7
AG91L1	4	1	-	-	-	5
AG91R2	2	4	-	1	-	7
AG91R3	3	-	-	-	-	3
AG91R5	2	-	-	-	-	2
AG91L5	17	1	-	-	-	18
E16 TOTAL	**46**	**11**	**4**	**1**	**-**	**62**
	(74.2%)	**(17.7%)**	**(6.5%)**	**(1.6%)**		**(100%)**
AF82L7	14	6	1	-	-	21
E15 TOTAL	**14**	**6**	**1**	**-**	**-**	**21**
	(66.6%)	**(28.6%)**	**(4.8%)**			**(100%)**
AG06L1	1	1	-	-	-	2
AG06R4	-	1	-	-	-	1
AG06R3	6	1	-	-	-	7
AG06L7	7	5	2	-	1	15
AG06L4	3	-	-	-	1	4
E14 TOTAL	**17**	**8**	**2**	**-**	**2**	**29**
	(58.6%)	**(27.6%)**	**(6.9%)**	**-**	**(6.9%)**	**(100%)**

(Price and Thurlow,1988). The average number of neuronal clones per cortex in the fifteen reconstructions was 7.3, with maximum and minimum values of 21 and 1 respectively. The E14 injections had a slightly larger mean clonal size (mean=2.0) than that from E16 (mean=1.4). There were 3.5 times more clones in the left cortex than the right, although the mean clonal size in the left and right cortices was equal at 1.5.

Of the 112 clones 28 (25%) were found either exclusively or partly within the PMBSF. This figure is relatively low because it was not possible to direct an infection to a specific area of the VZ, nor predict how labelled progeny would disperse. Furthermore of these 28 only 12 (43%) were multicellular clones which could be analyzed further with respect to clonal distribution in relation to barrel/columns. This decrease was expected as the majority of all clones were single cells (see above).

3.3.9 The Distributions of Individual 'BAG' Labelled Clones in Relation to Barrel/Column Boundaries

The following figures show examples of three-dimensional reconstructions which view how clones were spread in relation to barrel/columns. Several features common to all of the reconstructions were apparent. First, as expected from previous studies, individual clones were not strictly radially aligned with 'BAG' labelled cells stacked on top of each other. Second, clones were found throughout the reconstruction and were not restricted to say lateral areas of neocortex (e.g. figure 3.11), nor were they exclusively found within or outside of barrel/columns. For example the cortex in figure 3.11 contained seven neuronal clones and only two of them (29%) (containing five and two cells) were found in the PMBSF. Hence it appeared as though clones were effectively randomly distributed throughout the infected cortex.

The most interesting aspect of the data related to the distribution of clones in relation to barrel/columns. The data, although small in number, suggested that individual clones crossed the barrel/column boundaries. This, just like the preferred distribution reported in figure 3.13, was true irrespective of the time of infection. Clones crossed barrel/column boundaries when labelled early in neurogenesis (E14, E15) or even at later stages (E16) (Table 3.6). A total of 12 multicellular neuronal clones (11% of the 112 total) were found either totally or partly within the PMBSF. The vast majority of these 12 clones spread across barrel/column boundaries and had a distribution incompatible with the radial unit hypothesis. Only one clone, seen after an E15 injection, satisfied the criterion of the radial unit hypothesis as it was found exclusively within a barrel/column hollow. Exactly how the clones cross the boundaries is shown in the right of Table 3.6, with the majority of the clones found distributed across the hollow and septa. Two of the clones were found in different barrel/column hollows and one was found in the septa surrounding different barrel/columns.

TABLE 3.6

The Distribution of Clones of Neurones in Relation to Barrel/Column Boundaries.

	Clones Within Boundaries	Clones Crossing Boundaries		
		hollow /septal	hollow /hollow	septal /septal
E14	0	2	1	1
E15	1	1	0	0
E16	0	5	1	0
TOTAL	**1**	**8**	**2**	**1**
	(8%)	**(67%)**	**(17%)**	**(8%)**

In reconstructions below the eleven cases are presented where clones crossed barrel/column boundaries and the one case where a clone did not. Furthermore also addressed were specific questions that arose for each reconstruction. Such questions included the possibility of artifacts. For example, did the processing each reconstruction was subjected to result in a misinterpretation of barrel/column boundaries? Furthermore what happended to the data in Table 3.6 when some of the cortices containing a large number of clones were not included in the data pool?

The clonal distribution in figure 3.11 (already discussed in a number of respects above) was typical of many illustrated. In the entire reconstruction different clones were well separated from one another (figure 3.11), even within the PMBSF (figure 3.12). The two-cell clone in figure 3.12 spread from the barrel/column hollow to the septum separating different barrel/column rows. Furthermore the clone was oriented in the mediolateral plane although the clone was not included in the figure 3.13 as both cells were found in the same section. As discussed in 3.3.7 a mediolateral distribution was highly suggestive of the clonal nature of the two cells. By consulting both figures 3.11 and 3.12 it was clear that the barrel/column boundary which the clone crossed was not diffuse and not an artifact in figure 3.12. A similar spread was seen with the five cell clone, although the entire barrel/column boundary was not visible. In figure 3.12 it was suggested that the most medial member of this clone was found in the septal region and three cells located more laterally were found within the barrel/column hollow. Admittedly the distribution of the five cell clone was not as clear as the two cell clone. The barrel/column in which the clone rested was only found in two sections. The barrel/column boundary constituted of two halves which very nearly joined together and one cell of the clone was found near where they met. It was possible that, because of the short inter-hollow distance between rows described above, the cell was found within the same barrel/column hollow as all the other siblings. However it was perhaps equally valid to say that the cell was found within the hollow of an adjacent barrel/column. The deciding factor in reaching the distribution shown in figure 3.12 was the topography of the surrounding barrel/columns. These were strongly radially aligned and the disputed cell was very close to (but appeared not to cross) the adjacent barrel/column boundary. Hence it was assumed that the cell was positioned in the septum and that the clone had crossed a barrel/column boundary.

A similar distribution was also seen in cases where the PMBSF contained several clones. For example in figure 3.14 four multicellular clones were found in the PMBSF. Again they were all oriented mediolaterally suggesting that they were separate clonal events. Indeed two clones (both located posteriorly in figure 3.14) were separated by only 330μm at their closest point. However their distinct clonal nature was suggested by their independent mediolateral spread. The three cell clone was spread very little in terms of depth (all three cells were

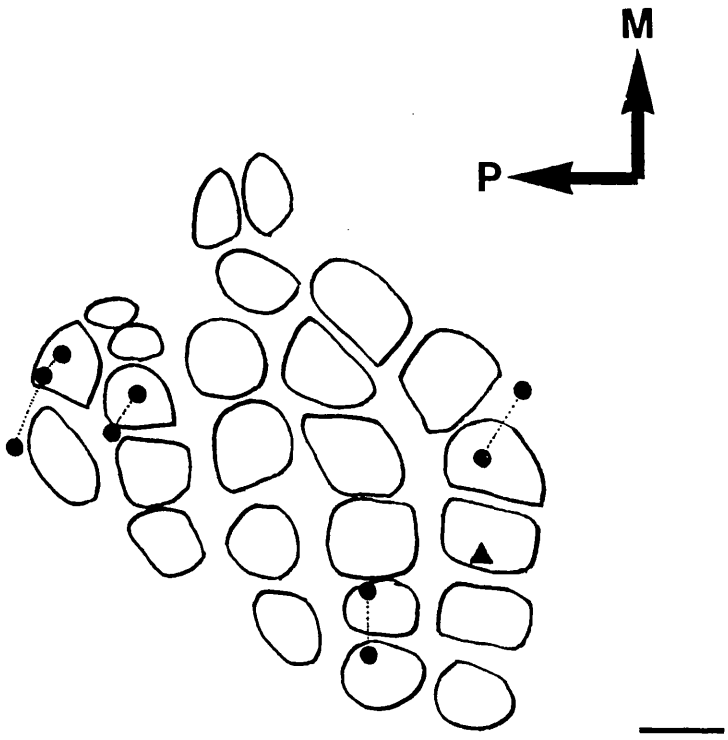


Figure 3.14. The Distribution of Clones in Sm1 From Animal AG09L1.

The animal was injected with 'BAG' virus when aged E16. Five clones were found within the PMBSF. Scale bar 300μm.

found in two adjacent sections) whereas the adjacent two cell clone was spread mediolaterally in accord with the data in figure 3.13. Both of these clones spread from a barrel/column hollow to septum. By refering to the original reconstruction the two cell clone was clearly spread hollow/septal, although the septum was that immediately surrounding the barrel/column and there was a slight chance that the position was misinterpreted.

Two, two-cell clones were found in the anterior portion of the PMBSF. The most anterior clone was clearly spread hollow/septal. Again it was oriented mediolaterally (in accord with data in figure 3.13). Furthermore the two cell clone and the single unknown cell located lateral to it formed a stepwise arrangement, in terms of depth, oriented mediolaterally. This tentatively suggests a possible clonal relationship even though the constituent cells had spread slightly more than 300μm apart. This putative three cell clone spanned two barrel/column hollows and septa. However as this putative clone did not satisfy the 300μm clonal domain the distribution was not included in Table 3.6.

The third two cell clone, found most laterally, was proposed to have spanned two barrel/column hollows. However the boundaries were not strongly radially aligned in this region and only after consulting *camera lucidas* was this distribution assigned. It appeared to be the most feasible although it was possible that the clone could have been restricted within one barrel/column hollow or even spread hollow/septal. This clone could be viewed as spreading across two barrel/column boundaries.

As mentioned above, it was not possible to predict before analysis which animal contained any multicellular clones in Sm1. For example figure 3.15 is a reconstruction where all multicellular 'BAG' labelled cells were found just outside of the barrel/columns. Needless to say it was possible to speculate on the possible distribution of the two cell clone (located most posteriorly). At first sight it would appear difficult to imagine that the clone would be constained within the barrel/column if the boundary was complete. Furthermore the two cell clone may have actually consisted of three cells and spread even further than anticipated. A third cell was located posteriorly to this clone, just outside of a 300μm clonal domain. Again this possibility is favoured by a stepwise depth distribution of all three cells, in accord with the vector distribution in figure 3.13.

All the three reconstructions shown above were from cortices injected at E16. However the clonal distributions reported above were not unique to this age but were also seen after E15 and E14 injection. For example figure 3.16 is a reconstruction from an animal injected at E15. This cortex contained 25 clones in total, the upper limit used in this study. Hence of all the material it was most likely that clonal identification in this cortex was flawed. However only seven clones were found within the PMBSF, and only three were multicellular. Both two cell clones were found in areas that were well radially aligned and their distribution in

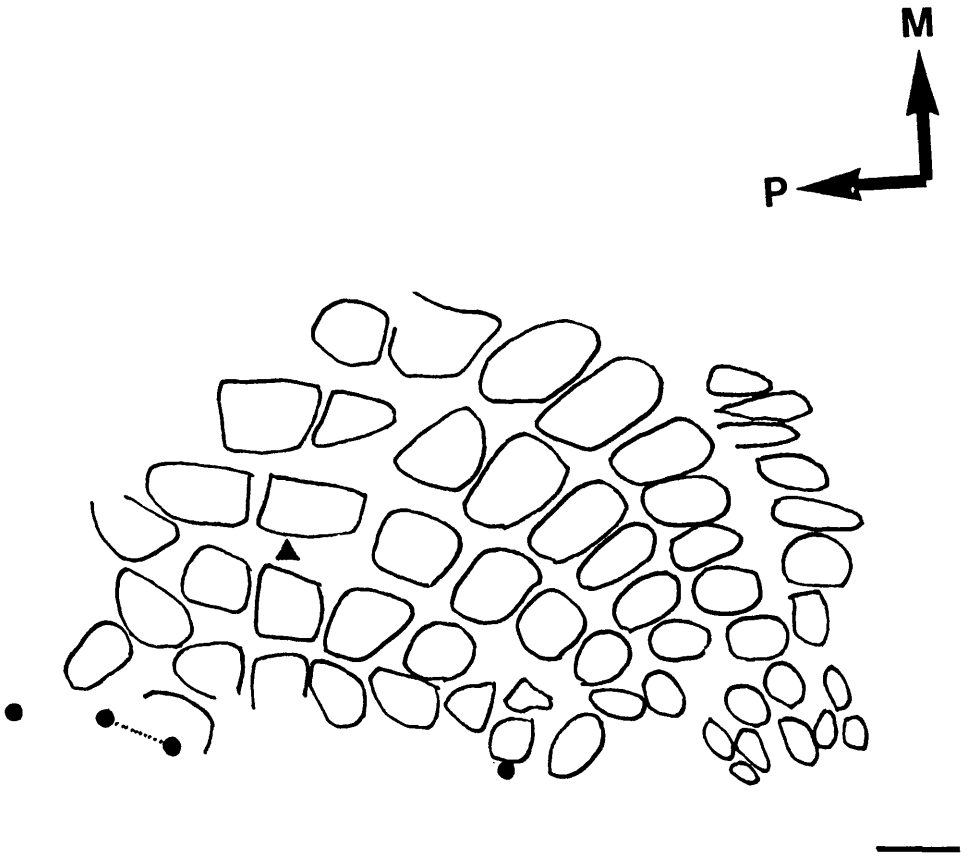


Figure 3.15. The Distribution of Clones in Sm1 of Animal AG91L1.

The animal was injected with 'BAG' virus when aged E16. Unfortunately none of the multicellular clones in the animal were found within barrel/columns. Scale bar 300μm.

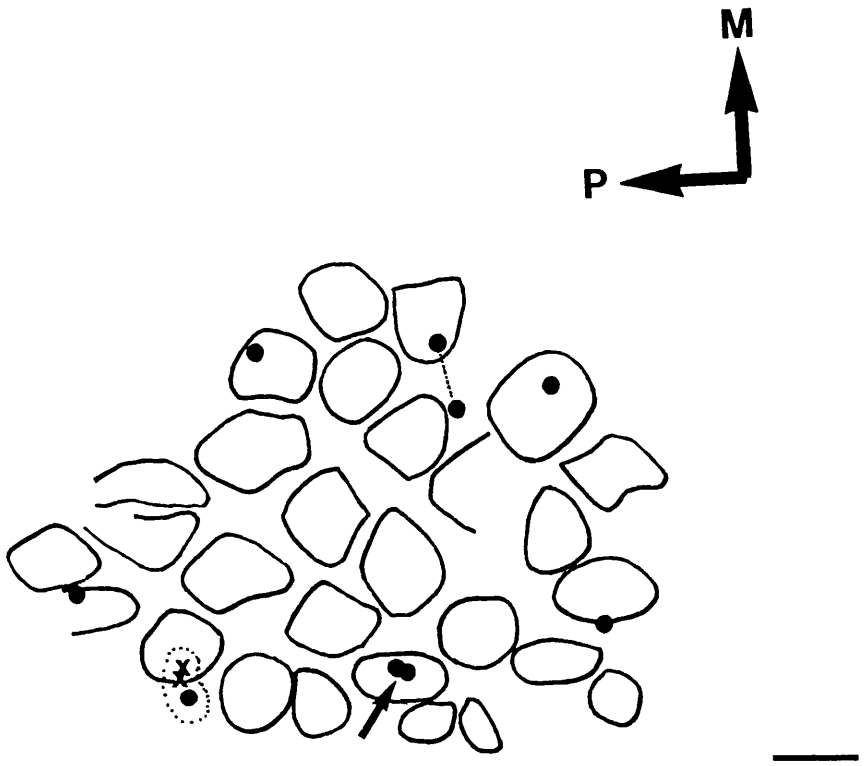


Figure 3.16. The Distribution of Clones Within Sml of Animal AF82L7.

The animal was injected with 'BAG' virus when aged E15. Three multicellular clones were found in the PMBSF. The arrow denotes one clone found exclusively within a barrel/column. Scale bar 300μm.

relation to barrel/column boundaries was unequivocal. The laterally located, two cell clone (arrowed in figure 3.16) was found exclusively within the barrel/column hollow. Thus it did not cross the barrel/column boundary. However, as summarized in Table 3.6 this was the only example of such a distribution detected in all of the reconstructions. Indeed a second two clone within the same reconstruction (located more medially) crossed a barrel/column boundary spreading from hollow to septum. However there are two aspects of dispute with this clone. First as one cell in this clone was very close to a barrel/column boundary, the clonal distribution could be hollow/hollow and not hollow/septal. Second, and perhaps more importantly, due to the large number of clones in the cortex this two cell clone may not be one clonal event but may represent a superimposition. This possibility cannot be excluded, but the clonal nature of these two cells is suggested by their mediolateral distribution (although both cells were found in the same section). A mixed neuronal/astrocyte clone, located at seven o'clock in the figure, was defined as spreading from hollow to septum.

Hence useful data could be derived from cortices containing the larger numbers of clones. This is further illustrated by the reconstruction in figure 3.17. This cortex also contained a total of 25 clones. A total of eight clones were found in the PMBSF. Most of the clones were relatively well spaced apart although they did tend to cluster laterally (towards the bottom of the figure). Both two cell clones spread from hollow to hollow. The clone containing two neurones (the two circles joined by a dotted line) was spread approximately mediolaterally, although both cells were found at the same depth. The second two cell clone was also spread mediolaterally but the deeper cell was found medially. The three cell clone was found in a barrel/column which only had a partial boundary. The clone spread from septa to hollow and then to presumed septa. The three cells were distributed in an approximate mediolateral plane, although the deepest cell was located medially. Perhaps this clone and the two cell clone discussed above represent spurious superimpositions, as they were not spread in the manner described in 3.3.7. This is possible considering the number of clones in the cortex and the fact that a number of clones are clustered in one part of the PMBSF.

In addition to cortices containing large numbers of clones some contained relatively few. As mentioned in 3.3.8.1 the lowest number of clones in a cortex was one. The reconstruction containing this clone, obtained after infection at E14, is displayed in figure 3.18. Only two stained cells were found in the cortex and as they were found in Sm1 separated by just under 300μm they constituted a two cell clone. The two cells were distributed in a mediolateral manner and the data was included in figure 3.13. This was further suggestive of their clonal nature. One cell in the clone was found in the septal region surrounding one barrel/column. The precise position of the other cell was not clear. In the reconstruction this cell appeared to be located in the septum and

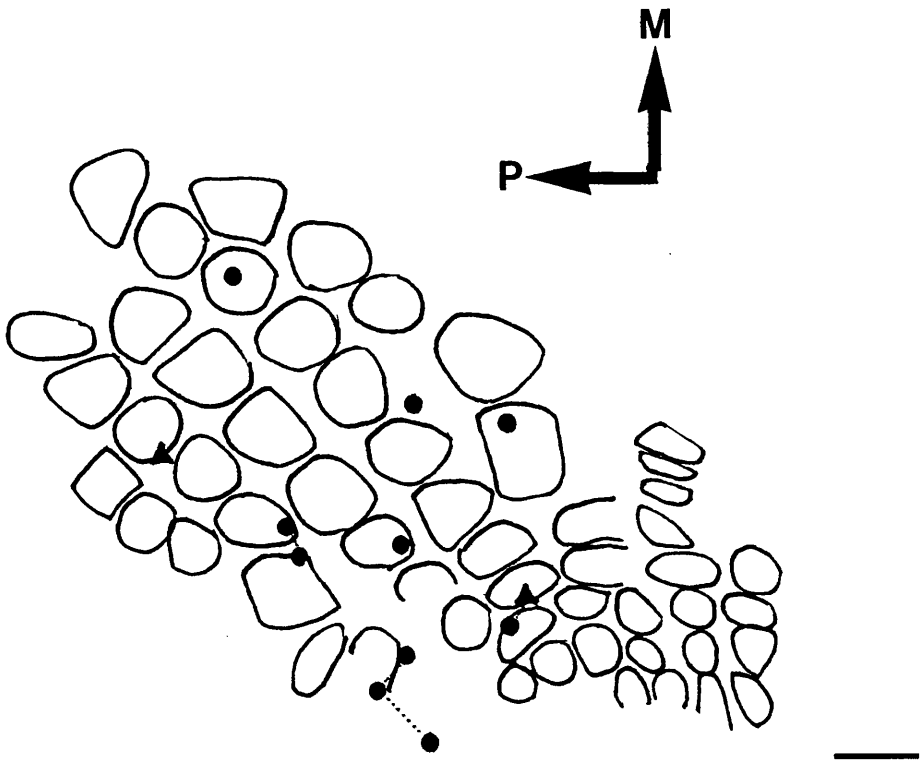


Figure 3.17. The Distribution of Clones Within Sm1 of Animal AG06L7.

The animal was injected with 'BAG' virus when aged E14. Several multicellular clones are shown in the PMBSF. Scale bar 300μm.

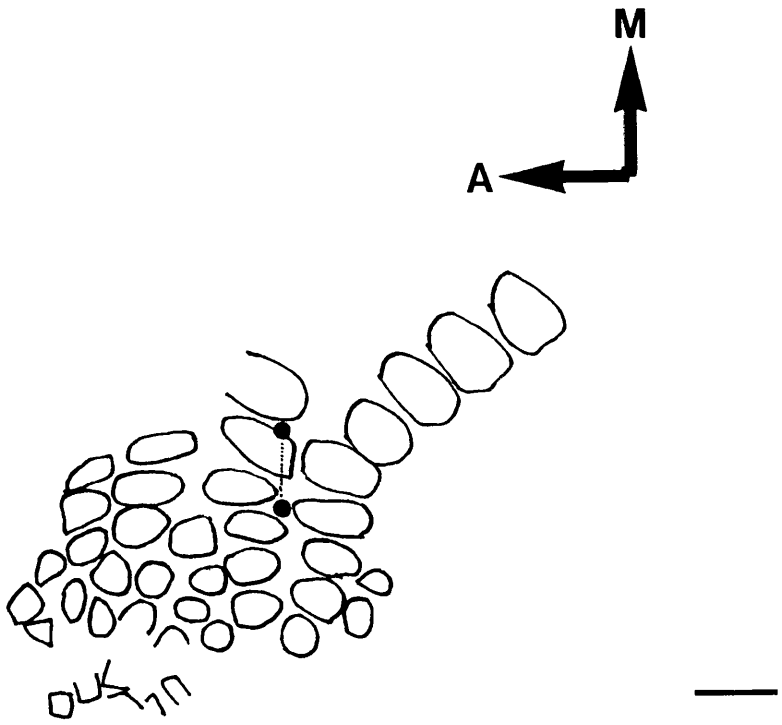


Figure 3.18. The Distribution of Clones Within Sm1 of Animal AG06R4.

The animal was injected with 'BAG' virus when aged E14. Only one clone was present in the PMBSF. Scale bar 300μm.

this was how this clone was included in Table 3.6. As the cell was found between two barrel/columns in a row it could have been positioned either in the hollow or septum. However irrespective of the precise distribution of this cell, the clone as a whole clearly spread in a manner incompatible with the radial unit hypothesis.

Perhaps the one case where the relationship of clones to barrel/column boundaries was most disputable is shown in figure 3.19. Both the two and three cell clones in the figure were classed as spreading from hollow to septum. This was based on the fact that one cell in each clone was positioned just outside of a barrel/column. However because of an inacurrate reconstruction or non-radial alignment both clones could have been confined within one barrel/column. Regardless of the distribution of the clones their clonal nature is suggested by their independent mediolateral spread. Indeed the two cell clone was included as data in figure 3.13.

As mentioned above the majority of 'BAG' labelled clones were found to contain exclusively neurones. However, as presented in Table 3.4 mixed clones were also detected. Such clones also did not respect barrel/column boundaries, although such data was not included in Table 3.6. Examples of this phenomenon have been illustrated in figures 3.16, 3.17 and 3.19. The mixed three cell clones in figures 3.16 and 3.19 were defined as spreading hollow/septal, the two cell clone in figure 3.17 was classed as spreading hollow/hollow.

3.3.10 Analysis of the Distribution of Clones of 'BAG' Labelled Neurones Using More Stringent Criteria

The figures in the preceeding paragraphs illustrate the distribution of clones in relation to barrel/column boundaries. Also discussed are the reasons why cells were assigned to their particular position during processing. However also mentioned, where necessary, were cases where the distribution of cells to a barrel/column boundary could be disputed. This was particularly common when cells were found very close to the barrel/column boundary or when the radial alignment of the barrel/columns was not particularly good. Furthermore some reconstructions contained over 20 different clones and the probability of superimpositions of different clones in such material was probably high. Such material was included in the data pool so that there was a large number of clones in total to consider. Indeed the non-random distributions in figure 3.13, which contains data from material containing a large number of clones, suggest that superimpositions were not a serious problem. However what happened to the distribution reported in Table 3.6 if only clones in reconstructions with a low clonal number, say less than twelve clones in total, and with a relatively clear distribution were considered? The figure of twelve was selected as an upper limit on a relatively arbitrary basis. All of the eleven clones in the reconstruction in figure 3.11 were well spaced apart and using this limit reconstructions containing 25, 25, 19 and 17

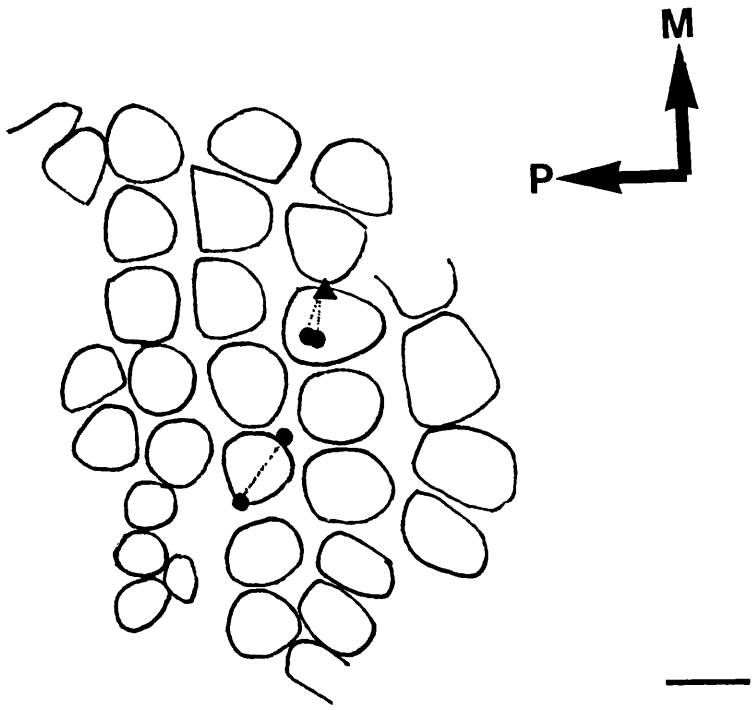


Figure 3.19. The Distribution of Clones Within Sm1 of Animal AG06L1.

The animal was injected with 'BAG' virus when aged E14. Two clones were found in the PMBSF. Scale bar 300μm.

clones were excluded from analysis. The mean number of clones in the remaining cortices was 5.2.

Figure 3.20 illustrates the criterion used to eliminate certain clones on the grounds of distribution near barrel/column boundaries. Until now the boundary of a barrel/column has been defined as the boundary of the shaded barrel/column in figure 3.20A. However some clones were found very near this boundary and their position could have been misinterpreted. Hence the data was reconsidered using a new enlarged boundary illustrated in figure 3.20B. This new boundary extends to the surrounding septum between barrel/columns, and was deliberately designed to be an overestimate of the true barrel/column boundary.

Hence in summary the data was subjected to selecting only those reconstructions where the following criteria applied :

(i) Reconstructions containing less than twelve clones in total.

(ii) Selecting only clones with an undisputed relationship to barrel/column boundaries using the criteria in figure 3.20B.

By applying these criteria to the clones in Table 3.6 several of them are eliminated. First, both clones that crossed from hollow to hollow (illustrated in figures 3.14 and 3.17) were excluded. In one case (in figure 3.17) the clone cannot be used because the reconstruction contained too many clones. The second clone (in figure 3.14) was found in a poorly aligned region of cortex and the distribution was not conclusive. However after applying the criteria to the hollow/septal clones four examples remained. These have been illustrated in figures 3.14 (three cell clone and one two cell clone), 3.12 and 3.18 (two cell clones). Incidently the four clones all spread mediolaterally, further suggestive of their clonal nature. Furthermore the one clone restricted within one barrel/column was excluded from the data. Thus the distribution of clones crossing boundaries to those not was four to zero.

Hence in summary the data pool contained eleven neuronal clones which were classed as crossing a barrel/column boundary. Using more stringent criteria to define clones and barrel/column boundaries, four of these clones were found in cortex that was both conclusively radially aligned and contained a very small number of clones. These four clones were likely to be clonal and appeared to have conclusively spread across a barrel/column boundary.

3.4 Discussion

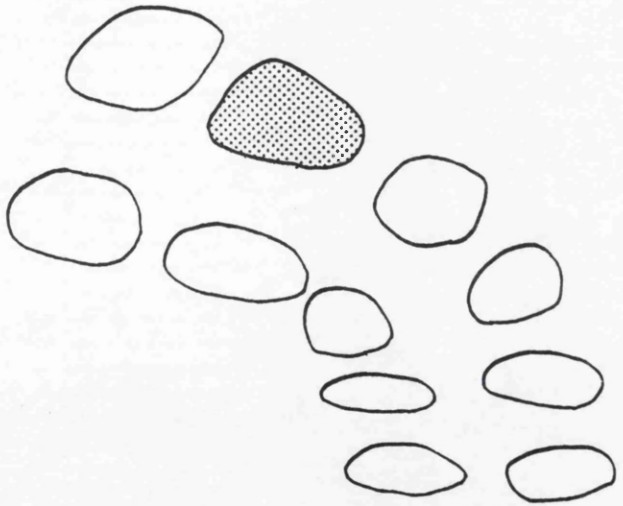
3.4.1 Surgical Procedures Required to Introduce BAG-psi Virus to the Target

Using surgical procedures the BAG-psi virus was successfully introduced into the lateral ventricles of rat embryos *in utero*. The procedure has also been successfully performed with mouse (Luskin *et al.*,1988; Austin

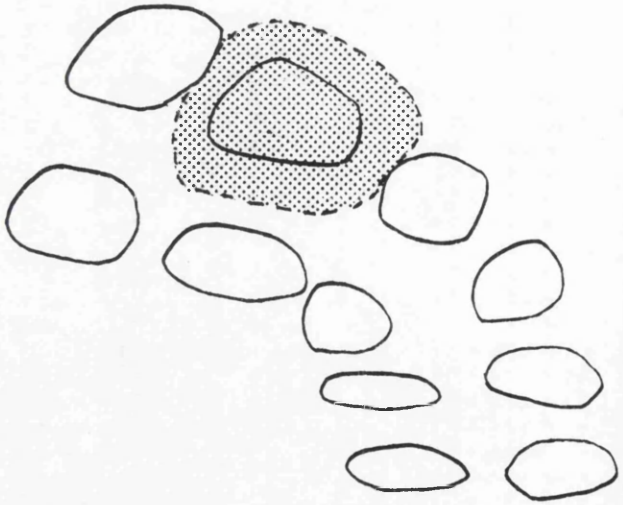
Figure 3.20. The Definitions of Barrel/Column Boundaries Used in this Study.

A. The boundary of a barrel/column used in most of this study was the border between hollow and septum (the outline of the shaded shape in A).

B. However in order to consider errors due to an inaccurate radial alignment or a misinterpretation of the position of a cell to the boundary, a second boundary was devised. This was larger than that in A and was the outline of the shaded shape in B.



A



B

and Cepko,1990) and macaque embryos (H.Kennedy, personal communication) and is thus not restricted to rat embryos.

The majority of the operated female rats recovered from the operation and gave birth to normal young. However the procedure caused some mortality to the operated females and their embryos, but the survival rates were much higher than those reported for the *exo utero* surgical technique used to deliver virus to the target very precisely (Turner *et al.*,1990; Cepko,1989a). The greatest embryonic mortality occurred after injecting into E14 embryos. Presumably the chances of irreversibly damaging tissues were greater at this age. Embryonic mortality could have been caused by a variety of factors from something as severe as gross tissue damage to a simple puncture of the amniotic membrane that did not reseal. The latter was believed to be the cause of the high mortality with the *exo utero* technique (Turner *et al.*,1990).

Stained cells were found in both cerebral cortices, even though the virus was injected unilaterally. No other nervous tissue was examined during this study but by following an identical experimental procedure, clones of cells have been found in the rat hippocampus (E.Grove, personal communication) and midbrain (G.Forster, personal communication). These results, together with those obtained with dye injections into the chicken neural tube (see Chapter Four), imply that once injected the virus is free to diffuse throughout the entire cerebral vesicle.

3.4.2 The Infection of Neocortical Progenitor Cells in Vivo

By following the surgical procedure outlined in 3.2.2 it was possible to successfully infect cortical progenitor cells in rat embryos, in utero. The vast majority, if not all, of these cells were probably located in the VZ. This region contains the vast majority of mitotic cells and surrounds the injection target, the lateral ventricle. However it is possible that some virus may have infected a mitotic SVZ cell or glial cell near the tissue penetration site, but due to the obligate requirement for DNA replication for 'BAG' integration (see 1.9.5) no post-mitotic neurones will have been infected. Since microglial are derived from monocytes (Perry and Gordon,1988) and not concentrated at the VZ it was unlikely microglial precursors were infected.

3.4.3 The X-Gal Staining and Fading of 'BAG' Infected Cells

The nuclear staining reported in 3.3.2.1 was assumed to be a characteristic of neurones. Although no electron microscopical analysis of these cells has been published yet, after intracellular dye injections nuclear staining is found exclusively in cells of neuronal morphology (J.Price, personal communication). Hence the nuclear staining was a reliable feature of neurones. The nuclear staining was suggested by Price and Thurlow (1988) to be a natural phenomenon and not a staining artifact and appeared to be a feature of most, if not all, 'BAG' infected CNS neurones. Certainly 'BAG' infected hippocampal

neurones and cultured cortical neurones *in vitro* also stain in this manner (E.Grove, personal communication). Price and Thurlow (1988) further suggested that the staining was nuclear localized as the bacterial ß-galactosidase protein contained a cryptic, neurone specific, nuclear localization signal. Such localization sequences are amino acid sequences of a protein that target it to a specific intracellular or extracellular location after synthesis (Verner and Schatz,1988; Finley *et al.*,1989; Roberts,1989). This phenomenon appears to be developmentally related as 'BAG' infected neurones in adult animals do not stain in this fashion (J.Price, personal communication). However the nuclear staining could be a histochemical artifact.

Astrocytes were stained very diffusely and this may have been due to inadequate fixation of the ß-galactosidase. However a more elegant explanation, in accord with the findings above, is that the ß-galactosidase protein contains cryptic amino acid sequence(s) that specify secretion from the cell, or does not contain sequence(s) which specify retention. For example the 'KDEL' sequence has been shown to be an amino acid sequence specifying retension in the endoplasmic reticulum (Munro and Pelham,1987). Whatever the nature of the sequence(s) involved in this secretion they must be astrocyte specific. As no diffuse staining was seen in chimeric embryos, where one genotype constitutively expressed *lacZ* (Beddington *et al.*,1988; Kadokawa *et al.*,1990; Suemori *et al.*,1990), the secretion must be tightly developmentally regulated. The two staining patterns obtained with X-Gal were also obtained with another chromogenic ß-galactosidase substrate (see 3.3.2.3). This suggests that the staining patterns described above were not artifacts attributable to the X-Gal histochemical technique. Furthermore similar examples of ectopic localization and secretion have been noted previously (Gould and Subramani,1988; Iturriaga *et al.*,1989).

The indigo precipitate gradually faded such that stained cells disappeared, sometimes over a matter of hours. However the rate of fading was different for stained cells, even when they were found in the same section. Possibly the indigo was soluble in the D.P.X. mountant. The differential rates of fading and the much slower rate of fading in thicker sections could be attributed to differences in local D.P.X. concentration and accessibilty of the D.P.X. to the indigo, respectively.

3.4.5 The Staining of Barrel/Columns by Cytochrome c Oxidase and Succinate Dehydrogenase Histochemistry

By both histochemical methods the barrel/columns were easily identifiable in tangential sections. However the PMBSF in the rat and other animals is relatively difficult to detect by cresyl violet, Nissl or thionin stains (e.g. Welker and Woolsey,1974; Land and Simons,1985; Waite *et al.*,1991). Indeed it has been suggested that because of this barrel/columns may be present in more species than has been previously supposed (Waite *et al.*,1991).

Blood vessels were easy to detect in tangential sections and appeared to be excluded from the barrel/column hollows and only found in the septal region. This finding may not be unique to Sm1. The macaque visual cortex contains cytochrome c oxidase blobs (Horton,1984) from which blood vessels are excluded (see figure 1 in Kuljis and Rakic,1990).

3.4.6 The Phenotype of Clonally Related Cells in Vivo

Although not the prime objective of this research the data generated gave some insight into cell fate in the neocortex. The data is compatible with previously reported studies (Price and Thurlow,1988; Luskin *et al.*,1988). The majority of clones in the grey matter consisted exclusively of neurones. Presumably some of these clones contained horizontal cells in the underlying white matter and resulted from an infection of the multipotential progenitor (discussed in 1.13). Perhaps the horizontal cells are prevented from entering the grey matter by a barrier in the cortical subplate. Indeed some barrier of an unknown nature prevents O2A cells from entering the retina (Miller *et al.*,1989). Clones of grey matter astrocytes were second in abundance. In view of the proposed transformation of radial glia to cortical astrocytes (discussed in 1.3.5.3) it is possible that these clones of astrocytes originated from radial glia. However in a previous study in the mouse (Austin and Cepko,1990), where cell morphology was analyzed shortly after 'BAG' infection, very few infected cells had the morphology characteristic of radial glia. Perhaps then the astrocytic parent was an undifferentiated VZ cell. Hence by E14, E15 and E16 the majority of neuronal and glia progenitors had segregated. Obviously an important question to study is exactly when so that the mechanisms by which this is achieved can be investigated. Perhaps they segregate by interacting with each other; a suitable analogy is the segregation of neuroblasts from the neuroectoderm in the developing insect nervous system (Doe *et al.*,1985).

3.4.7 The Spatial Distribution of Clones of Neurones in Sm1

Using a retroviral vector as a cell lineage marker, clonal spread was examined in relation to morphological/physiological structures termed barrel/columns. If one model of neocortical development, the radial unit hypothesis (see 1.8), applied to the rodent telencephalon then clones would have be distributed in a very specific manner i.e. within one barrel/column.

Two novel observations arise from analysis of the data:

(i) One progenitor in the VZ can contribute neurones to more than one barrel/column, and moreover can contribute cells to both hollows and septa (Table 3.6).

(ii) The deeper cells of a clone tend to be found lateral to their more superficial siblings (figure 3.13).

Thus the radial unit hypothesis cannot apply to Sm1. The VZ is not pre-specified with an areal fate as individual clones transgress a border between barrel/column hollows and septa. Neurones within these areas have very different physiological and anatomical characteristics (Armstrong-James and Fox,1987; Koralek *et al.*,1988). Indeed it has been suggested that hollows and septa should be considered as separate cortical areas (Koralek *et al.*,1988). Moreover two cases were found where individual VZ cells contributed to more than one barrel/column. This finding complements the work of Goldowitz (1988) who showed that more than one VZ progenitor contributed to one barrel/column. The two clones spread across the boundaries following a mediolateral distribution. A similar mediolateral distribution of clones was seen in the chick hindbrain (Fraser *et al.*,1990). However in this case, after a certain point in development, clones did not cross the boundary between rhombomeres in the hindbrain. But at three different infection points in the cerebral VZ no restrictions upon migration were seen. Furthermore all three points are quite late in development and it is improbable that clonal restrictions may have applied later e.g. E18 for example.

3.4.8 Interpreting Clonality in the Developing Neocortex

Using retroviral vectors as lineage markers solves one problem for the developmental biologist. Fortunately the virus does all the hard work by introducing the *lacZ* marker into small and, as in this study, inacessible progenitor cells. However this creates a problem for the developmental biologist as the number and position of infected cells are unknown. Thus individual clones need to be inferred from analysis of the mature tissue. Few problems arise if the labelled progeny cells are distributed very closely together, as occurs in the retina (Turner and Cepko,1987; Turner *et al.*,1990) and optic tectum (Gray *et al.*,1988; Galeileo *et al.*,1990). Clones of cells formed very strict radial arrays. Indeed both statistical (Turner and Cepko,1987) and experimental evidence (Galeileo *et al.*,1990) supported a clonal origin for each radial array.

However radial arrays of 'BAG' labelled cells have not been seen in the developing neocortex, a finding supported by several independent studies (Walsh and Cepko,1988, Luskin *et al.*,1988; Price and Thurlow,1988; Austin and Cepko,1990). Although cells tended to be found in different laminae they were rarely radially arrayed. Instead they formed dispersed clusters which tended to be restricted laterally within 300μm (Price and Thurlow,1988). The dispersed clusters were interpreted as clonal events for several reasons:

(i) First, they were a common finding even in experiments where clones were very rare (e.g. figure 3.18).

(ii) Second, more discrete clusters of neurones were very rarely seen. The only example of a discrete cluster was the two cell clone remaining within a barrel/column hollow (arrowed in figure 3.16).

(iii) Third, if the dispersed clusters were not clonal then each cell would have to constitute a single cell clone. However there are no known reasons why single cell clones would cluster in such a fashion, nor why only single cell clones would be produced.

(iv) Finally the data in figure 3.13 suggests that clones are oriented non-randomly. If the pairs of cells in the figure were not clonally related then the lines should point in all directions like the spokes on a wheel. This was clearly not the case.

There is one aspect however where the clonal definition was inadequate. The 300μm clonal definition was inappropriate for describing clonal spread from E14 or E15. Since more cell divisions should have taken place then clones of cells should be larger. This is indeed the case with when comparing the mean size of clones from E14 (mean=2.0) to E16 (mean=1.4). However the difference should be greater since there is a 48 hr difference between the two infection points. Assuming asymmetric divisions and a cell cycle time of 12 hrs then cells labelled on E14 should have, on average, four more than those labelled at E16. This suggests that only a subset of clones were detected at E14 and clonal spread at this age was even greater than presumed. Furthermore clonal sizes at all ages were probably larger than reported as a number of sections in each series were stained for cytochrome c oxidase. However this was unlikely to alter any of the findings.

3.4.9 Are Blood Vessels an Accurate Judge of the Radial Dimension in Sml?

Blood vessels were used to align the sections radially. An obvious question arising is, are they radially aligned and do they remain so throughout the entire series of sections reconstructed? Bär (1980) showed a striking radial orientation of the cerebral vasculature. The majority of the blood vessels are radially aligned in the superficial layers but some do start to sprout laterally as they approach the deeper layers. However the larger diameter blood vessels, like those used to align sections in this study, remained radially aligned throughout all of the neocortical layers. The technique of radial alignment with blood vessels is well established and has been used in several publications, some of which involved barrel/columns (e.g. Woolsey and Van der Loos,1970; Kuljis and Rakic,1990; Bernardo *et al.*,1990; Koralek *et al.*,1990).

3.4.10 A Model of Clonal Spread in the Developing Neocortex

The data presented above do not support one very specific aspect of the radial unit hypothesis; at least in the rat Sm1 there appears to be a significant lateral spread of clones. Radial glia were proposed to prevent the lateral spread of individual clones by providing a substrate for migration (Rakic,1988a). My data suggest that the VZ is not intrinsically specified as a protocortex. For example a cell in the VZ cannot be specified to producing only neurones that will occupy one barrel/column hollow. Cells derived from one progenitor clearly spread across the boundary between hollows and septa. Moreover two clones spread from one barrel/column hollow to another and could be viewed as spreading across two barrel/column boundaries. Hence it appeared as though neurones were generated in the VZ irrespective of their future fate in the CP. Presumably cortical specification occurs after the proliferative phase of neural development. Hence specification is not intrinsic but probably extrinsic to neocortical neurones.

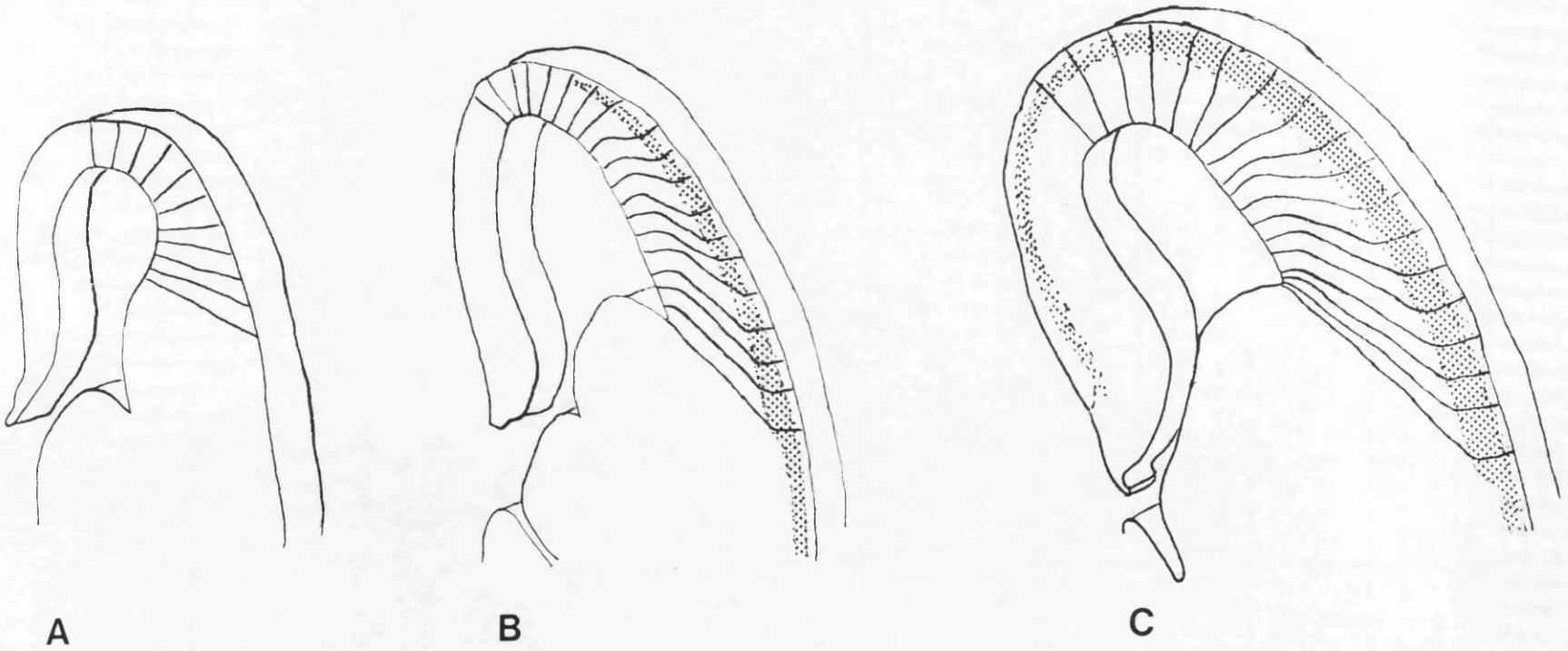
My data do not directly cast doubt on the modular hypothesis of columnar organization (see 1.7). Specifically the data presented above suggest that if ontogenetic columns do exist during development then they are not derived from a single or small groups of VZ cells determined to forming a particular minicolumn or cortical column. A transient precursor of a minicolumn such as an ontogenetic column may still be formed from clonally unrelated cells. However although the modular organization hypothesis was attractive for some time several pieces of circumstantial evidence, including the absence of clonal ontogenetic columns, suggest that it may not be correct (reviewed by Swindale,1990).

The results presented in this study are perhaps surprising to many who supported the radial unit hypothesis (e.g. Leise,1990; Rakic,1988a). However the hypothesis has remained unchallenged and untested for several years. Furthermore the data presented above are compatible with, and can be accounted for by gross anatomical changes that occur naturally during the development of the cerebral cortex.

During normal development there are a series of morphological changes occurring in the forebrain (figure 3.21). Specifically there is a difference in growth of the VZ and CP, with the CP expanding far more laterally than the VZ (Smart and McSherry,1982). As a result the two structures lose their topological correspondence (Smart and McSherry,1982). This is clearly seen with radial glia which in the telencephalon are truely radial at the beginning of neurogenesis but within a few days become skewed and deflect laterally (e.g. Gadisseux *et al.*,1989; Smart and Sturrock,1979). This distortion is most pronounced at the lateral edges of the cortex (figure 3.21). However it has always been assumed that despite this distortion, radial glia maintained an exact 1:1 correspondence between the VZ and CP so that clones were constrained within a cortical column as suggested by the radial unit hypothesis (figure 3.22A). However the data presented above suggest that

Figure 3.21. The Changing Relationship Between the VZ and CP During Rodent Cortical Development.

All of the sections are coronal and reproduced from the work of Smart and McSherry (1982). The CP is shown by stipple shading. Radial glia are shown as cells spanning the entire length of the cerebral wall. A is a coronal section from an E12, B from an E14 and C from an E15 mouse cortex. Note that the radial glia are progressively distorted laterally during normal development to accommodate the lateral growth of the CP.


A
B
C

clones are not restricted to a cortical column and tend to spread mediolaterally. In view of the fact that there appears to be no lateral displacement of infected VZ cells during development (Austin and Cepko,1990) there are two ways in which this distribution could arise (figures 3.22B and 3.22C).

Model A (Figure 3.22A). This is the predicted distribution of clones expected if the radial unit hypothesis was true. However this is not how clones to spread in relation to barrel/columns.

Model B (Figure 3.22B). This model suggests that a mediolateral distribution of clones occurs during histogenesis. As outlined above the CP is distorted laterally during development. A 'BAG' labelled cell in the CP (the larger triangle in figure 3.22B) will be moved passively laterally relative to its progenitor in the VZ. This could occur after migration and detachment from the radial glial cell or upon entry in the CP. Hence the direct radial correspondence between the progenitor and its sibling is lost. When the next cell is produced by the same VZ progenitor (the larger triangle in figure 3.22B) it will migrate to the CP, but will be found in a position more medial to its sibling. Furthermore because of the 'inside-out' neurogenic gradient it will occupy a more superficial position relative to its sibling already in the CP. This mode of dispersion can account for the clonal distribution seen in the vector diagrams (figure 3.13) with the deeper neurones situated laterally to the more superficial ones.

Model C (Figure 3.22C). This model also suggests that a mediolateral displacement occurs, but the method by which it is achieved is totally different from that described in Model B. Model C suggests that radial glia play little, if any role, in neuronal migration. Although unlikely it is possible. For example Model C suggests that neurones migrate strictly radial from the VZ, perhaps by moving away from a chemorepulsive signal produced by the VZ. Furthermore perhaps the MZ neurones act as a barrier and instruct CP neurones to cease migration. This later fact can account for the 'inside-out' pattern of migration.

Model C also can account for mediolateral spread. The model suggests that the first cell generated is moved laterally by the same passive forces described above. However, model C differs from model B in that the second neurone generated simply migrates radially away from the VZ to a superficial position, irrespective of the fact that the radial glia and the CP have been distorted laterally.

For several reasons model B is probably correct. As described in 1.3.5 there is strong *in vivo* anatomical and *in vitro* culture evidence suggesting that radial glia do guide neurones to the CP. Radial glia are present only during the phase of neuronal migration; they are closely opposed to migrating neurones; and they have a morphology suitable for

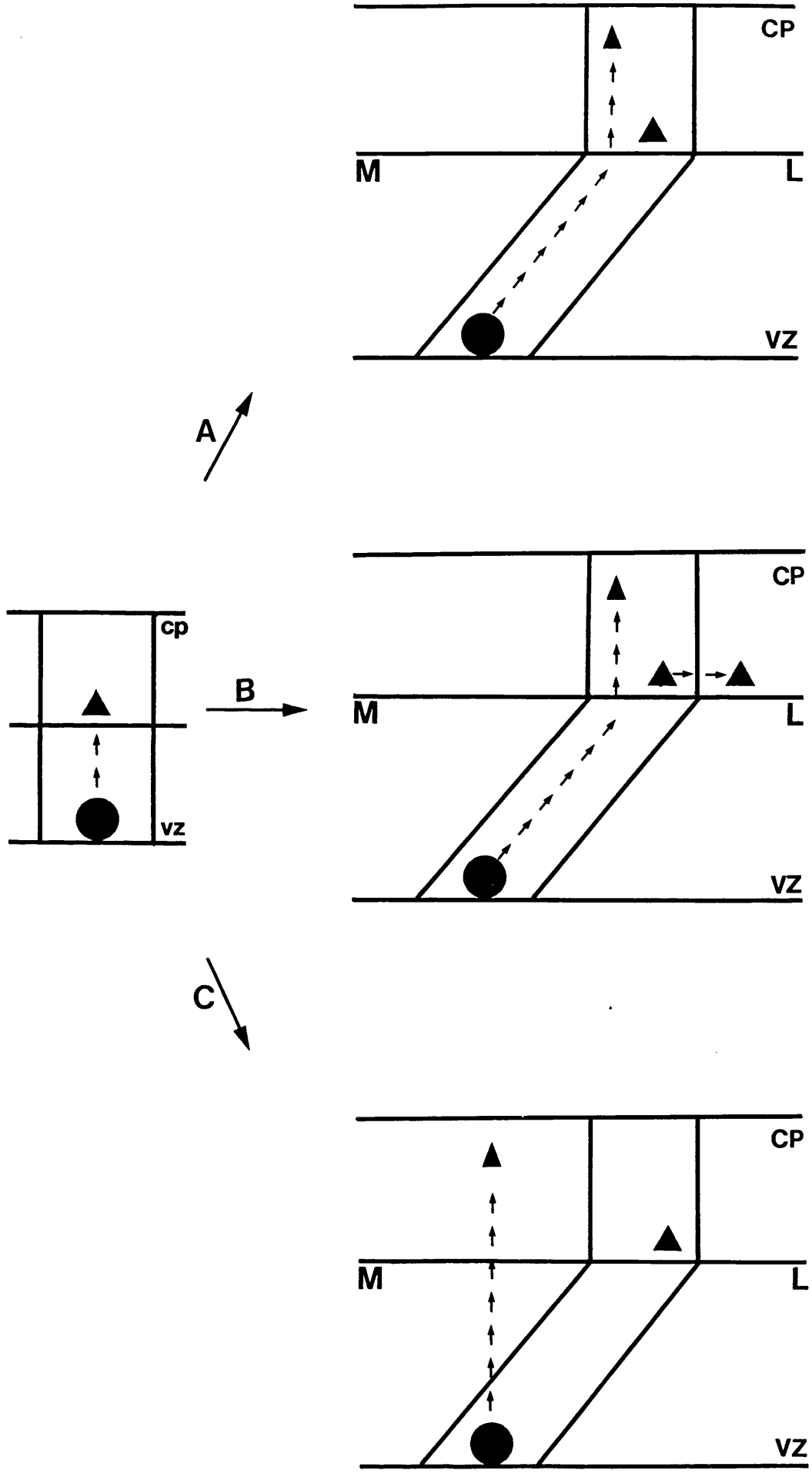
Figure 3.22. Three Models To Account for the Mediolateral Distribution of Neuronal Clones During Neocortical Development.

In all three cases a 'BAG' labelled VZ cell (represented by a black circle) is shown in relation to its progeny (illustrated by triangles) in the CP in relation to the mediolateral axis. The first cell generated by the labelled VZ cell is represented by a large triangle whereas the second is represented by a smaller triangle. Radial glia are shown initally perpendicular to the two limiting surfaces. Subsequently however they are deflected laterally. For simplicity I have only shown two siblings in the CP (the two triangles), but the models could also apply to larger clones.

A. The expected distribution of clones according to the radial unit hypothesis.

B. The expected distribution of clones if the constituent cells were passively separated from one another (see text).

C. The expected distribution of clones if radial glia were not a substrate for neuronal migration (see text).


CP
M
L
VZ
A
cp
B
vz
CP
M
L
VZ
C
CP
M
L
VZ

their implied function. Furthermore transplantation experiments (McConnell,1988b) suggest that radial glia could perhaps signal prospective lamina position to a migrating neurone as they did not migrate to the end of the glial fibre but actively stopped some distance from it. Moreover several independent studies suggest that clones of migrating neurones initially start migrating along one radial glia fibre or fascicle (Balaban *et al.*,1988; Austin and Cepko,1990). These observations are all consistent with model B.

Model B suggests that all clones irrespective of their size could be distributed mediolaterally. As reported in 3.3.7 this appeared to be the case for two cell clones and the relatively smaller number of larger clones. Certainly some of the larger clones were not positioned in a strict mediolateral line as Model B suggests. For example the five cell clone in figure 3.12 was circular in shape. Perhaps a mediolateral displacement was followed most precisely by a slowly dividing VZ. In comparison the progeny of a more rapidly dividing VZ cell may be inserted into the CP before any noticeable mediolateral spread occurs. As noted in 1.3.1 there is evidence for two different cell cycle times in the VZ. Furthermore as noted in 1.3.2 the 'inside-out' gradient of neurogenesis in rodents is not as precise as that in primates. Hence VZ cells dividing faster and generating larger clones may not be dispersed in a precise 'inside-out' fashion.

None of the published studies addressing clonal migration in the developing neocortex (Luskin *et al.*,1988; Price and Thurlow,1988) have reported a mediolateral distribution of clones. One possible reason could be that none of these published studies, unlike this one, analyzed the neocortex in the tangential plane. Cells found in different cortical layers appeared in different sections and thus data relating to depth was easy to quantitate in a vector diagram.

3.4.11 The Tabula Rasa Hypothesis of Neocortical Development

Apparently the radial unit hypothesis cannot account for the development of the rodent neocortex. In view of the results presented above, and the model proposed above to account for mediolateral spread, the alternative proposition of Creutzfeld (1977) appears to be a more attractive proposition. This hypothesis has gained in popularity in recent years and is supported by a wide variety of data (reviewed by O'Leary,1989) and can account for some aspects of phylogeny (Killackey,1990). The model, termed the '*tabula rasa* hypothesis' (Kuljis and Rakic,1990), suggests that the CP is a *tabula rasa*, devoid of any intrinsic genetic program. The hypothesis is attractive as it simplifies considerably the hugh problem of the genetic determination of a structure as complex as the neocortex. According to the *tabula rasa* hypothesis the different cortical areas are not specified at the time of neurogenesis but by subsequent extrinsic, epigenetic interactions of a molecular and/or electrical nature. The exact nature of these interactions and between who they occur remains obscure, but they might be supposed to involve

thalamocortical afferents. The barrel/columns, according to the *tabula rasa* hypothesis, would be specified via signals extrinsic to the cortex, originating in the periphery. Thus the specification of the columnar structure of the neocortex may result from only one specified event during embryogensis - the guidance of afferents to their appropriate location in the neocortex.

The *tabula rasa* hypothesis is supported by several anatomical observations in rodent Sm1. Barrel/columns are first detected histologically by Nissl staining early in postnatal life (Rice and Van der Loos,1977). This is several days after neurogenesis has ceased and suggests that neurogenesis and barrel/column formation are not directly coupled. However barrel/column formation is closely preceded by the arrival and sorting of thalamocortical afferents in the CP (Erzurumlu and Jhaveri,1990). Furthermore by removing a vibrissa it is possible to prevent the formation of the corresponding barrel/column (see 1.6.2). These data suggest that barrel/column formation is dependent upon input from the periphery.

Perhaps the strongest evidence supporting the *tabula rasa* hypothesis comes from strains of mutant mice which differ in the number and configuration of their mystacial vibrissae. Some strains have supranumerary vibrissae (Van der Loos *et al.*,1984), innervated by trigeminal ganglion axons (Welker and Van der Loos,1986), with a physiologically active input into Sm1 (Welker *et al.*,1985). Moreover these animals develop a barrel/column homologue at the appropriate somatotopic position in Sm1 (Van der Loos *et al.*,1984). However, not all supernumerary vibrissae are represented as barrel/columns in Sm1. A suprathreshold number of nerve fibres innervating the vibrissae follicles is needed for the appropriate barrel/column homologue to form in Sm1 (Welker and Van der Loos,1986). Welker and Van der Loos (1986) suggested that this value is near 40 as follicles innervated by less than 40 axons are not represented by barrel/columns. Moreover the density at which vibrissae are innervated is proportionally related to the number of neurones within a cortical barrel/column; the largest vibrissae are innervated by the most sensory neurones and are represented by the largest barrel/columns (Lee and Woolsey,1975). These observations suggest that a barrel/column is only formed if a suprathreshold of electrical/molecular factor(s) are supplied to the cortex. Furthermore the areal extent of a barrel/column, once the threshold is exceeded, is proportional to the amount of factor(s) supplied.

These conclusions are elegantly supported by the work of Andrés (1990), who transplanted a vibrissal follicle into the muzzles of newborn mice and found that a characteristic barrel/column was induced in Sm1.

Indeed in addition to the evidence involving vibrissae and barrel/columns, the periphery is known to influence the CNS in a variety of experimental systems. Typically enriched peripheries result in larger

domains in the CNS; frogs with supernumerary limbs (Hamburger,1939; quoted by Welker and Van der Loos,1986) and chick embryos with supernumerary legs (Hollyday and Hamburger,1976). However perhaps the most striking example of an enriched periphery influencing the CNS was demonstrated by Constantine-Paton and Law (1978). By implanting a third eye into embryonic frogs they induced eye specific columns within the optic tectum that were alternatively innervated by the host and supernumerary afferents (Constantine-Paton and Law,1978). These columns had an anatomy much like the mammalian ocular dominance columns. However normally the eye specific columns are not seen in the frog optic tectum. The segregation of afferents within these eye specific columns appears to depend on electrical activity as either cutting the optic nerve or tetrodotoxin treatment desegregates the stripes (Reh and Constantine-Paton,1985). Indeed ocular dominance column formation in cats is dependent upon electrical activity (Stryker and Harris,1986). The afferent segregation in three eyed frogs is dependent on the activity of the NMDA receptor (Cline *et al.*,1987). Again this feature has also been described for the development of the mammalian ocular dominance columns (Kleinschmidt *et al.*,1990).

Hence collectively these observations suggest that electrical factors are of prime importance in the establishment of columnar structure. However, because of technical difficulties, it has not been possible to examine if barrel/column formation is dependent upon either electrical activity and/or the NMDA receptor *per se* (N.G.F.Cooper, personal communication). It is also feasible that soluble molecules, originating from the afferents, are involved in the specification although none have been characterized to date. However there are many examples of a similar phenomenon in the PNS. For example, soluble molecules can influence neuronal transmitter phenotype (Patterson and Chun,1977). Moreover soluble molecules derived from neurones can induce several effects in a postsynaptic cell e.g. clustering of membrane proteins (Wallace,1989) and induction of gene expression (Merlie and Sanes,1985).

The *tabula rasa* hypothesis is consistent with several well known features and perturbations of neural development. One well known phenomenon in neural development, discussed in 1.3.3, is the loss of axonal projections and selective stabilization of others. Collateral exuberance is of wide occurrence (has been found in all areas and species examined) and has been particularly well studied with callosally projecting neurones (reviewed by Innocenti,1988). Although the exuberance is not random, the phenomenon could be interpreted, in view of my findings, as an error correcting mechanism. For example, a neurone not intrinsically determined at the time of birth to one areal fate may make several projections. However, as a result of (postnatal) cellular interactions, axon retraction and stabilization result in the assembly of the correct adult pattern.

As mentioned in 1.3.3 collateral exuberance is a well known phenomenon in neocortical development. Pyramidal neurones in the neonate

often project to several targets, but subsequently some projections are stabilized and others retracted. For example both rostral (R) and occipital (O) cortex in the neonate project to the pyramidal tract. However in the adult only R cortex maintains this projection. Recently O'Leary and Stanfield (1989) examined the collateral exuberance within heterotopic cortical transplants between rostral and occipital cortex i.e. R→O and O→R. They found that in both cases the transplanted neurones initially made projections to the pyramidal tract. However subsequently certain projections were retained but the projection stabilized depended upon the position of the transplant within the neocortex. For example in the R→O transplants, rostral cortex maintained projections characteristic of occipital cortex. Hence the neurones within the transplants retained the projection typical of their new position and not origin. Moreover these heterotopic transplants have been performed between Sm1 and visual cortex (Schlagger and O'Leary,1988). Visual cortex transplanted into the heterotopically into Sm1 develops 'barrel-like' morphologies. These experiments suggest that newborn cortex is not regionally specified at birth and is in accord with the *tabula rasa* hypothesis. Indeed the transplantation experiments are analogous to those performed in the PNS; as outlined in 1.3.5.4 the fate adopted by the neural crest depends upon the position of neural crest cells within the embryo and not upon lineage relationships.

A similar experiment was that in which Seo and Ito (1987) placed small lesions in Sm1 during the critical period and found that neurones in the presumptive barrelfield were able to reorganize into the correct somatotopic pattern of barrel/columns. However it was not clear how this change was accommodated in Sm1. For example Sm1 could have shrunk in size or expanded into other cortical areas to avoid the ablation. This question could not be precisely addressed in the Sm1 due to the small size of individual barrel/columns. However fetal binocular enucleation results in a considerable shift in the border between area 17 and 18 (Dehay *et al.*,1988; Rakic,1988a). A region of cortex, normally contained within area 17, undertook a number of area 18 characteristics. These observations suggest that not only columns but also cortical areas are specified by afferent input.

The tabula rasa hypothesis is also supported by observations in mice with the reeler mutation (Caviness *et al.*,1988). Reeler mice have a grossly abnormal sequence of neocortical histogenesis, but despite this, they possess a barrelfield at the appropriate lamina position. The barrelfield shows the correct somatotopic relationships of barrel/columns (Welt and Steindler,1977).

3.4.11.1 The Tabula Rasa Hypothesis and Glial Cordones

Whatever the nature of the factor(s) responsible for the formation of barrel/columns, the interactions between the afferents and cortical neurones result in the formation of glial cordones. As discussed in 1.6.2, cordones are transient structures formed from radial glia that

demarcate, in late embryonic/early postnatal life, the prospective barrel/column boundaries. The glial cordones are believed to form a scaffold for the crystallization of the afferents (Steindler *et al.*,1989), and appear to be dependent upon afferent input for formation (Cooper and Steindler,1989; Waters *et al.*,1990). In addition to outlining the barrel/columns, glial cordones also transiently outline a variety of subcortical structures, and thus may be a universal phenomenon in the developing CNS (Steindler *et al.*,1990).

A similar glial boundary is also found transiently outlining developing olfactory glomeruli in the moth *Manduca sexta*. The glomeruli are loci in which synaptic connections are concentrated and are thought to operate as functional units in the processing of olfactory information, much like the cortical columns of mammals. Furthermore, like the barrel/columns, removal of afferent input disrupts the formation of the glial boundary (Oland and Tolbert,1987). Moreover because of the simplicity and accessibility of the moth system it is possible to totally ablate the glia by X-ray elimination; this procedure disrupts the formation of the glomeruli even though the afferents remain intact (Oland *et al.*,1988).

Collectively these experiments suggest that the glial boundaries are induced by afferents and are responsible for later stages of glomeruli and barrel/column development, possibly refinement of the afferent projections. Evidence supporting this comes from the fact that glia in the barrel/column cordones express J1/tenasin (Steindler *et al.*,1989), a molecule known to influence neurite outgrowth (Chiquet,1989). Indeed the segregation of afferents from an initial diffuse pattern (in both the radial and lateral dimensions) is preceeded by J1/tenasin expression (Erzurumlu and Jhaveri,1990). Perhaps the cordones are the source of the 'stop signal' proposed to encourage afferent arborization within layer IV (Blakemore and Molnár,1990). Thus it appears that, at least in mammals, radial glia not only mediate neuronal migration but are also necessary for later events leading to afferent segregation.

3.4.11.2 When Do Afferents and Target Neurones Interact in the Developing Neocortex?

If the *tabula rasa* hypothesis is a credible developmental mechanism then afferents responsible for the formation of cortical columns must arrive prior to column formation. As outlined in 1.6.2 this is certainly the case with barrel/columns and ocular dominance columns.

However several pieces of evidence that suggest that afferents and their future target neurones interact before they meet in the CP. Studies in three different species have demonstrated that in the visual cortex thalamocortical afferents arrive in the IZ at the time their targets, presumptive layer IV neurones, are migrating from the VZ (Lund and Mustari,1977; Rakic,1977; Shatz and Luskin,1986). Thus afferents and their target cells could interact while in the IZ. Certainly synapses are formed in the IZ during this waiting period (Chun and Shatz,1988),

so at least interactions of an electrical nature can occur at this time. However any interactions must be transitory as the afferents subsequently wait for several days in the IZ region (Lund and Mustari,1977; Luskin and Shatz,1986; Rakic,1977) while the target cells continue unabated on their migration. The interactions occurring in the IZ have been proposed to alter the migration patterns of a target cell (Austin and Cepko,1990). However removal of afferents *in utero* from the developing visual cortex does not alter several aspects of visual cortex architecture (Rakic,1988a), suggesting that any interactions in the IZ are not essential for normal cortical histogenesis.

It is not clear if interactions of this type could occur in Sm1 as the timing of afferent arrival in relation to the generation of layer IV neurones has not been studied in this region. Furthermore the issue is confounded by recent studies using carbocyanine dyes which suggest that afferents arrive earlier than previously reported (discussed in 1.6.2).

It is also possible that afferents and target cells could interact even though physically separated. At least some of the VBN afferents wait in the IZ prior to invasion of the CP (Wise and Jones,1978) and could form transient synapses with the SP neurones. Many SP cells project to the CP (Friauf *et al.*,1990) and could relay electrical/molecular signals from the thalamocortical afferents to prospective layer IV. Hence any interaction between thalamocortical afferents and their future targets may be indirect, via SP neurones.

3.4.12 The Radial Unit Hypothesis and the Primate Neocortex

The radial unit hypothesis was originally proposed after an anatomical study of neuronal migration in the macaque visual cortex. My data suggests that it cannot account for rodent Sm1 development where development is most convincingly accounted for by the *tabula rasa* hypothesis.

However there are many features of neocortical development that are not in line with the *tabula rasa* hypothesis. For example, in the rodent there is evidence for separate astrocyte and neuronal precursors (Price and Thurlow,1988; Luskin *et al.*,1988; Temple,1989) and clustering of apical dendrites in embryonic neurones (Hirst *et al.*,1991). Both of these events occur before afferent invasion of the CP. Hence some developmental phenomena (albeit not the development of physiological columnar structures) occur normally in the absence of afferent input. Furthermore there is some experimental evidence that cannot be accounted for by the *tabula rasa* hypothesis. Possibly in the macaque, where the radial unit hypothesis was first suggested, there is a specification at the VZ, as suggested in the radial unit hypothesis. For example the macaque visual cortex contains structures termed cytochrome c oxidase blobs. These resemble rodent barrel/columns in a number of respects (Horton,1984) and just like the barrel/columns, the blobs are formed *in utero* after afferent penetration of the CP (Horton,1984; Rakic,1977; P.Rakic, personal communication). However after removing afferent input

from an embryo *in utero*, the blobs are formed in area 17 approximately normally. The blobs have the same size, the same density and lamina position and non-random distribution of NPY containing neurones, as is found in control embryos (Kuljis and Rakic,1990; Kennedy *et al.*,1990). This suggests that the blobs are specified intrinsically at the VZ. These results are the opposite to those obtained by ablating vibrissae in the rodent (see 1.6.2 and Parnavelas *et al.*,1990).

Neocortical histogenesis in the macaque takes 60 days to complete (Rakic,1974). Hence although both the macaque and rat cortices contain basically the same number of neurones in a radial traverse (Rockel *et al.*,1980), they differ in the time taken to generate them. This protraction may be a reflection of a longer cell cycle time at the VZ (although unfortunately no cell cycle time estimates are yet available for the macaque VZ; I.H.M.Smart, personal communication). A long cell-cycle time at the VZ may be necessary for cellular interactions to occur which result in specification. Furthermore the 'inside-out' gradient of generation of the cortical layers (see 1.3.2) is very precise in the macaque (Rakic,1974; Rakic,1977) and perhaps this is a consequence of the protracted generation time. This lack of scatter in the horizontal plane tentatively suggests that there is less lateral clonal spread within the vertical plane.

However conclusive proof of specification of columnar structures in the macaque VZ awaits a study of cell lineage. This may not be too far in the future as high titre retroviral vectors capable of infecting primate cells have been constructed (Wilson *et al.*,1989).

Chapter Four : Studies on Forebrain Development in the Avian Embryo

4.1 Introduction

An attempt was made to infect precursor cells in the early chicken neural tube with a retroviral vector in order to examine clonal spread and phenotype. As reported in Chapter Two, a retrovirus containing the 'BAG' genome and capable of infecting avian cells was generated and successfully concentrated. This was successfully injected into the neural tube of early chicken embryos. Previous studies using a retroviral vector in the chick revealed that there were strong impositions to lateral migration in different parts of the CNS during development (Gray *et al.*,1988; Galileo *et al.*,1990; Leber *et al.*,1990). Unfortunately however none of these studies addressed clonal migration in the forebrain. Hence specifically an examination of clonal spread in the developing forebrain was performed.

Unfortunately after many attempts, even with different retroviral vectors, no X-Gal stained cells were detected in the injected embryos after histological staining. This was due to either insufficient titre and/or inefficient *lacZ* expression within the cell.

4.2 Materials and Methods

4.2.1 Procurement of Chicken Embryos

Fertilized Rhode Island Red chicken eggs were obtained from J.K.Needle and Co.,(Herts,UK). If they were not incubated immediately on the day of arrival they were stored at 10°C. Eggs were incubated, lying on their sides in metal trays, at 37°C in an Astell Hearson incubator. The start of incubation was termed day E0 and the next day as E1.

The eggs were windowed immediately prior to the experiment. A syringe was used to remove 2ml of egg white from the blunt end of the egg. A piece of Sellotape was placed over the top of the egg to prevent fragments of shell dropping onto the embryo. A small circular window was then made with a pair of curved scissors. After surgery (see below) a few drops of Hanks MEM F12 supplemented with 50iu/ml penicillin and 50μg/ml amphotericin-B (Fungizone) were dropped onto the exposed embryo to prevent infection. The window was then resealed with Sellotape.

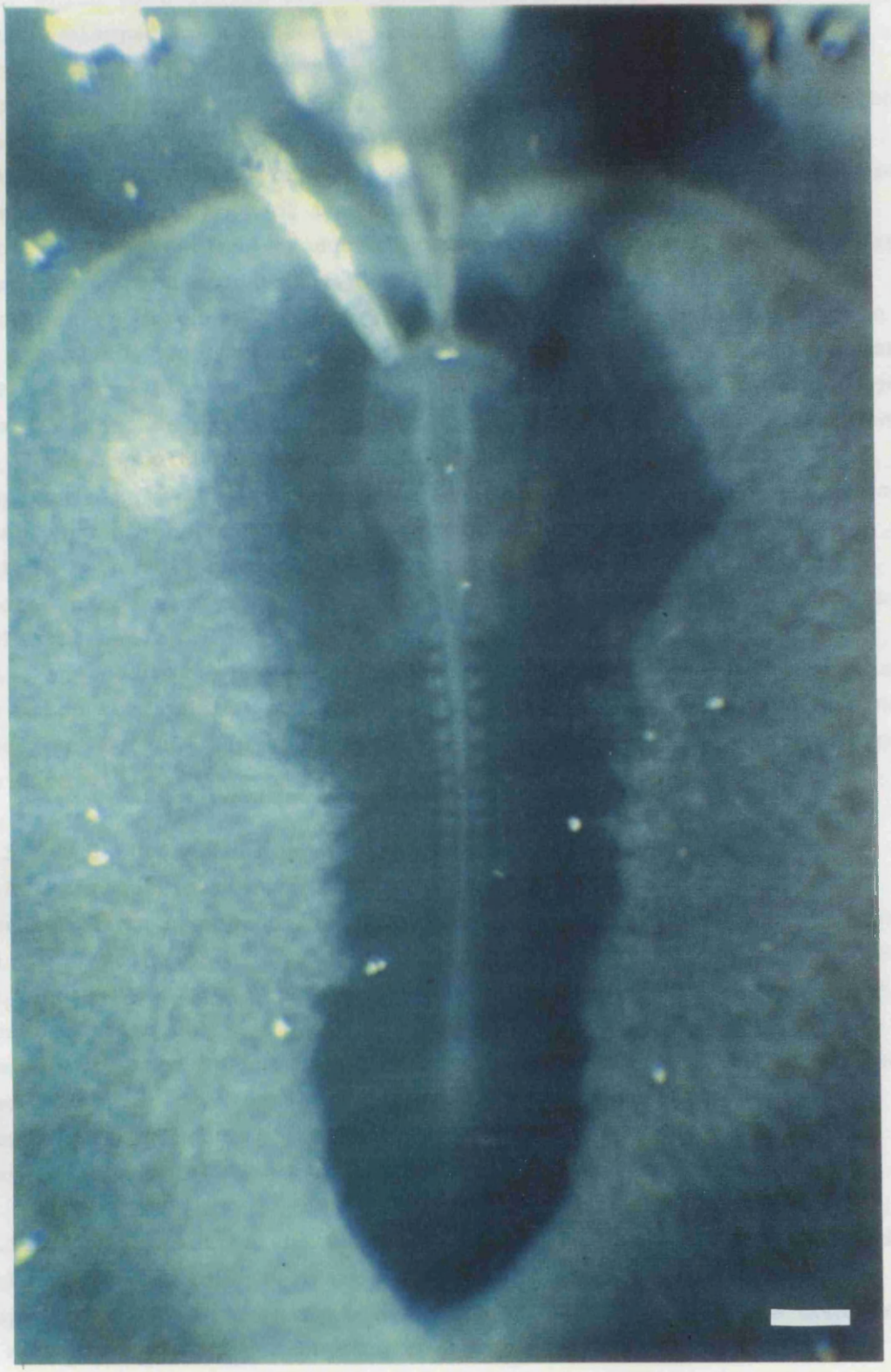
4.2.2 Injection of 'BAG' Viruses into the Embryonic Chick Neural Tube

Chicken embryos after either 2 or 3 days of incubation (Hamburger Hamilton (HH) stages 11/12 or 18/19) were used in these experiments. After windowing they were visualized by injecting a small volume (between 50 and 100μl) of 10% (v/v) solution of black Pelikan Fount India Ink (Pelikan AG,FRG) in DMEM underneath the blastoderm (figure 4.1). The penetration site was just outside of the margin of the *area opaca*. A 1ml syringe fitted with a slightly curved 30G needle ('Microlance', Becton Dickinson,UK) was used to introduce the ink. All embryos were staged, both before viral injection and histochemical processing, according to the criteria of Hamburger and Hamilton (1951).

Needles for injection were prepared by pulling 1mm diameter glass capillaries (Supracaps,FRG) on a heated microelectrode pulling machine

Figure 4.1. A Chicken Embryo Prior to Injection of Virus.

Ink has been injected underneath the blastoderm and a needle, containing the virus suspension, is resting above the target for viral injection, the forebrain. Scale bar 500μm.



(SRI Ltd.,UK). The temperature and traction settings producing the point most suited for these experiments were determined experimentally. The needle was connected to a flexible tube linked to a Hamilton syringe, all of which were filled with liquid paraffin (Rusco Pharmaceuticals,UK) and then clamped to a micromanipulator apparatus (Micro Techniques (Oxford) Ltd.,UK). The embryo was illuminated using fibre optic illumination (Schott KL 1500-T; Schott Glaswerke,FRG) and viewed with a dissecting microscope. The needle was filled with virus suspension (containing 10μg/ml polybrene) by withdrawing on the Hamilton syringe. The virus used for most of the chicken injections was concentrated amphotropic BAG-C virus produced by the SL88 cell line. The virus was thawed from -70°C storage immediately prior to use. Unlike the rat injections reported in Chapter Three, a much smaller diameter needle was used for the injections. Hence in order to remove any particulate matter which may have blocked the needle, the virus suspension was microfuged for a few seconds prior to loading. An identical protocol was followed when injecting either BAG-P or vLZ10.

Approximately 10μl of virus suspension was pressure injected into the dorsal part of the presumptive forebrain. Care was taken to avoid passing the needle through the forebrain into the yolk below. The injected suspension of BAG-C virions contained 1000 virions, as assayed on NIH 3T3 cells.

All injected eggs were returned to the incubator and left for three days prior to fixation and staining. All embryos received only a single injection. By this time they had reached HH stages 28/30. Any embryos that were visibly deformed were discarded at this point.

4.2.3 The Histochemical Detection of 'BAG' Labelled Cells

4.2.3.1 Tissue Fixation

After reaching an appropriate stage the embryos were fixed by placing in a 2% (w/v) solution of paraformaldehyde in 0.1M PIPES containing 2mM $MgCl_2$ and 1.25mM EGTA, pH 6.9. Usually only the head was removed and processed, although the brain was not dissected from the head. The tissues were kept in the paraformaldehyde solution at 4°C for two to three days. The paraformaldehyde solution was made fresh every time prior to use by heating (not boiling) and stirring polymerized paraformaldehyde (BDH,UK) in the PIPES solution, in a fume hood.

4.2.3.2 Cryoprotection and Embedding of Tissue for Cryostat Sectioning

Chicken heads or embryos intended to be cryostat sectioned were cryoprotected by an overnight immersion in 30% (w/v) sucrose containing 2mM $MgCl_2$, at 4°C. The following morning, when the tissues had sunk they were embedded and frozen in O.C.T., exactly as described in Chapter Three. However the heads were embedded such that the forebrain was sectioned coronally. The embryo number was inscribed on the bottom of this holder prior to freezing.

4.2.3.3 Cryostat Sectioning of Injected Forebrain

The forebrain was sectioned with a cryostat described in Chapter Two, initially at 25μm. The sections were thaw mounted on subbed slides, prepared as in Chapter Three. In some experiments, sections of 100-200μm were taken. However the detergent solution used in X-Gal histochemistry caused some of these sections to float off the slide so this practice was discontinued.

4.2.3.4 The Histochemical Detection of 'BAG' Infected Cells Using X-Gal

Sections of the chick forebrain were stained with X-Gal exactly as reported in Chapter Three. All sections were stained in the presence of a positive control slide, as outlined in 3.2.3.7.

4.2.3.5 Whole Mount Staining of Injected Chicken Embryos

Whole mount staining of chick embryos was performed essentially as previously described (Allen *et al.*,1988). After removal of all extraembryonic membranes the embryos were fixed for 30 min in a solution of 1% (w/v) paraformaldehyde, 0.2% (v/v) glutaraldehyde and 0.02% (v/v) NP40 in PBS pH 7.4, at room temperature. They were then washed well for 3x15 min in a large excess of PBS containing 2mM $MgCl_2$, and then left overnight in the ferrocyanide and ferricyanide solution (see 3.2.3.5) containing 1mg/ml X-Gal, at 30°C. A positive control slide (3.2.3.7) was taken through these steps with the embryos. The following morning the embryos were examined under a dissecting microscope for clusters of X-Gal stained cells in the neural tube.

This whole mount method of histochemical ß-galactosidase staining has been used successfully by several groups (Kothary *et al.*,1988; Gossler *et al.*,1989; Bonnerot *et al.*,1990) and probably is just as sensitive as staining sections.

4.3 Results

The vast majority (approximately 90%) of chick embryos survived the windowing, ink localization. One common fault which was reponsible for some mortality was an inadequate sealing of the Sellotape window upon returning eggs to the incubator. Of those surviving the injection approximately 80% recovered and developed normally.

Trial injections using only 0.05% (v/v) Trypan Blue (Sigma, from 0.4% stock) were performed to see how precise the injections were and how far the virus spread within the neural tube. This dye was used in a similar manner previously in rat retina injections (Price *et al.*,1987). However 0.05% or even 0.005% solutions of Trypan Blue were found to be toxic to the embryos used in this study. Substitution with Pelikan ink showed that the injected solution was not restricted to the forebrain and slowly diffused throughout the neural tube.

The neural tube of control embryos did not stain with X-Gal when tissue was stained either as sections or as whole mounts. The retinal pigment epithelium sometimes stained however.

Infected tissues were processed as either sections or as whole mounts. However staining was never detected in the neural tube of injected embryos regardless of whether they were injected with either BAG-C, BAG-P or vLZ10 viruses at HH stages 11/12 or 18/19. In some experiments as much as 20μl of BAG-C virus suspension (containing 2000 virions) was introduced into the neural tube. However X-Gal staining was never detected upon histological examination.

4.4 Discussion

The 'BAG' retroviral genome was successfully packaged into an amphotropic virion, by two different packaging lines. This virus was then successfully concentrated and injected within the neural tube of early chicken embryos. However no infected cells could be detected histologically upon subsequent histological examination of injected tissue. Certainly due to a ten hour cell cycle time in the chick VZ (Fujita,1964), clones should have been quite large and easy to detect in whole mount, regardless or whether they remained spatially coherent or not.

There were several reasons why the attempt at infecting avian VZ cells failed, and they may not have been independent. Perhaps the simplest was that no integration of the retroviral vector occurred. This could have been caused by an inadequate titre of virus i.e. after injection the virus was simply diluted too much by diffusion. Indeed the titre of virus used in these injections was much lower than used for the rat injections in Chapter Three, although this could be compensated for by injection of a much larger volume of virus. The problem could have been further compounded by a low affinity of the amphotropic *env* protein for the chicken cellular receptor(s). Indeed, a recent study suggested that both the PA317 and CRIP packaging lines produce viruses which can infect avian cells, but at a low efficiency (Delouis *et al.*,1990). Leakage from the neural tube may have been a problem. Certainly at the earlier stages the injected virus suspension clearly leaked out of the anterior neuropore. In comparison, the neuropore in rodents had been sealed for several days prior neocortical histogenesis (Jacobson and Tam,1982).

The second and third reasons assume that titre was not limiting and that integration did occur but expression of ß-galactosidase did not because of limitations associated with the MoMLV enhancer. First, the MoMLV enhancer may not be sufficiently active in avian cells. This was probably not the case as I and others (Delouis *et al.*,1990) were able to infect primary cultures of chicken fibroblasts with a MoMLV based vector.

The second and perhaps the more feasible and interesting explanation relates to the expression of the ß-galactosidase. As outlined in 1.9.6 it is well established that the MoMLV enhancer is inactive in early rodent embryos. The LTR is also inactive in the pregastrulating chicken embryo (HH stages 10-11) (Mitrani *et al.*,1987). However this study did

not address when, or in which tissue(s), the block to transcription was removed. It is conceivable that at the stages injected the vast majority of cells in the neural tube were not permissive to viral expression. Indeed attempts at infecting preimplantation mouse embryos with BAG-psi virus were uniformly unsuccessful (Savatier *et al.*,1990; J.Price, personal communication). However chick embryos have been successfully infected between stages 11 and 18 with retroviral vectors, including vLZ10 (Gray *et al.*,1988; Galileo *et al.*,1990; Leber *et al.*,1990). However the reported titre of vLZ10 used in these studies was higher than achieved here.

Chapter Five : Future Studies

5.1 The Role of Cell-Cell Interactions in Neocortical Development

The data presented in Chapter Three suggest that cell lineage does not play a significant role in the topological assembly of the rodent neocortex. Presumably the characteristic columnar organization of the neocortex arises from cell-cell interactions. Thus although it appears that the prospective lamina position of a neurone is specified shortly after its birth (McConnell,1988b; McConnell,1989b), the columnar organization is determined much later in development.

My findings, although unexpected, are not without precedent. The role of epigenetic interactions in neural histogenesis has been most successfully studied in the *Drosophila* ommatidium (reviewed by Tomlinson,1988). Indeed this system shows a number of similarities with neocortical histogenesis and physiological organization. First, like in the VZ, the neurones are generated deep and migrate superficially before differentiating. Second, the lattice like arrangement of ommatidia and their constant size resemble the proposed model of the columnar organization of the neocortex (see 1.7). Furthermore, like demonstrated in Chapter Three for the neocortex, cell lineage plays little, if any part, in fate determination in the ommatidium (Lawrence and Green,1979). However, unlike the neocortex, several of the genes involved in the fate determination process have been characterized (Basler and Hafen,1988; Tomlinson *et al.*,1988; Mlodzik *et al.*,1990; Banerjee and Zipursky,1990). It is an interesting proposition to examine if any of these genes have vertebrate homologues involved in fate determination in the neocortex.

5.2 Limitations of and Improvements to the Retroviral Lineage Marker Technique

As discussed in 1.12.2 retroviral vectors do have limitations as lineage markers during development. They suffer from the disdvantages that the number and location of infected cells are unknown. Hence all clonal relationships need to be inferred. Furthermore the limitations of retroviral expression during embryonic development are unknown. These problems were known at the inception of the technique (Herrup,1987) and have not been conclusively addressed to date. However there are several ways in which these problems can be circumvented or reduced in severity.

The first involves simultaneously infecting tissue, at a low multiplicity of infection, with two or more different retroviral vectors that express different marker genes. This could be followed by simultaneous or sequential histochemical detection of different clones of cells infected by the viruses. Individual clones derived from one precursor infected by one retrovirus, will be stained in the same manner. Hence the chance of misinterpreting individual clones is reduced, but not eliminated. There are several marker genes with chromogenic substrates which could theoretically fulfil this role e.g. horseradish peroxidase (Smith *et al.*,1990), ß-glucuronidase (Sittertz-Bhatkar,1990; Jefferson,1989). A very similar approach is to use the same marker gene but to constitutively target it to different

subcellular locations e.g. the nucleus (Galeileo *et al.*,1990; Hughes and Blau,1990). Unfortunately this approach could not be used in this study as the 'BAG' virus ß-galactosidase is targeted to the nucleus of neurones (see 3.3.2.1) but remains cytoplasmic in other cells.

The second technique is perhaps the only way to irrefutably assign clonal relationships. As mentioned in 1.9.5 all retroviruses integrate into host DNA essentially at random. Hence different clones will contain the 'BAG' genome at different locations in the genome, although cells belonging to the same clone will all share a common integration site. Hence the sequence of host DNA adjacent to the 'BAG' genome will be unique to individual clones. It is possible to specifically amplify this flanking DNA using a modification of the polymerase chain reaction (PCR) (Saiki *et al.*,1988), termed the inverse PCR (IPCR) (Triglia *et al.*,1988; Silver and Keerikatte,1989). With this technique it is possible to amplify DNA of unknown sequence (DNA flanking the provirus) provided it is adjacent to DNA of known sequence (the 'BAG' provirus). If it is possible to isolate individual stained cells from sectioned tissue, then extract the DNA and perform the IPCR reaction, clonal relationships between stained cells could be examined by the IPCR reaction.

However this specific application of the IPCR requires the utmost sensitivity. Whether this can be achieved routinely, on fixed tissue is questionable. For it to be feasible, it would have to be as sensitive as the normal PCR reaction which can be performed on just a single copy template and even on fixed tissue (Lai-Goldman *et al.*,1988).

The third approach is to genetically modify the 'BAG' vector so that it contains an extra length of genome. After infection, histochemistry and DNA extraction it would be possible to perform the PCR reaction (Saiki *et al.*,1988) and examine the size of the amplified product. Clones from different vectors would generate different sized PCR products. The advantage of this technique is that the genomic insertion could be any piece of DNA (i.e. not encoding a histochemical marker) and possibly could result in an enormous number of different vectors.

Furthermore both of the PCR techniques mentioned above could also be performed on histologically 'negative' tissue in which no stained cells were detected to test for limitations of viral expression.

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