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Barriers of the Developing Brain; *In Vivo* and *In Vitro* Studies

by

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Declaration:

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UNIVERSITY OF TASMANIA
FACULTY OF MEDICAL SCIENCES
PHYSIOLOGY

BARRIERS IN THE DEVELOPING BRAIN: *IN VIVO* AND *IN VITRO* STUDIES

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ABSTRACT

The work presented in this thesis addresses certain aspects of brain development using two models; one *in vivo* and the other *in vitro*. The main theme of the work presented centres around the importance of plasma proteins contained in the cerebrospinal fluid and found in high concentration early in development, and the barriers which separate this fluid from the blood and the brain.

The *in vivo* model was based on the marsupial species *Monodelphis domestica*, or grey short tailed opossum, which was used to study the transfer mechanism for the plasma protein albumin, transported from the blood to the CSF during the very early period of brain development at the time when the cortical plate of the neocortex first starts to appear. Previous studies have not examined this transfer at such an early stage of brain development. This thesis shows that during the earliest ages studied the movement of albumin from the blood into the CSF appeared to be via a selective transport mechanism which was able to distinguish between different species of albumin. The barrier between the CSF and the brain was shown to be well developed.

Measurements of total protein concentration in fetal rat lateral ventricular CSF showed that the peak in concentration occurred at E15, coincident with the appearance of the cortical plate, as has been described in other species.

The *in vitro* model used fetal rats (gestational day 15). The entire central nervous system was isolated and maintained in culture for up to 40 hours. This second model showed that the commonly used culture medium supplement, fetal calf serum (FCS), causes rapid growth and proliferation of cells of the neocortex. Cell division occurred at the ventricular zone as *in vivo*. Evidence provided by the light and electron microscope showed that the barrier between the CSF and the brain was maintained throughout the culture period. Immunocytochemical staining demonstrated that the fetal protein fetuin is specifically taken up by certain cells of the neocortex both *in vivo* and *in vitro*. After a 24 hour period in culture, fetuin positive cells could be seen in a region of the cortical plate similar to those found in development *in vivo*.

The models and techniques described have shown that the blood-CSF and CSF-brain barriers to protein are both well developed even at the earliest stages of brain differentiation, and that transport of proteins between blood, CSF and brain is dependent on specific transport mechanisms.

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Chapter One

General Introduction

Introduction and Background

From the very earliest stages of development the brain is surrounded by a fluid, cerebrospinal fluid (CSF), which bathes and protects the central nervous system (CNS). The blood has access to both the CSF and the brain and in more recent years definite barriers have been recognised between the blood, brain and the CSF (see Davson *et al*, 1987). The term barrier is used to describe a restriction against the diffusion of lipid insoluble molecules between the different compartments. The barriers are:

1) A blood-brain barrier created by the cerebral vessel walls.

2) A blood-CSF barrier created by choroid plexus epithelial cells, between the extracellular fluid of the choroid plexus and the CSF secreted by these cells.

3) A pia-arachnoid-brain barrier separating the CSF in the sub-arachnoid space from the extracellular fluid of the brain.

4) A CSF-brain barrier present only in developing animals which separates the CSF in the ventricular system from the extracellular fluid of the immature brain.

The work in this thesis studies three of these barriers: the barriers on the outer and inner surface of the brain at the pial surface and ventricular surface respectively (3 and 4 above) and the barrier between the blood and the CSF (2 above). The CSF-brain barrier which separates the CSF in the ventricular system from the extracellular fluid of the brain has only recently been described and is present in the early stages of brain development (Møllgård and Saunders, 1986). Its importance to brain development is not known but it is interesting to note that it is present when cells close to the ventricular surface, the ventricular zone, are generating only neurons.

The CSF of fetal brains is characterised by a high concentration of protein compared to the adult (Dziegielewska and Saunders, 1988). The nature, origin and possible functions of the increased protein concentration have been the subjects of numerous studies in the last few decades. At present it is known that the majority of the proteins in the CSF are immunologically identical to those in the plasma. The origin of these proteins in the CSF seems to be from blood plasma, at least in the later part of brain development and especially in the adult (Davson, 1967; Felgenhauer, 1974; Davson *et al*, 1987). The possible functions of such an increased concentration of protein in brain development are still unknown, although several possible suggestions have been made (Saunders *et al*, 1992b):

i) A contribution to CSF formation by exerting colloid osmotic pressure between ventricular spaces and the brain extracellular fluid.

ii) Providing binding proteins with which a number of ligands essential for brain development are brought to the CNS.

iii) Providing an environment essential for the high levels of cell division occurring within the ventricular zone regions of the brain that are in contact with the CSF within the ventricles.

In all the animal species studied so far the concentration of protein in the CSF in the immature brain is not only higher than in the adult but is also developmentally regulated. Thus in all species, at the earliest stages studied, the protein concentration is appreciably higher than in the adult (see Table 2.1), and early on in brain development it increases to a peak and then declines, at first rapidly, and then more slowly towards the adult concentration. The mechanisms responsible for the peak concentration of protein in the CSF and the developmentally regulated increase in CSF total protein concentration are occurring at a time of rapid brain growth. So far, this mechanism has not been studied at around the time of the peak due to technical difficulties because of the

extremely small size and fragility of the available animal models (eg. sheep fetus of gestational age of 30 days, E30).

In the present thesis a new model, the marsupial *Monodelphis domestica* (grey short-tailed opossum), will be introduced for a study of the development of the peak of total protein concentration in the CSF.

The peak of protein concentration in CSF does not seem to be related to any particular stage of general animal development. Table 2.1 (page 44) illustrates the available data on the timing of the peak of protein concentration in the CSF, in days after conception. This table shows that there is no relationship between the timing of the peak and the time of birth. The only obvious similarity is the fact that the peak occurs early in gestation.

Analysis of the available data on the studies of the brain development in different species and on the CSF composition in these species suggests one possible link; the peak protein concentration in the CSF seems to be occurring at the time when cortical plate formation in the neocortex first occurs (see Page 6). Table 2.2 summarises available data on neocortical development on different species and the time of peak CSF protein concentration in the same species early in brain development. These studies show a correlation between the peak in CSF protein concentration and the initial formation of the cortical plate. The exception appears to be the rat. In previous studies it was established that the peak of CSF protein concentration in the rat occurs at around the time of birth (E20-E22) but the cortical plate begins to form earlier, at about E15-E16. Several possible explanations for this discrepancy are discussed below. In the present work this problem is re-examined in the light of available information on differences in protein concentration in different regions of the ventricular system.

An important outstanding question is the functional significance of such a large protein concentration in CSF of the developing brain. Introducing experimental changes *in vivo* is technically impossible at present. A new *in vitro* model has been set up in the present work to study some aspects of the influence

of externally applied (or exogenous) proteins on brain growth and differentiation. This model also provides a convenient way of studying the CSF-brain barrier at both the pial and ventricular surfaces.

The analysis of previous work shows that early in development, when the concentration of protein is high in the CSF, these proteins are prevented from diffusing into the brain, due to the CSF-brain barrier. Additionally, at least in the sheep fetus, during this period neurogenesis is also occurring (Åstrom, 1967). It is these observations from previously published work that provide the main focus for the work presented in this thesis.

Outline

This thesis is presented in two main parts: *in vivo* studies using *Monodelphis* and fetal rat and *in vitro* studies using the entire central nervous system of the fetal rat isolated and maintained in culture. Each part has its own introduction followed by sections on results and discussion.

The first part is concerned with both the protein composition of the CSF and its changes during development, as well as regional differences in the CSF compartments (lateral, IIIrd and IVth ventricles). In this part experiments are presented that also investigate the mechanism which transfers proteins from the blood to the CSF at a developmental stage earlier than has been studied before, by using a marsupial species.

The second part focusses on the *in vitro* model and how it has been manipulated to study the influence of protein on the development of the forebrain *in vitro*. This part compares the morphological changes *in vitro* with those *in vivo* using various techniques.

Finally at the end of this thesis a general discussion is presented combining aspects of both *in vivo* and *in vitro* experimental work and raising further questions to be answered in future experiments.

Development of the Neocortex

During the development of the CNS an enormous diversity is created between the different cell types. Cells formed during development undergo maturation and during this process show altered characteristics which define the particular cell phenotype. The timing of phenotype appearance is specific for particular cells. Progenitor cells form two daughter cells, these cells may then migrate out to a particular region of the CNS (Sauer, 1935a, b). In the case of the neocortex the migration continues until the formation of the different layers within the cortex. During the course of development maturing cells within the central nervous system will express specific molecules: receptors, neurotransmitters and structural proteins which give the cell its specific molecular identity closely related to the function it will serve. As development continues axonal projections interconnect with other cells either from the same region or elsewhere in the nervous system forming synaptic contacts and communication between cells. During this period of axiogenesis the formation of shorter processes, dendrites, form dendritic arbors which add to the overall cytoarchitecture within the brain. Throughout the modelling of the central and peripheral nervous system certain cells or groups of cells will die, having already differentiated and established contacts with other regions (Jacobson, 1991).

For many years research into the development of the brain has resulted in a variety of terms being used to describe the morphological differences in regions of the developing brain. Early in development the histology of the neocortex is very different from that of the adult. In order to eliminate confusion created by the various terms being used for the same regions, the Boulder Committee (1970) proposed the names: ventricular zone, subventricular zone, intermediate zone, marginal zone and cortical plate (Figure, 1.1). These are the regions which appear early in development and differentiate into the adult cortex. The following chapter describes the changing morphology within the developing neocortex of the fetal rat either *in vitro* or *in vivo* using the Boulder Committee's

recommended terminology.

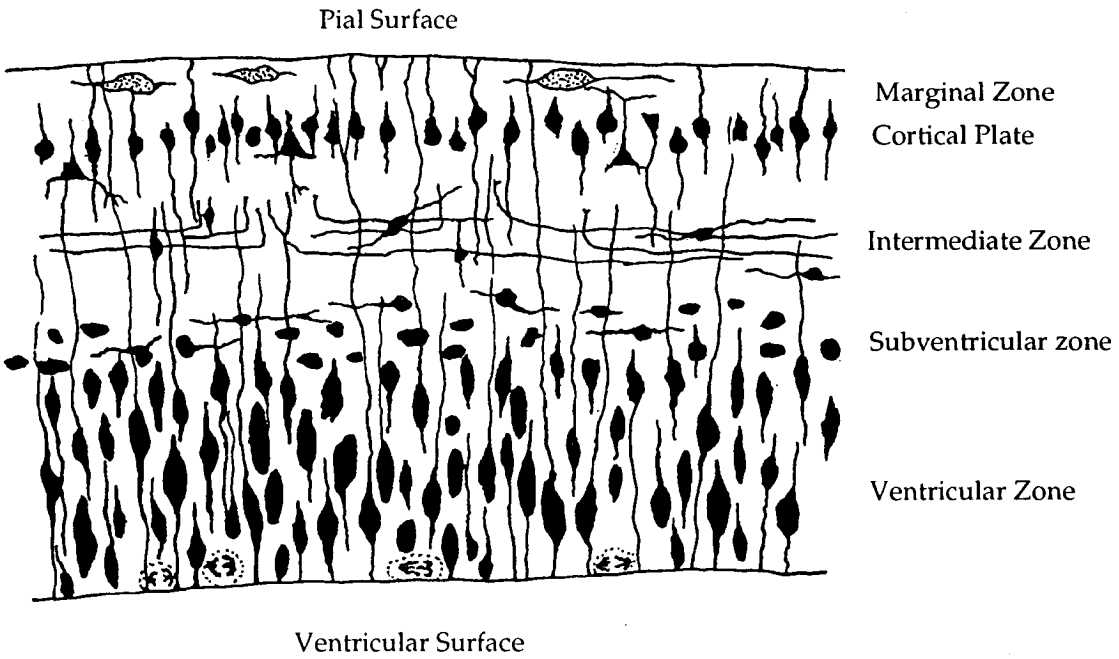


Figure 1.1: Diagrammatic representation of a transverse section through a region of vertebrate neocortex during development showing the five layers. As described by the Boulder Committee (1970).

Ventricular Zone

The ventricular zone, frequently referred to as the germinal layer, has the appearance of a pseudostratified columnar epithelium and is continually undergoing mitotic division. At E14 in the rat, the ventricular zone of the cerebral neocortex is a prominent, cell dense region which forms the boundary layer between the ventricles and the more superficial regions (Bayer and Altman, 1991). During development, the neocortex thickens and differentiates into numerous layers which form superficially to the ventricular zone. Once the period of cell division and migration out of the ventricular zone is over, the remaining cells form a single layer of cells, the ependyma, which lines the

ventricular system of the adult brain. The ventricular zone has been the source of much interest to the early anatomists (Vignal, 1888; Ramón Y Cajal, 1891, reviewed by Jacobsen, 1991), especially the mechanism of cell division in that region. Cells of the ventricular zone appear to be morphologically identical and undergo a process of interkinetic nuclear migration (Sauer, 1935b; Sidman *et al,* 1959; Sauer and Walker, 1959) during their cell generation cycle. This cell division occurs at the ventricular surface and the DNA replication (S phase) occurs on the outer border of the ventricular zone (see Figure 1.2). The nuclei in this region are continually moving perpendicular to the ventricular surface, backwards and forwards, across the ventricular zone. More recently this movement and mechanism of division has been studied extensively using a variety of techniques.

The most commonly used technique is based on the radioactive nucleotide ^{3}H-thymidine. This molecule is incorporated into the DNA of dividing cells and can be detected within cell nuclei in subsequent progeny. This was only possible provided that the number of divisions was not too great as to dilute the amount of label beyond the limits of the detection mechanism. After fixation and tissue processing, the positions of labelled nuclei were determined using autoradiography. This method has been used to trace the movements of nuclei within the ventricular zone of fetal mice and rats (Sauer and Walker, 1959; Angevine and Sidman, 1961; Lewis and Lai, 1974; Bayer and Altman, 1990). Nuclei of the ventricular zone in the early stages of neocortical development, which had been exposed to ^{3}H-thymidine for 1-2 hours, were situated in the superficial side of the ventricular zone, away from the lumen of the ventricle. At subsequent time intervals these labelled nuclei had moved closer to the mitotic zone at the ventricular surface where they divide, forming two daughter cells and therefore diluting the label. Each of these two new cells can then either leave the ventricular zone and migrate to another region or continue this process of cycling and division. The time which it takes for a nuclei to undergo one complete cycle varies according to the gestational age and the region in the brain in which this germinal region is located. At E11 in the cerebral ventricular zone of the rat, the

cycling time is 11 hours, at E18 this cycling time is 19 hours (Waechter and Jaensch, 1972). This intermitotic nucleic cycling has also been confirmed using other methods such as cytophotometric measurement of DNA content of nuclei obtained from different depths of the ventricular zone (Sauer and Chittenden, 1959).

As cortical development progresses, dividing cells are later seen (E15) outside the ventricular zone, the proportion of these more superficial cells to those at the ventricular surface increases during development (Lewis, 1968).

In the rat (Bayer and Altman, 1991) the majority of cell proliferation occurs within the ventricular zone at E15/E16, with many fewer dividing cells seen within the subventricular zone (see later). These cells are the ultimate progenitors of all the neurones and glia. The proliferation will give rise to the various different types of cells which migrate to more superficial regions of the cortex. Many different cells types exist within the mature cerebral cortex, classification depending on factors such as: distribution, class of transmitter released, membrane properties and cellular morphology.

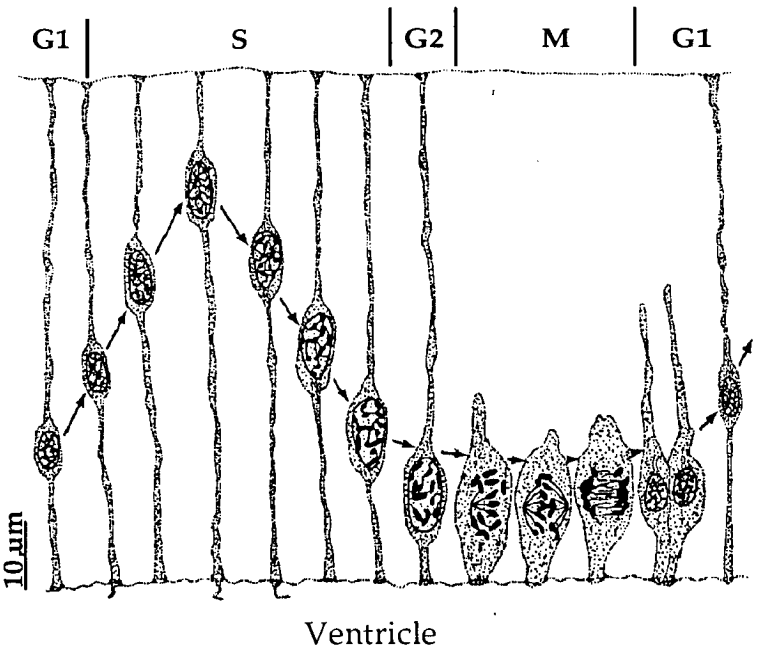


Figure 1.2: The movement of the nuclei to different levels of the ventricular zone during the mitotic cycle. From Jacobson (1991).

Marginal Zone

At E14 in the rat there are only two distinguishable layers of the neocortex which have been classically described as: the ventricular zone and the marginal zone (Boulder Committee, 1970). However, this superficial, cell sparse layer, the marginal zone, was later termed the primordial plexiform layer (Marin-Padilla, 1978) and contains as well as cells at various stages of differentiation, the terminally differentiated Cajal-Retzius cells. Marin-Padilla (1978, 1983) proposed that the term "marginal zone" should refer to the outermost and most superficial layer of the neocortex after the appearance of the cortical plate. He suggested the term primordial plexiform layer for the outer zone prior to the appearance of the cortical plate. The primordial plexiform layer therefore has a distinctive structure and only a short duration, immediately before the cortical plate appears.

The primordial plexiform layer contains cells with a neurogenetic gradient which is "outside in" (Raedler and Raedler, 1978; Bayer and Altman, 1990).

The first cells to move out of the ventricular zone settle furthest away from the ventricular surface with the later, young neurons of the subplate staying in the less superficial region closer to this germinal layer. Therefore the oldest neurons in the cortex are the large Cajal Retzius cells which reside in the outer layer I. However, subsequent neurogenesis forming the cortical plate has a neurogenetic gradient which is "inside out". In other words, neurons generated in the ventricular and subventricular zones migrate in a radial fashion through the intermediate zone to occupy a region just below layer I. This means that younger cells will migrate through layers of older neurons before reaching their final position in the cortex.

Cortical Plate

Cells of the ventricular zone extend their processes out to the pial surface, the main part of the cell body and nuclei remaining in the well defined, densely stained band, next to the ventricles. This primordial plexiform layer contains cytoplasmic filaments from cells of the ventricular zone. As these cells elongate, the primordial plexiform layer increases its thickness until the formation of other layers. As well as the Cajal-Retzius neurons, which have a horizontal orientation, there are other, younger neurons deeper in the layer which will become subplate neurons forming a subplate zone (Kostovic and Molliver, 1974; Marin-Padilla, 1983). These two regions of the primordial plexiform layer will become split into the outer marginal zone (or layer I) and the inner subplate with the formation of the cortical plate between these two layers; this occurs at E16 in the rat (Marin-Padilla, 1983) and E31 in the cat (Luskin and Shatz, 1985).

Using the results from ^{3}H-thymidine autoradiographic studies in fetal rats, Bayer and Altman (1991) proposed the hypothesis that subplate neurons temporarily reside in the cortical plate. Pregnant rats at various gestational ages were pulse-labelled with ^{3}H-thymidine and the position of labelled cells examined at different times afterwards. Rats injected on E14, the peak time of

subplate neurogenesis, appeared in the cortical plate at E16, however the subplate itself did not become distinct until E18 (Bayer and Altman, 1991). This population of neurons residing in the cortical plate has been described in other species. Kostovic and Rakic (1980, 1990), also using the ^{3}H-thymidine technique, showed that interstitial cells of white matter in neocortex of humans and monkeys originated from early subplate cells. Luskin and Shatz (1985), working on the cat neocortex, referred to these cells not as a cortical plate but the "upper subplate". Saunders *et al* (1992b) showed that the neurons in the region of the cortical plate, which contribute to the formation of the primordial plexiform layer, the initial subplate and early cortical plate, are positive for the fetal protein fetuin in the sheep. This was shown using immunocytochemical methods combined with ^{3}H-thymidine autoradiography. Therefore it seems that fetuin, in certain species, is a suitable marker for the early neurons in the cortical plate and subplate (eg., sheep, Møllgård *et al*, 1984; cow, Reynolds *et al*, 1987; wallaby, Jones *et al*, 1991).

McConnell *et al* (1989) showed in the cat that it is cells of the subplate which send out the initial projections to subcortical regions, such as the thalamus. These findings have been supported by more recent studies (Shatz *et al*, 1990; Erzurumlu and Jhaveri, 1992). These projections are however only temporary and disappear soon after permanent thalamocortical projections are established from layer 5 and 6 (Shatz *et al*, 1988). Kim *et al* (1991) provided evidence based on morphology of growth cones growing out of the subplate region that suggested that these projections are the pioneering fibres for the later forming corticothalamic pathway.

As well as fibres leaving the neocortex, early in its development, fibres from other regions such as the thalamus, reach this region by E15 in the rat (Catalano *et al*, 1991). These fibres grow in a tangential direction parallel to the ventricular surface and then turn and grow radially towards the cortical plate as early as E16 (Catalano *et al*, 1991). Studies of developing thalamocortical projections in other species have shown that incoming fibres from the thalamus

do not grow tangentially into the cortical plate as soon as they arrive at their appropriate position below the subplate but appear to wait for varying periods of time, depending on the species, in the subplate (Molliver and Van der Loos, 1970; Lund and Mustari, 1977; Rakic, 1977; Shatz and Luskin, 1986; Ghosh and Shatz, 1992). Using "DiI" tracing methods Ghosh and Shatz (1992) found that afferent fibres arrived in the subplate of the cat at E36 and invaded the cortical plate at E55. These dates were much earlier than originally thought (Shatz and Luskin, 1986), from studies using a less sensitive technique. The emergence of better fibre tracing methods (such as DiI) has meant that the waiting periods in different species has been better defined. In rats the rate of development is comparatively fast making it difficult to establish whether a waiting period exists or not (Catalano *et al,* 1991).

The region of the subplate is therefore known as the "waiting zone" and is suggested to occur because the cells in the cortical plate are too immature to attract or permit the ingrowth of thalamocortical fibres until the end of the waiting period (O'Leary and Koester, 1993). Ghosh and Shatz (1993), using fetal cats, removed the subplate neurons at the onset of the waiting period and found that the number of cells (ie. in the lateral geniculate nucleus) sending incoming fibres into the cortical regions was markedly reduced. This work suggests the importance of subplate neurons in formation of the thalamocortical pathway before the appearance of cells in the cortical plate. Synapses between thalamocortical fibres and subplate zone cells in the rat have recently been described (Kageyama and Robertson, 1993).

Intermediate zone

From the classical definition, illustrated and described by the Boulder Committee (1970), this layer of cells is situated between the cortical plate and the subventricular zone (see Figure, 1.1). During the process of radial migration (radial movement of cells out of the ventricular zone to more superficial layers)

cells will pass through the intermediate zone before settling in more superficial regions (Rakic, 1972). Fibres growing to or from the cortical layers will pass through the intermediate zone before projecting up to cells in the cortical layers.

Subventricular zone

The subventricular zone appears first at E15 in the rat in the more ventrolateral portion of the neocortex above the ventricular zone. This region is sometimes referred to as the secondary mitotic region, although cells here remain in a fixed position throughout their cell cycle. They continue to divide long after the disappearance of the ventricular zone and have a smaller, more rounded appearance compared to the cells of the ventricular zone (Boulder Committee, 1970). This region is thought to give rise to certain specific classes of neurons as well as most of the macroglia in the CNS (Boulder Committee, 1970).

Migration of Cells during Neurogenesis

The majority of cellular migration in the cerebral cortex appears to occur in a radial fashion (Rakic, 1988) guided by radial glial cells whose processes extend to the outer pial surface of the neocortex (Rakic, 1972; Gadisseuz *et al*, 1989). The idea of radial migration has arisen through a large body of evidence using ^{3}H-thymidine pulse labelling (Sauer and Walker, 1959; Angevine and Sidman, 1961; Rakic 1972, 1988; Raedler and Raedler, 1978; Kostovic and Rakic, 1980; Caviness, 1982; Bayer and Altman, 1990) and has been termed the radial unit hypothesis (Rakic, 1988). This hypothesis proposes that terminally divided neurons leave the mitotic region, close to the ventricular zone, and migrate to the more superficial neocortical layers in a radial movement. Across the neocortex span radially orientated glial filaments which, it has been suggested, have a high affinity for the migrating cells (Rakic, 1972). The autoradiographic technique, using radioactively labelled thymidine, is able to detect nuclei at large distances from the ventricular zone after long periods of time

(eg. Valverde *et al*, 1989). The difficulty of this technique is that labelling all cells undergoing DNA replication during a short period of time, using a pulse label, does not show from which region of the ventricular zone labelled nuclei in the cortex originated. In other words, the position of labelled nuclei sometime after the exposure to radioactive thymidine does not give any information as to the original position of the nuclei at the time of labelling.

Another difficulty with this method is due to marker dilution, caused by further cell divisions after labelling. One cell division halves the quantity of the radioactive thymidine within the nuclei of the daughter cell.

In recent years more sophisticated techniques, using inheritable markers, have been used to trace the movements of dividing cells as they leave the ventricular zone. The use of retroviruses to introduce "reporter genes" into cells eliminates the problem of marker dilution such as ^{3}H-thymidine (Price *et al*, 1987). When a retrovirus infects a dividing cell its genome integrates into a chromosome of the infected cell and is inherited by progeny of that cell (Sanes, 1989). Clonal analysis and lineage studies in the cerebral cortex appear to show that radial migration is not the only mechanism through which the cortex is built (Walsh and Cepko, 1992, 1993; Price and Thurlow, 1989). Cells migrating tangentially after leaving the ventricular zone have been shown to disperse widely across functional areas of the cortex (Walsh and Cepko, 1993). The relationship between the number of cells migrating tangentially and the stage of development of the neocortex is unclear. Walsh and Cepko (1993) showed a large amount of clonal dispersion (43%) occurring between E14 and E20 in the rat neocortex whereas Nakatsuji *et al* (1991) suggested, on the basis of experiments using mouse neocortex, that the majority of the dispersion and mixing of cells between radial columns was occurring later in development.

The mechanism of radial dispersion and migration of neurons along radially arranged glial cells seems to be dominant early in neocortical formation (Rakic, 1988) and a movement of neurons perpendicular to this glial cell

framework appears well understood. However, certain *in vitro* studies have shown that cells destined for the cortical plate exhibit both perpendicular and parallel contact guidance on parallel aligned neurite bundles (Nakatsuji and Nagata, 1989; Nagata and Nakatsuji, 1990).

During this proliferation, migration and formation, the CNS is constantly surrounded by the CSF in the ventricular system inside the brain and subarachnoid space surrounding the brain. This fluid compartment is separated from the brain during development due to the presence of a cellular barrier (CSF-brain barrier). As the CNS grows, angiogenesis increases the network of blood vessels which penetrates the nervous tissue. These vessels maintain a barrier between the blood and the brain extracellular fluid. Therefore, during development the neocortex forms while isolated from the CSF and the blood.

Barriers of the Developing Brain

The term blood-brain barrier is used widely in the literature and encompasses a number of barriers which separate and protect the brain from the very earliest stages of development. These are; the blood-brain barrier, the blood-CSF barrier and the CSF-brain barrier.

The Blood-Brain Barrier

Saunders (1992) describes the barrier which prevents the movement of proteins within the plasma from entering the brain and the CSF as being the most fundamental. Blood vessels which first invade the developing brain (blood-brain interface) and form within the choroid plexus (blood-CSF interface) provide tight and well developed barriers to protein from the outset (Saunders, 1992).

The exact nature of these barriers, between the blood and the brain, is the tight junctions between cerebral endothelial cells (cells lining the blood vessels) making the blood-brain barrier and between choroid epithelial cells making the blood-CSF barrier. These restrict diffusion of molecules from the blood into the brain or its environment therefore protecting it from rapid changes which may occur in the rest of the body.

These barriers have been studied using a variety of methods: electron microscopy (eg. Møllgård and Saunders, 1977, 1986; Saunders and Møllgård, 1984), protein permeability studies (eg. Dziegielewska *et al*, 1979, 1980b, 1991; Habgood *et al*, 1992), measurements of transendothelial resistance *in vivo* (Butt *et al*, 1990) and *in vitro* (Rubin *et al*, 1991). These studies have been able to determine the nature of the barrier (to proteins) in the adult as well as the developing young. Other studies had concluded incorrectly, that the blood-brain barrier (to protein) is immature in the developing brain (eg. Wakai and Hirokawa, 1979, 1981; Risau *et al*, 1986). However, Saunders (1992) and

Dziegielewska and Saunders (1988) describe a number of shortcomings in these early studies which have led to incorrect interpretations of results. Studying the morphology of the endothelial and epithelial cell tight junctions in the very young brain require extreme care in the tissue fixation. Poor fixation may lead to an unnecessary amount of tissue shrinkage and disruption of the junctions between cells. Studying the permeability of this barrier to marker proteins introduced into the adult as well as the young has been used in a number of studies to examine the nature of the barrier. However, before the results from these type of experiments can be interpreted the physiological state of the animal and degree of disruption to which it is exposed must be considered, ie. the conditions during the experiment must be kept within physiological limits.

In spite of some claims to the contrary, the tight junctions between endothelial and epithelial cells have been shown to be well formed in the earliest brains using freeze fracture techniques (Møllgård and Saunders, 1975, 1986; Saunders and Møllgård, 1984).

The first part of this thesis uses the marsupial species *Monodelphis domestica* to examine the nature of the barrier (to albumin) between the blood and the CSF at a very early stage of brain development not previously investigated in physiological experiments.

CSF-Brain Barrier

The second half of this thesis introduces a new and innovative approach to investigate the barrier between the CSF and the brain. This is a more recently discovered barrier which restricts the movement of substances between the CSF and the brain (Fossan *et al*, 1985; Møllgård *et al*, 1987). This interface between the CSF and the brain, is known as the neuroependymal cell layer which lines the ventricles, and has been shown to provide a diffusion barrier to the movement of proteins during only the very early stages of development (Fossan *et al*, 1985). The pial surface on the outside of the brain appears to provide a similar barrier

but its ultrastructure has not been adequately studied. Some preliminary information is included in this thesis.

In the past, chemical markers such as horseradish peroxidase (HRP), injected into the ventricles of adult brain have been shown to penetrate into the extracellular space of the brain between the ependymal cells lining the ventricles (Brightman and Reese 1969). However, Fossan *et al* (1985) looked at this barrier in the fetal sheep at 2 different gestational ages, E60 and E125 (term is 150 days). This experiment involved the perfusion of the same marker protein, HRP, into the ventricles, as well as ^{125}I labelled human albumin, ^{3}H sucrose and ^{14}C inulin. The total protein concentration of the perfusate was adjusted to be around that which would normally be found in the CSF at these two different ages, thus avoiding disturbance of the surrounding tissue due to osmotic effects. The entry of the horseradish peroxidase into the brain from the CSF, at E125, was time dependent, ie. the longer the perfusion time the greater the penetration of protein from the CSF and into the brain. This was a similar result to that of Brightman and Reese (1969) in the adult mouse although the levels of CSF protein concentration in Fossan's experiment closely matched the endogenous levels, whereas those in Brightman and Reese's experiments were much higher than found in adult mouse CSF. However in the younger fetus, E60, the penetration of the HRP into the brain was very much restricted. This barrier, present only during these early stages, was shown to be coincident with the occurrence of morphological structures ("strap junctions") not present in the older fetuses. There appeared to be specialised junctions between cells of the ventricular zone not normally present between ependymal cells that line the ventricles in the adult. These junctions and the appearance of this barrier has been subsequently studied at various stages of development in a number of different species (human, Møllgård and Saunders, 1986; sheep, Møllgård *et al,* 1987; wallaby, Dziegielewska *et al,* 1988).

Cells throughout different organs of the body display a variety of junctions: tight junctions (zonula occludens), intermediate junctions (zonula

adherens) and desmosomes (macular adherens), (Farquhar and Palade, 1963). These and the junctions between early ependymal cells were studied at different stages of development by Møllgård *et al* (1987). Apposed normal ependymal cells at the ventricular zone of the E125 sheep were linked close to the luminal surface by intermediate junctions, with 20 nm intercellular gaps and gap junctions were also seen (Møllgård *et al*, 1987). This same study examined closely the morphology of the junctions present in the much younger fetuses (E19-E40). These were described as "complex" with the two adjacent membranes folded and exhibiting a "tortuous configuration" with a narrow intercellular gap. Intermediate-like junctions were apparent although there was no evidence of any desmosomes or definite gap junctions. At the apical surface, adjacent cell membranes were fused with a tight junction-like appearance. These junctions however did not show a typical belt-like structure but were shown to spiral around the cells membrane forming an average of 4 "kissing points" with the adjacent cell. These junctions were not characteristic of any other type of junction in an early developmental stage and the authors describe this new type of junction as a "strap junction" (Møllgård *et al*, 1987).

This study (Møllgård *et al*, 1987) draws attention to the correlation between the disappearance of these strap junctions, the fall in the protein concentration in the CSF and the decline in the blood-CSF permeability to a variety of markers. The functional significance of the junction is therefore likely to be involved in controlling the environment which surrounds the developing neuroblasts, as well as providing a mechanical stabilising effect on the rapidly expanding neocortex (Saunders, 1992). This raises questions about the functional significance of high protein levels within the CSF during the early developmental stages. If this is to provide substrates required by the rapidly dividing neuronal tissue which lacks significant vascularisation at this early stage, then it is surprising that a barrier exists between the CSF and the extracellular space of the brain. How much access do cells of the neocortex have to the proteins within the CSF, if at all, and if they do by what mechanism?

Blood-CSF Barrier

The basis of the blood-CSF barrier is the presence of intercellular tight junctions between the epithelial cells of the choroid plexus. These junctions prevent the passage of proteins and similar sized macromolecules from the blood into the CSF. There is however a transcellular route through the epithelial cells of the immature brain which bypasses these junctions and enables plasma proteins to accumulate within the CSF early in brain development (Møllgård and Saunders, 1977; Dziegielewska *et al*, 1991) (see Chapter Two).

General Methods

In Vivo Methods

Monodelphis domestica, a marsupial species, has an average litter size of between 6 and 10 young which are born at an extremely early stage of brain development. Thus each litter provides a number of young which are an accessible model in which to study the barriers in the CNS.

(i) Handling and Injection of *Monodelphis domestica*

Animals supplied for this work came from the local colony (Animal House, University of Tasmania, Hobart). Details of breeding and care of these animals have been published previously (VandeBerg, 1983; Adam *et al*, 1988).

Animals from between 3 days old and adulthood were used in these experiments. From birth (P0) until about 15 days old (P15) the young are permanently attached to the mother and then detach periodically until nearer the time of weaning, at around 48 days. The younger animals had to be injected whilst still attached to the mother as they would not return back on to the mother's teat and would be eaten by the mother within minutes of removal. Once the young were injected IP they were then left on the mother for a period of time (until steady state was reached), and were then carefully removed taking care that no damage was done to either the teat or the mouth of the young. For these barrier permeability experiments (see below) individual animals were injected with standardised volumes (8% of estimated total blood volume).

Since the circulating volume of blood in these young animals is small (*Monodelphis* pup, approximately 7 days old, weighing 2g will have a circulating blood volume of approximately 200µl, assuming blood volume of 10% body weight) an intravenous injection of a single bolus dose could cause large effects on its first pass through the microcirculation, before adequate mixing has occurred with the rest of the blood. Therefore it was important that

the amounts injected did not increase the circulated blood volume enough to cause any disruption to blood vessels. This was achieved by limiting the injected volume and by giving the injection into the peritoneal cavity from where it entered only slowly into the circulation. It was also important that the amount of foreign protein injected did not markedly change the existing concentration of protein already circulating in the plasma. A litter based model was used for determining the time to reach steady state (Habgood, 1990; see also section (iv) below), ie. individual animals or pairs of animals represent a single time point. With this method it is essential that each animal had the same concentration of foreign marker circulating in the plasma irrespective of its size. The volume injected was 8µl/g of body weight which was equivalent to 8% of its total blood volume, assuming the total amount of blood in each animal was equivalent to 10% of its body weight. The different exogenous albumin markers (see below), which were injected IP, were made up as 20% (w/v) solutions in sodium chloride (0.9% w/v). In the youngest animals a 30 gauge needle was used for the injection, taking care that there was no leak of solution back through the entry hole. This was done by displacing the skin to one side immediately before inserting the needle into the peritoneal cavity. After removing the needle the skin relaxes back into its original position moving across the entry hole into the peritoneum. If a substantial leak of solution occurred back through the entry hole (greater than 40%) then the animal was not included in the experiment.

(ii) Injected Albumins

Bovine serum albumin (BSA);Sigma; code No. A 2153.

Human serum albumin (HSA); Sigma; code No. A 1653.

Succinylated bovine serum albumin (Succ BSA), supplied by K.M. Dziegielewska (see Habeeb *et al*, 1958, for methods).

^{125}I radiolabelled human serum albumin (^{125}IHSA), Amersham; code No. IM 17P.

(iii) Sampling of CSF and Blood from the Young *Monodelphis*

After careful removal from the mother, individual animals were killed by an overdose of halothane anaesthetic prior to the sampling of CSF and blood. Blood was collected by two methods, either by direct cardiac puncture in the older animals or by cutting the left subclavian artery as it emerged from the chest cavity in the very young. This ensured that pleural fluids of the chest cavity were not removed with the blood (which was found to be a problem with cardiac puncture in the youngest animals). Samples were removed using gentle suction into fine, glass, heparinised micropipettes. CSF was then removed by carefully cutting away the muscles at the back of the neck between the base of the skull and the first vertebra. This exposed the *dura mater* covering the CSF space of the *cisterna magna*. The *dura mater* was pierced with a fine glass micropipette and by gentle suction a sample of CSF was removed, taking care not to disturb any of the blood vessels in the region. CSF and blood samples were also checked under a stereo microscope for blood and any showing detectable signs of contamination were discarded. Levels as low as 0.05% could be detected in this manner by comparison with reference samples with known levels of contamination. CSF and blood samples were stored in microglass sealed capillary tubes at -20°C until used for further analysis.

(vi) Determination of Steady State

As discussed by Dziegielewska and Saunders (1988) results from permeability experiments can only be valid if the levels of markers in the blood were maintained or estimated to be, at an approximately constant level, steady state. Experimentally, it is not possible in such small animals to control and monitor both the blood and CSF concentrations of markers. By measuring the

plasma concentration at regular intervals (or estimating the concentration in the litter based model) it is possible to obtain a time weighted mean of the plasma concentration over a period prior to obtaining a CSF sample. To ensure that the concentrations of marker (exogenous albumin, BSA) in the plasma and CSF of the young *Monodelphis* were at a constant level at the time of sampling the litter based model was used (Habgood *et al*, 1992). Each pup from a litter of *Monodelphis* was given a standardised IP injection (see above) so that each littermate was estimated to have the same amount of marker injected per unit weight. This would result in each animal having the same marker plasma concentration. Individual animals were then subsequently removed from the mother after different time intervals and their CSF and plasma marker concentration measured. Individuals were sampled after 6, 11, 15 and 24 hours. The concentration of protein in CSF was estimated and results expressed as a ratio, CSF/plasma. Once CSF/plasma ratios were no longer rising significantly, then steady state is approached.

The following sections describe methods using the fetal rat to investigate certain aspects of the changes in CSF protein concentration during development. This animal was chosen as previous studies have examined similar changes (Dziegielewska *et al*, 1981) and also because the CSF in the ventricular system of the fetal rat was easily removed using a micropipette. The midbrain vesicle and the lateral ventricles are large in relation to the rest of the brain and easily seen under the dissecting microscope.

(v) Handling and Anaesthesia of Pregnant and Fetal Rats

Time mated female rats were anaesthetised as below (see section (xii)) and laparotomy performed to expose the fetuses. The fetuses were either removed for CSF sampling (see section (vi) or left *in utero* for a period of time after small volumes of protein solution were injected into the lateral ventricles (see section (vii)).

(vi) Sampling of CSF from Fetal Rats.

Between 13 and 20 days gestation CSF from within ventricles of the fetal rat was removed as described for *Monodelphis* (see section (iii)). The fetus was separated from the mother and immediately exsanguinated by the removal of the heart. Fine, drawn-out glass micropipettes were then inserted through the skin and skull and through the thin layer of neural tissue. Once the tip of the micropipette was located inside either the IIIrd ventricle or the region of the *cisterna magna,* by gentle suction through plastic tubing attached to the end it was possible to withdraw exact amounts of fluid. These samples were then centrifuged and checked for contamination. Any samples showing any signs of blood contamination were discarded.

(vii) Injection of Solutions into Ventricles

The pregnant rat was anaesthetised (see section, xii) and the uterus exposed with a medial incision in the lower abdomen. After exposure the fetuses were kept damp throughout the experiment using gauze swabs soaked in sodium chloride solution (0.9% w/v), to ensure that they remained viable throughout. Fetal rats (E15-E16) were injected with different protein solutions (200mg/100ml bovine albumin + 200mg/100ml bovine fetuin) into one of the two lateral ventricles. Care was taken not to inject too large a volume (<5µl) which may cause damage to the tissues. For these experiments the fetus remained alive and *in utero* for a period of 30 minutes after foreign proteins were injected into the ventricles.

Using fine glass micropipettes (see above) a small volume of CSF was removed from either of the two lateral ventricles. This was immediately replaced by the same volume of protein solution using a similar micropipette.

After injection the fetus was then covered in "Parafilm" and damp gauze swabs (see above). After 30 minutes the fetus was removed and fixed in Bouin's fixative and processed for paraffin histology ready for further analysis.

Analysis of Plasma and CSF Samples

(viii) Measurements of Total Protein Concentrations.

The total protein concentration in the culture medium, CSF and plasma was measured using the method of Bradford (1976). One ml of protein reagent (100mg. Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. 100ml of 85% w/v phosphoric acid was added and the solution was then made up to 1litre) was added to tubes containing 1 to 10 µg of protein standard (Sigma, protein standard, code No. 540-10) in 100µl volume. The protein standard contains 5.0g/100ml of albumin and 3.0g/100ml of globulin, the major protein components present in concentrations similar to those found in the adult human plasma. Although the protein composition of the CSF and plasma changes with age and the dye used in this method may react differently with different proteins, this protein standard was found most satisfactory, as has been discussed by Dziegielewska *et al* (1991) who used this method for estimating the total protein levels in fetal sheep and recognised differences in values obtained from previous studies where only one protein (ie. albumin) was used as the standard. In the present study the same protein standard was used throughout.

After thorough mixing of the solutions their absorbance was measured, at 595nm, in 1ml cuvettes against a reagent blank using a spectrophotometer (Pye Unicam, UV, SP 6-500). The blank contained 100µL of buffer or saline and was used to zero the absorbance readings after every sample. The absorbance was plotted against the corresponding concentration of protein standard and this graph was used to determine the amount of protein contained in an unknown sample.

(ix) Measurement of Individual Proteins in Plasma and CSF Samples

The concentrations of individual proteins, either endogenous or exogenous were measured using radial immunodiffusion assays (Mancini *et al,* 1965).

The different anti-albumin antibodies used are known to have some cross-reactivity with albumins of different species. Therefore all the antibodies used were preabsorbed against all other possible cross-reactants. For example, if human serum albumin was the marker injected into the young *Monodelphis*, anti-HSA antibody was firstly preabsorbed overnight with an excess of antigen (*Monodelphis* control plasma). Finally, lack of cross-reactivity was checked using immunodiffusion assays in the case of the Mancini method and on the appropriate histological sections in the case of immunocytochemistry (see section (xvii). If cross-reactivity had not been eliminated this would have shown on these controls. Only samples of absorbed antibodies that showed no cross-reactivity were used.

Significant differences between *Monodelphis* and exogenous albumins were calculated using unpaired Student's t-tests.

(x) Identification of Individual Proteins

Different proteins within CSF samples, culture media and plasma samples were identified in crossed immunoelectrophoresis using the method of Laurell (1965) which separates different proteins in an electric field (20volts/cm for 1 hour in one dimension, then at 4 volts/cm overnight at right angle in the 2nd dimension which contains appropriate antibodies). The individual proteins can be identified using monospecific antibodies (see section (xix))

The gel used for both dimensions was 1% (w/v) agarose (Litex) in Tris-barbitone buffer at pH 8.6. After the electrophoretic run the plate was washed, dried and stained with Coomassie Brilliant Blue (Weeke, 1973). To differentiate

the staining of the proteins, acetic acid and ethanol were used as a destainer (eg. Dziegielewska *et al*, 1979).

(xi) Counting of ^{125}I Labelled Samples

Samples from animals that had received ^{125}I labelled human serum albumin were diluted (CSF X2 and plasma X10) and known quantities pipetted into tubes containing 6ml of scintillation fluid (Optiphase Hisafe, LKB). After thorough mixing, samples were counted for 5 minutes in the window appropriate for the ^{125}I isotope in the scintillation counter (Rackbeta, LKB). Quench correction was automatic using the LKB external standard technique. To correct for background activity two vials containing the same volume of distilled water as the plasma and CSF samples and 6 ml of the scintillant were counted and the reading averaged, this was subtracted from the experimental readings. The results were expressed as disintegrations per minute per µl of sample (dpm/µl) and the CSF/plasma ratio was again calculated.

In Vitro Methods

(xii) Dissection of the CNS

Female rats were time mated to ensure that the fetuses were of the correct age. Males and females (Hooded Wistar) were put together for a 24 hour period only. The day they were separated was designated as E0. The age of the fetus was additionally checked against the crown rump length (Butler and Juurlink, 1987).

Pregnant rats were anaesthetised intraperitoneally (I.P.)with 0.6ml/100g body weight of 25% urethane (ethyl carbamate, Sigma, lot. 69F0633) and maintained on a heated pad at 35°C. Fetuses, at day 15 of gestation (E15),were removed by caesarean section immediately prior to the dissection and killed by rapid exsanguination. The whole fetuses were then completely immersed in culture medium in a small Petri dish lined with a clear silicone polymer (SYLGARD, Seneffe, Belgium). The silicone polymer allows for a more overall illumination during the operation as well as being able to accept dissecting pins, keeping the specimen anchored throughout. Initially the fetus was pinned out so that its dorsal surface was uppermost and kept for the duration of the dissection in basal medium Eagle's (BME) and bubbled continuously with 5% carbon dioxide in oxygen.

First, the skin covering the skull and spinal cord was removed and using fine watchmakers forceps and iris scissors the calvarium was peeled away, exposing the developing brain. Careful blunt dissection was used to expose the spinal cord with dorsal root ganglia attached. The ganglia at this age lie more ventrally and are situated closer to the cord than later on in development.

Removing the brain away from the base of the skull inevitably led to damage of the nervous tissue around the area of the optic chiasm. This could be

kept to a minimum by carefully cutting away the brain in the area of the optic nerves as well as the trigeminal ganglia. Any serious damage to the brain caused a collapse of the ventricle due to the loss of CSF. Preparations with such distortions were discarded as the culturing process led to further damage of these fragile preparations.

The dissection procedure was performed in clean but asterile conditions, using a dissecting microscope (Wild M8) and media bubbled continuously with 5% CO_2 in oxygen that was between 5°C and 12°C. Once removed, the preparations were then transferred to fresh media under sterile conditions in a laminar flow cabinet ready for culture.

(xiii) Culturing the Preparation

Under sterile conditions of a laminar flow cabinet, preparations were sequentially washed, with sterile medium (BME, Gibco, Life Technologies, Scotland, UK, see below for medium composition) 10-15 times, using sterile plastic pipettes (Falcon, 1 ml). They were then transferred individually to culture flasks containing 15 ml of medium with 0.1% gentamycin with or without 10% fetal calf serum (FCS).

These flasks were stoppered using sterile rubber bungs and bubbled with 5% carbon dioxide in oxygen through sterile, surgical needles (21G, $1\frac{1}{4}$"). Sterile filters (Sigma, 0.2μm) were attached at both the gas inlet and outlet of the flask reducing the chances of infection during culture.

The rates of bubbling were kept to a minimum (approximately 3 per second) firstly to limit the mechanical disturbance of the nervous tissue, and secondly to minimise the foaming action of the solutions containing protein. Foam rising up the flask saturates the exhaust filter, blocking it and stopping aeration of the fluid. Flasks were immersed in a water bath with thermostatically controlled heater and maintained at either 29°C or 33°C.

(xiv) Survival of the Preparation

Electrophysiological recordings from individual cells within the cortex were not attempted. The preservation of cell structure and the overall state of the preparation were determined histologically. Preparations which did not survive the culture, for example when the gas supply to the medium stopped, quickly deteriorated and soon broke up into smaller fragments. The tissue under the dissecting microscope had a granular appearance and was more fragile than the original and those which survived.

(xv) Sampling of Fluid within the Ventricles.

At various times during the culture of the whole central nervous system the protein concentration and composition of the fluid within the ventricles of the brain was measured. The preparation was first removed from the culture vial and washed in fresh, protein free medium; this was to remove from its surface any protein which were present during culture. A fine glass micropipette (inside tip diameter, approximately 80μm; outside diameter, approximately 100μm), drawn out over a bunsen flame, was then inserted in turn into each of the ventricular spaces. By gentle suction through a tubing (Portex, ref;800/010/125/800), connected to the micropipette, fluid from within the ventricles of the brain was withdrawn. Samples from different ventricular spaces within the same brain were collected together into Eppendorf tubes and stored at -20°C until further analysis.

Samples were removed from the IIIrd ventricle at all the fetal ages and also from a region of the *cisterna magna* at two fetal ages (see Introduction).

Morphological Methods

(xvi) Tissue Fixation for Light Microscopy

Bouin's fixative.

Composition of Bouin's fixative: 75 ml of saturated aqueous solution of picric acid, 25ml of 40% formaldehyde and 5ml of glacial acetic acid (Culling, 1963).

The preparations were immersed in Bouin's fixative overnight. After several washes in tap water they were then dehydrated through 70%, 95% and then 100% ethanol (minimum 6 hours in each with 2 changes) followed by two changes of chloroform, one overnight. This was followed by 3 changes of paraffin wax at 56°C for at least 4 hours in each. Once embedded and cooled to room temperature the blocks were then cut serially in 3μm sections and placed on gelatin coated slides (Culling, 1963).

(xvii) Immunogold Silver Staining

This immunocytochemistry technique was used to localise specific marker proteins (endogenous or exogenous) within fixed tissue sections which have been mounted on slides, as described above. The principle of the technique was based on firstly, exposure of the mounted prepared sections to an antibody specific to the marker in question (eg. bovine albumin, ie. rabbit anti-bovine albumin). Sections were then incubated with a gold labelled secondary antibody (Auroprobe One™, Amersham, code No; RPN 470-473) which was specific to the first antibody. Visualisation of the gold labelled antibody was achieved using a silver enhancement technique (Holgate, 1983).

Procedure;

a) Dewax Sections

i) Xylene 2 changes.

ii) Absolute alcohol (2 changes, 10 minutes each).

iii) 2 ml 100% hydrogen peroxide in 400ml absolute alcohol (20-30 minutes).

iv) Graded alcohols, 95% and 70%, 5 minutes in each.

v) Tap water (5 minutes)

b). Staining of Sections

i) Phosphate buffer solution, (PBS, phosphate buffered saline, 3 changes, 20 minutes each) pH 7.2 with 0.2% Tween detergent (polyoxyethylene sorbitan monolaurate, Sigma; code No. P1379).

ii) Blocking solution for 1 hour (solution used; 10% gelatin in PBS with 0.2% Tween.

iii) Incubated in primary antibody at 4°C overnight. Antibody was diluted appropriately and applied in blocking solution.

iv) Washed 3 times in PBS/Tween.

v) Gold conjugated secondary antibody applied for 2 hours at room temperature. Antibody was diluted (X50) and applied in blocking solution.

vi) Washed 3 times in PBS with Tween for a total of 15 minutes.

vii) Washed in distilled water, 2 times for a total of 10 minutes.

viii) Enhanced for 20 minutes using silver enhancement reagents (Amersham, code number; RPN 491), A:B, mixed 1:1.

ix) Running distilled water for 15 minutes.

x) Counterstained in toluidine blue for 30 seconds.

xi) Washed in distilled water until appropriate level of staining remains.

xii) Dehydrated in graded alcohols, cleared in xylene and mounted in DPX.

(xviii) Incorporation of BrdU

Dividing cells *in vitro* and *in vivo* were detected using immunocytochemistry. The method involves introducing a small quantity of the thymidine analogue, bromodeoxyuridine (BrdU, Sigma code No. B 5002), into the culture medium for a short period of time, immediately after the CNS has been isolated from the fetus. The nucleotide analogue will be incorporated into newly made DNA and can be detected using the immunocytochemical technique and antibodies specific to BrdU (Schutte *et al,* 1987).

Preparations were exposed to 30uM BrdU (MW 307.11) for 1 hour, 29°C or 33°C, in fresh medium. After labelling they were thoroughly washed in sterile medium before being separated into sterile culture vials as described above.

Localisation of the BrdU incorporated into DNA was done on fixed and paraffin embedded preparations as described above. Acid or enzyme digestion procedures were found to be unnecessary in the present work. Significant staining of the labelled cells was achieved using a X20 dilution of the BrdU antibody (Sigma).

(xix) Antibodies

Antibodies to *Monodelphis* plasma proteins were produced in New Zealand white rabbits by serial, intracutaneous injections of 0.05ml plasma with Freund's

adjuvant (Sigma Code No. F-5506) (Dziegielewska *et al,* 1989). Complete adjuvant was used initially followed by incomplete adjuvant for all the subsequent injections (Harboe and Ingild, 1973).

Antisera to other species of albumin (human serum albumin, bovine serum albumin), with the exception of *Monodelphis* albumin, were obtained from Dako (see below).

(xx) Primary Antibodies

Rabbit Anti-Monodelphis Albumin (Supplied by Dr. K.M. Dziegielewska)

This antibody was preabsorbed with against BSA and HSA. Each albumin (10mg) was mixed with 1ml of the antibody, left overnight at 4°C, centrifuged for 10 minutes and separated from the supernatant. The antibody was used at concentrations between 1:20 and 1:100.

Rabbit Anti-Calf Fetuin (DAKO, Denmark, Code No. Z 249).

This antibody has been shown not to cross-react with rat fetuin (Dr. K. M. Dziegielewska, personal communication) and therefore making preabsorption unnecessary. The concentrations used as the primary antibody were between 1:50 and 1:200 with a 10% gelatin blocker.

Rabbit Anti-Rat Serum (DAKO, Denmark, Code No. Z 179).

This antibody cross reacts with fetal calf serum. 100 µl of antibody was therefore preabsorbed with 10 ml of 10% FCS in PBS. This solution was left overnight at 4°C centrifuged for 10 minutes and separated from the supernatant.

This solution was used as the primary antibody (diluted 100 times) and blocker combined.

Rabbit Anti-Cow Albumin (DAKO, Denmark, Code No. Z229)

This antibody was found not to cross react with rat albumin; however as a precaution it was preabsorbed with rat plasma (100µl plasma to 1 ml of antibody). The antibody was used at concentrations between 1:200 and 1:300 in a 10% gelatin blocker solution.

Rabbit Anti-Rat Albumin (NORDIC, Netherlands, Code No. RARa/Alb).

This antibody cross reacts with albumin in fetal calf serum. Antibody (100µl) was mixed with 10 ml of 10% FCS in PBS, left at 4°C overnight, centrifuged for 10 minutes and separated from the supernatant. This solution was used as the primary antibody and blocker combined (dilution X100).

Monoclonal Mouse Anti-BrdU (DAKO, Denmark, Code No. M744)

This monoclonal mouse antibody shows no cross reactivity with any fetal rat proteins and was diluted 1:20 in PBS and blocker.

(xxi) Fixation and Preparation for Electron Microscopy

Immediately after removal from the animal (or removal from culture), the entire CNS was washed in 0.1M cacodylate (pH 7.4) for 30 seconds, approximately. Brains (cerebral vesicles only) were immersed in 2.5% glutaraldehyde (Polysciences) in 0.1M cacodylate buffer (Fluka), pH7.4 overnight at 4°C. They were then washed in cacodylate buffer (3 times) before

the pieces of fixed tissue were cut, using scalpel blades, into small blocks (not more than 5mm in any dimension).

Post Fixation

These fixed pieces of tissue then underwent post fixation in an osmium-potassium ferrocyanide mixture (De Bruijn and Den Breejen, 1975; De Bruijn and Den Breejen, 1976; Goldfischer *et al,* 1981). The solution was made up as follows; 0.21g ferrocyanide (0.5M) in 10ml of 0.2M cacodylate buffer with 10µl of NaOH. This solution was mixed with an equal volume of 4% reduced osmium ($Os0_4$). Therefore the final solution is 2% reduced osmium, 0.025M $K_4Fe(CN)_6$ and 0.1M cacodylate buffer. The pieces of tissue were postfixed in this solution for 2 hours at 4°C, agitating continuously.

At room temperature the tissue was then added to 1% uranyl acetate (in water) for 1 hour before being dehydrated in alcohols (4°C); 70% for 15 minutes, 96% for 15 minutes, 2X 100% for 15 minutes. Once dehydrated the tissue was then prepared for embedding;

i) Propylene oxide (2X 15 minutes at 4°C)

ii) 1: 1 Propylene oxide: Epon. 30 minutes at 4°C.

iii) 1: 2 Propylene oxide: Epon. 2 hours at room temperature.

iv) Overnight in 100% Epon.

v) Change of fresh Epon and in oven for at least 12 hours at 65°C.

The blocked tissue was sectioned at approximately 300 Å and viewed using a Philips CM100 transmission electron microscope.

(xxii) Basal Medium Eagle (BME) Formula

COMPONENT	CONCENTRATION mg/ml
Inorganic Salts	
Calcium Chloride .$2H_2O$	0.185
Magnesium Sulphate(anhydrous)	0.098
Potassium Chloride	0.4
Potassium Phosphate Monobasic (anhydrous)	0.06
Sodium Bicarbonate	0.35
Sodium Chloride	8.0
Sodium Phosphate Dibasic (anhydrous)	0.048
Amino Acids	
L-Arginine.HCL	0.021
L-Cystine.2HCL	0.01585
L-Histidine (free base)	0.008
L-Isoleucine	0.026
L-Leucine	0.026
L-Lysine.HCL	0.03647
L-Methionine	0.0075
L-Phenylalanine	0.0165
L-Threonine	0.024
L-Tryptophan	0.004
L-Tyrosine.2Na.2H2O	0.026
L-Valine	0.0235
Vitamins	
D-Biotin	0.001
Choline Chloride	0.001
Folic Acid	0.001
myo-Inositol	0.002
Niacinamide	0.001
D-Pantothenic Acid (hemicalcium)	0.001
Pyridoxal.HCL	0.001
Riboflavin	0.0001
Thiamine.HCL	0.001
Other	
D-Glucose	1.0
Phenol Red	0.011

Chapter Two

CSF Proteins during Development

Introduction

Background to Plasma Proteins in CSF and Relation to Early Brain Development

From the very earliest stages of development the inner surface and later also the outer surface of the central nervous system is bathed in a fluid (cerebrospinal fluid, CSF) which is constantly being secreted and reabsorbed. Its composition, protein and electrolyte content is highly specific for particular stages of brain development and has been investigated extensively in various species (for reviews see Saunders and Bradbury, 1973; Davson *et al* , 1987; Dziegielewska and Saunders, 1988; Saunders, 1992).

CSF Protein Concentration (mg/100ml)	Sheep	Pig	Rat	Rabbit	Chicken	Wallaby	Opossum*
Peak during Development	1143 ± 83 (31 days)	961 ± 95 (31 days)	317 ± 15 (22 days)	529 ± 18 (20 days)	523 ± 16 (11 days)	405 ± 21 (42 days)	499 ± 25 (23 days)
Adult CSF	26 ± 2	31 ± 3	24 ± 8	29 ± 3	141 ± 15	66 ± 3	23 ± 3.8
Age (in days) post-conception at birth	150	115	22	32	21	28	14

Table 2.1; Total protein concentrations in CSF of 7 species. The comparison is between the levels of protein at the peak of CSF concentration and the levels in the adult. Timing of the peak in days post conception in brackets. Also shown are the ages of the different species at birth. Mean ± SEM. (from Dziegielewska and Saunders, review 1988). * from Dziegielewska *et al* (1989).

The protein concentrations within the CSF have been measured in many different species and in all those studied so far the levels during the early stages of brain development are much higher than in the adult. The variety of animal species studied so far is wide-ranging in both size and evolutionary terms. However in all species studied, a peak of protein concentration in the CSF occurs early in brain development (see Table 2.1) and the timing of this peak's appearance appears to have no correlation to the time of birth.

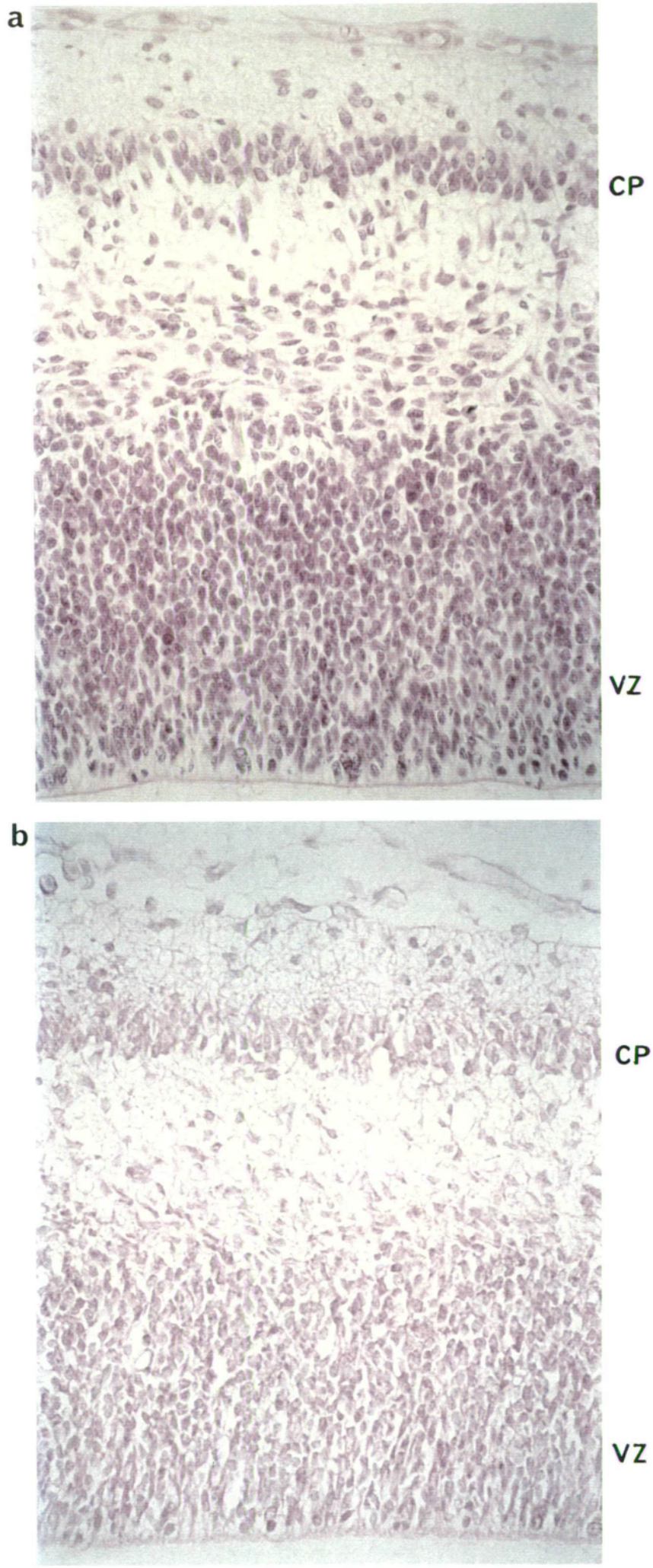
In the eutherian species studied (see Table 2.1) the peak of CSF protein concentration appears in the fetal stages of development. This is compared with the marsupial species (wallaby and opossum), born at an extremely early stage of development, in which the peak occurs postnatally.

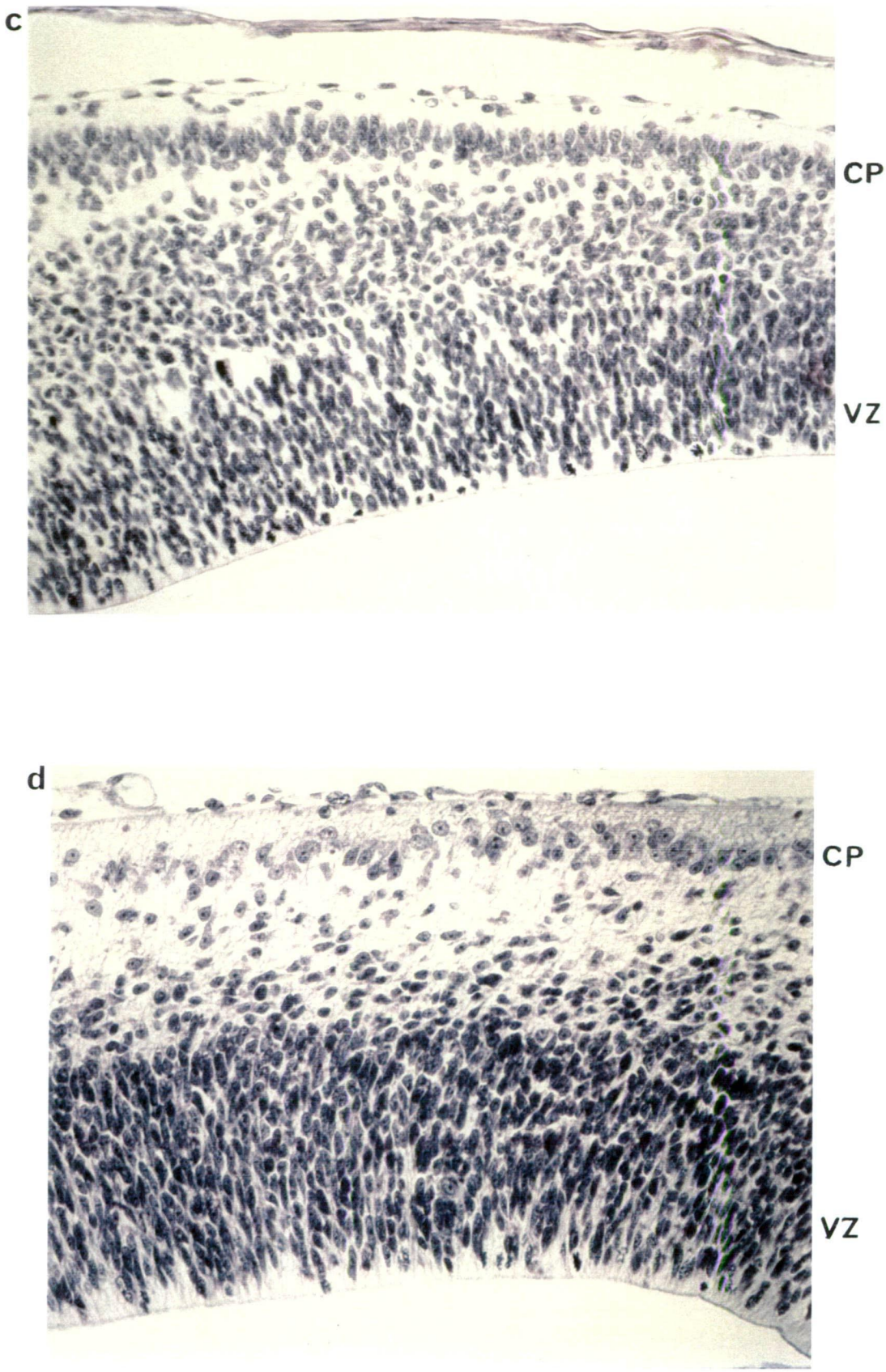
In all these species studied, it appears that the timing of the peak of protein concentration in the CSF coincides with the first appearance of the cortical plate in the neocortex (see section, 1.2; Development of the Neocortex and Table 2.2). The exception seems to be the rat. The rat, which shows very rapid brain development, has a gestational period of 22 days and the cortical plate first appears on day E16 (Bayer and Altman, 1991). From the studies of Dziegielewska *et al* (1981), the peak of protein concentration in the CSF in the rat occurs between E20-E22. The human has a gestational period of 40 weeks and the cortical plate appears during the 7th week (Marin-Padilla, 1983). The concentration of the CSF early in development is also high but it is not known when the peak of total protein occurs. In the marsupial species the appearance of the cortical plate and the CSF protein peak occurs *postpartum.*

Figure 2.1 shows cross sections of the neocortical wall from 4 different species at the age of the peak of protein concentration in CSF (sheep, pig, opossum and wallaby). In all cases the cortical plate has begun to differentiate into a distinct layer of cells.

Figure 2.1

Photomicrographs of cross sections through the neocortical wall from 4 different species at approximately the time when the cortical plate (CP) first appears in the dorsal region of the neocortex; (a), sheep (E34); (b), pig (E31); (c), opossum (P9, 23 days post conception); (d), wallaby (P15, 43 days post conception). VZ; ventricular zone, see Chapter One; Development of the Neocortex. Scale bar = 100μm.


a
CP
VZ
b
CP
VZ


c
CP
VZ
d
CP
VZ

In general, the cortical plate, which is the beginnings of the cortical gray matter, forms within the primordial plexiform layer splitting this region into the outer marginal zone and the inner subplate region (Marin-Padilla, 1983) (see General Introduction). This primordial plexiform layer, which is present before the formation of the cortical plate lies superficially to the ventricular zone, immediately below the pial surface (see section, 1.2, Development of the Neocortex).

Only in the rat does the timing of the peak of total protein concentration appear to be well after the first cells of the cortical plate begin to differentiate. However there are two points which need to be considered:

(1) From which region was the CSF sampled and how does this region compare with other regions during this critical time of development?

(2) If there is indeed a difference in the rat, then what could be the reasons for it?

The study of Dziegielewska *et al* (1981) sampled CSF mostly from the *cisterna magna*, a region close to the IV ventricle (see Figure 2.6), and they found the peak to coincide with the time of birth. A more extensive investigation of the nature of the raised peak of protein concentration within the CSF in different regions of the brain may give a different developmental pattern and a clearer picture as to the changes occurring throughout the CSF.

Cavanagh *et al* (1983) studied the regional differences of protein concentration at the earliest stage of development, using E30-E40 sheep fetuses. Using these young fetuses they examined the composition of the CSF within two different compartments of the brain, the lateral ventricle and the IVth ventricle. At the earliest age examined (E31) the concentration of protein within the two ventricles was similar and at its highest (lateral ventricle, 1533 ± mg/100ml (n=2); IVth ventricle, 1284 ± 105 mg/100ml (n=3)). By 40 days there was a marked difference between the two ventricles: the concentration in the lateral

ventricle fell sharply while that in the IVth ventricle remained similar (lateral, 373 ± 72 mg/100ml; IVth, 1184 ± 133 mg/100ml). At later stages, the CSF protein concentrations within the two different regions have both dropped considerably, the lateral ventricular CSF concentration was always lower than the IVth, even in the adult, as has been known for many years for adult humans (see Davson *et al*, 1987). Therefore, there is a much sharper drop of total protein concentration within the lateral ventricles than in the IVth ventricle. Regional differences like these may account in the rat for the apparent difference in the timing of a protein concentration peak after the appearance of the cortical plate.

	Sheep[1]	Pig[2]	Rat[3]	Wallaby[4]	Opossum[5]
Timing of the Protein Peak in CSF(in days after Conception) and total Protein Concentration (mg/100ml) in CSF	31 days (1143 ± 83)	31 days (961 ± 95)	22 days (317 ± 15)	41 days (405 ± 21)	23 days (499 ± 25)
Time (days after conception) of cortical plate appearance	33-34	26-31	15-16	34-43	17-21

Table 2.2: The timing of the CSF peak of total protein concentration in five animal species (in days after conception) compared to the time in days after conception when cells of the cortical plate first start to differentiate. [1], Reynolds and Møllgård (1985); [2], Cavanagh and Møllgård (1985); [3], Bayer and Altman (1991); [4], Reynolds *et al* (1985); [5], Saunders *et al* (1989).

The nature and origin of the peak of protein concentration within the CSF could be more accurately described if more was known about the characteristics of possible transfer mechanisms which may be present around this time.

Previous permeability experiments have been successful in determining the origins of proteins in CSF at developmental stages after the concentration of protein in CSF has declined from its peak values.

Results so far suggest that the peak of protein concentration is more related to a specific stage in brain development than to the time of birth. To date, no studies have investigated the mechanism(s) which brings about the peak at this early stage.

Origin of Proteins in the CSF

Felgenhauer (1974) compared the molecular radius of individual proteins within adult CSF and compared these with their CSF/plasma ratios. These studies in the adult showed that many proteins in the CSF, which are present in much lower concentration than in the fetus, could be accounted for by simple diffusion through the barrier which separates the blood from the CSF.

The idea of a barrier providing a protection against non-specific leak of molecules from the rest of the body is not a new one. Reviews by Dobbing (1969), and Davson (1967) describe in detail the various underlying mechanisms which collectively are known as the "blood-brain barrier" and more recently reviews have been published by Bradbury (1979), Davson *et al* (1987), Møllgård and Saunders (1986) and Saunders (1992).

The individual proteins within the CSF are immunologically identical to those found in the plasma (Dziegielewska *et al*, 1980a). This has misled many to believe that raised concentrations of protein in CSF early in development are due to an immaturity of the barriers between the blood and the central nervous system (eg. Birge *et al*, 1974; Wakai and Hirokawa, 1981; Adinolfi, 1986). An immature barrier was thought to be unable to stop the non-specific leak of proteins from the blood into the CSF. However, recent evidence which will be summarised below, shows that the brain tissue is protected from proteins in the

circulating blood from very early in its development, but developmentally regulated mechanisms result in a high concentration of protein in fetal CSF.

Proteins within the CSF during the early stages of development are thought to have contributions from four sources:

(1) A component from the plasma itself by passive transfer.

(2) By a specific transport mechanism from the plasma.

(3) From synthesis by epithelial cells of the choroid plexus.

(4) From synthesis within cells of the brain itself.

The quantitative contribution from these sources may be different at different stages of development and also for different proteins.

Transfer of Proteins from the Blood into the CSF

Over the years the nature of the "blood-brain barrier" and the permeability of different kinds of molecules have been investigated. These experiments have usually involved the introduction of a foreign "marker molecule" into the blood of an animal followed by the use of various techniques to detect the marker on the other side of the barrier, within the CSF or brain. In their review of the development of the blood brain barrier, Dziegielewska and Saunders (1988) describe in detail the criteria which must be met in such permeability experiments so that the results are valid. Important points concerning experimental detail are described, the omission of which in some of the previous work has led to much confusion concerning the development of the blood brain barrier in the fetus and newborn.

However, a number of experiments have examined the transfer of proteins from the blood into the CSF early in the development of the central nervous system (CNS).

Dziegielewska *et al* (1980a) estimated the concentrations of different proteins within the CSF of fetal sheep at different gestational ages. Despite similar molecular radii there were significant differences in the CSF/plasma concentration ratios between certain proteins in the younger fetuses studied. Felgenhauer's comparison of serum/CSF steady state ratios for different proteins with their hydrodynamic radii showed that most of the proteins within the adult CSF can be accounted for by passive diffusion from the blood (Felgenhauer, 1974). However, the study by Dziegielewska *et al* (1980b) showed that early in brain development there appeared to be increased concentrations of some proteins in the CSF which could not be accounted for by passive diffusion alone. One possible explanation for this was a greater penetration from the plasma, mediated by a transfer mechanism.

Fetal Age (days)	Albumin	Fetuin	AFP
35	26.9 ± 3.2	63.0 ± 3.8	52.6 ± 9.8
60	11.6 ± 1.1	15.1 ± 2.4	15.4 ± 2.0
Adult	0.30 ± 0.05	0.5 ± 0.2*	—
Diffusion Coefficient ($x10^{-7}$, $cm^2.sec^{-1}$)	6.10	6.20	5.73

Table 2.3; CSF concentrations (mg/100ml) for albumin, fetuin and α-fetoprotein (AFP) in fetal sheep at two gestational ages 35 and 60 (days) and in the adult; mean and S E of the mean. Diffusion coefficients at 20°C in water (from Dziegielewska *et al* 1980b). Note the different steady state levels of all three proteins with similar diffusion coefficients. AFP is not detectable in the adult. (* from Dziegielewska, 1982)

This was investigated by keeping a steady state level of different marker proteins within the plasma of sheep fetuses of gestational ages between E57 and E86 (Dziegielewska *et al*, 1980b). At E60 different proteins of similar molecular size penetrated the CSF to a different extent, and resulted in different steady states. In the older fetuses however this penetration had been reduced with no indication of differential penetration for different proteins. The increased penetration in the younger fetuses was greater than could be accounted for by simple diffusion.

Other studies have also found there to be regional differences in the ability of certain marker molecules to penetrate into the CSF (Cavanagh *et al*, 1983). They found that CSF from the lateral ventricles reached barely detectable levels of the marker protein (CSF/plasma ratio (%): ^{125}I-HSA 0.35%, 5-6 hours after the IP injection of the marker protein) whereas the natural endogenous albumin was at a much greater level (CSF/plasma ratio: 15%) This compared with much higher levels of exogenous albumin penetration into the area of the IVth ventricle, the region contained within the hindbrain, (14% CSF/plasma ratio ^{125}I-HSA, 41% CSF/plasma ratio for endogenous albumin). There were however some limitations to this study. The time period allowed for penetration of the marker protein into the lateral ventricle was only 2 hours; it is not clear if this was long enough for steady state to have been reached in this region. The authors point out that penetration into this region may take much longer. They also go on to mention the other probable contributors to the protein concentration, namely synthesis by the brain itself and also by the choroid plexus. This is the only study which has looked at the penetration of plasma proteins into the CSF close to the time of the peak protein concentration. All subsequent studies have concentrated on this transfer at some time after the peak and well after cortical plate formation.

The choroid plexus, a major site of CSF secretion and the main interface between the blood and the CSF, is found in each of the main brain ventricles throughout the ventricular system. Jacobsen *et al* (1983), using fetal sheep, had found that there were marked differences in the proportion of choroid plexus epithelial cells which contain different plasma proteins at different ages. They

found that a number of cells stained positively for various plasma proteins. The number of cells staining for fetuin, AFP and albumin in the choroid plexus from the telencephalic vesicle was between 30-50% of the total number of cells at E40. This had fallen to 10% by E60. Differences in staining for plasma proteins within epithelial cells of the choroid plexus have also been reported in the human embryo (Jacobsen *et al* 1982a, 1982b). There appears to be no direct correlation between this proportion of epithelial cells in the choroid plexus which are positive for intracellular plasma proteins and the concentrations of these proteins in the CSF. Differences in the regional concentrations are known in the adult, although the actual concentrations are much lower than in the fetus and the newborn (see Davson *et al*, 1987). Early in development however, these regional differences have only been investigated in the sheep (Dziegielewska *et al*, 1980a, 1991; Cavanagh *et al*, 1983; Reynolds *et al*, 1983).

Dziegielewska *et al* (1991) used the fetal sheep at E60 in order to investigate whether the differences in protein concentration of the CSF in different regions were due to either differences in the non specific permeability or due to a specific transfer mechanism which behaved differently in different regions of the CSF system. Small, inert molecules, sucrose and inulin, were used to assess the passive levels of permeability and the plasma protein albumin was used to investigate the transfer mechanism. Different species of albumin (goat serum albumin, GSA; human serum albumin, HSA; and bovine serum albumin, BSA) were measured in different regions of the CNS after a steady state level had been reached following intravenous infusion (IV). Three compartments of the brain: dorsal subarachnoid space, lateral ventricle and the *cisterna magna* (a subarachnoid region outside the IVth ventricle, immediately below the cerebellum) were sampled. With the exception of GSA, concentrations of the exogenous albumins were lower than the natural endogenous one. The foreign albumin markers were at lower concentrations within the lateral ventricles than in the other two compartments, as was also the case for endogenous albumin.

The study of Dziegielewska *et al* (1991) also showed however, a more striking finding, that of a significant difference of steady state levels between different species of exogenous albumin. Goat serum albumin penetrated to the same extent as sheep's own albumin yet bovine albumin reached only 50% of the steady state level. HSA showed the least amount of penetration as did the SSA which had been chemically modified (iodinated or succinylated). These series of experiments clearly demonstrate the presence of a species specific transport mechanism for albumin across the blood-CSF barrier early in development. These marker proteins are identical in terms of their molecular radius and yet show different permeabilities across the barrier.

The nature of this species specific transfer mechanism for albumin was further investigated by Habgood *et al* (1992) in 3 and 20 day old rats (P3 and P20). They were able to show that this phenomenon is not only particular to sheep but also the rat and that the species specific transport mechanism changes during development. At P3 Habgood *et al* (1992) estimated that only about a quarter of the albumin enters the CSF by diffusion from the blood with the remainder appearing to be transported actively. This value was calculated by using the inverse correlation between steady state CSF/plasma ratios and molecular size for plasma proteins in adult human CSF (Felgenhauer, 1974) and adult sheep CSF (Saunders, 1992). Therefore the larger the molecular radius the lower will be the levels of penetration into the CSF. This correlation holds, in fetal sheep, for inert markers such as sucrose and inulin as well as some foreign proteins (Dziegielewska *et al*, 1979). However, in the more immature animals the levels of penetration of endogenous plasma proteins into the CSF are greater than can be accounted for by simple diffusion (Dziegielewska *et al*, 1980b, 1991; Habgood *et al*, 1992). Habgood *et al* (1992) found that at P20 in the rat the penetration of albumin had declined significantly, with no distinction between different species of albumin or between endogenous and chemically modified albumin (succinylated albumin).

Dziegielewska *et al* (1991) showed, by using immunocytochemistry, that the route of entry for albumin is across the choroid plexus epithelial cells and the authors suggested that the different number of choroid plexus cells stained for albumin, both endogenous and exogenous, in different regions of the CNS may account for the striking differences in the concentrations of different species of albumin. This may also account for the differences in regional total protein concentrations. Total protein concentrations within various regions of the CNS reported by Dziegielewska *et al* (1991) were similar to those obtained previously by Cavanagh *et al* (1983).

In summary then, the raised levels of protein within the CSF early in the development can be entirely accounted for by entry from the blood in the case of albumin. This is facilitated by mechanisms present during the early stages of brain development which are able to differentiate between different species of the same protein (ie. albumin) and also between different proteins (Dziegielewska *et al*, 1980b). Differences in the overall concentration of protein between different regions of the CSF spaces may be due to changes of the specific transfer mechanism between areas. Another possible source of protein in the CSF early in development could be due to *in situ* synthesis ie. production of certain proteins within regions of the CNS. In all species studied so far, apart from the rat, the increased concentration of protein seems to reach a peak at similar stages of brain development.

Synthesis of Plasma Proteins in the CNS

The contribution made by the *in situ* synthesis of proteins to the level of protein within the CSF became clearer when it was shown that the levels of prealbumin within the CSF of the adult were much higher than could be accounted for by simple diffusion from the plasma (Felgenhauer, 1974). More recently the detection of mRNAs for plasma-like proteins (Levin *et al*, 1984; Dickson *et al*, 1985; Bloch *et al*, 1985; Dziegielewska *et al*, 1985, 1986b, 1993;

Møllgård *et al*, 1988) and evidence provided by amino acid incorporation experiments (Ali *et al*, 1983; Dziegielewska *et al*, 1984) indicates that *in situ* synthesis within the brain and choroid plexus may contribute to the high protein concentration in the CSF early in development.

Prealbumin, albumin, transferrin and AFP have all been shown to be synthesised by choroid plexus cells, cultured *in vitro* with ^{3}H-leucine (Dziegielewska *et al*, 1984). The radiolabelled proteins were detected using immunoprecipitation. This detection method uses antibodies to the particular plasma proteins which are able to bind to the equivalent protein produced in the brain or choroid plexus. Therefore immunologically the proteins are identical and described as plasma-like. Studies using techniques which detect mRNA of these plasma-like proteins have shown the synthesis of the proteins such as AFP, fetuin, and transferrin in cells of the choroid plexus of rat fetuses (Dickson *et al*, 1985; Dziegielewska *et al*, 1984).

As well as choroid plexus cells producing proteins which are also present in the CSF, cells of the brain itself appear to be able to synthesise proteins at various stages during their development and maturation. Dziegielewska *et al*, (1984), Møllgård *et al* (1988) and Dziegielewska *et al* (1993) found evidence for *in situ* synthesis of such proteins as AFP, albumin, transferrin, fetuin by brain cells of rat, human, sheep fetuses. The range of plasma proteins found in cells within the brain appears to be large. Møllgård *et al* (1988), using immunocytochemical techniques, studied regions of the human fetal brain and identified a total of 23 plasma proteins in different areas and different cell types. Some proteins were cell type specific while others were present in all cell types.

This evidence together with that of amino acid incorporation studies (Ali *et al*, 1983; Dziegielewska *et al*, 1984) in which cultured brain cells were exposed to radioactive-labelled amino acids followed by immunoprecipitation of proteins secreted or extracted from cells, suggests that proteins within the CSF may be important during the development of the CNS. However, the contribution that synthesis *in situ* makes to the overall total CSF protein levels in the developing

CNS is not clear and in at least some cases, eg. albumin in CSF, concentration can be accounted for by penetration from blood at ages so far investigated.

CSF Sink Effect

An important consideration concerning the higher concentration of protein within the CSF is the rate at which it is turned over, produced and removed from the CSF spaces in the CNS. Amtorp (1976) suggested that the high accumulation of ^{125}I labelled albumin into the CSF of young rats was due to the low rate of CSF production, rather than an increased rate of transfer from the blood. Few experiments have studied the rate of CSF production during the early stages of development. Bass and Lundborg (1973) using the rate of inulin clearance found that at 5 days postnatal the rate of clearance from the CSF was 40% of that measured in 30 day old animals. Johanson and Woodbury (1974) made estimates of the CSF production in young rats and found a correlation between the CSF production rate and age of the animals. Irrespective of the relative permeability of the barrier between the blood and CSF to proteins, the CSF/plasma ratio would eventually reach unity if the CSF production rate was zero. Therefore, the rate of turnover of CSF should influence the actual CSF/plasma ratio. Stastny (1974) inhibited the CSF production in rats and showed that during this time the CSF/plasma ratio for inulin increased.

The influence that the rate of CSF production has on the CSF/plasma ratios is termed the "sink effect".

Present Work

In the present thesis the protein concentration within the CSF of different regions and the mechanism of transfer from the blood to CSF have been studied using two different species: the fetal rat and the neonatal opossum. The aim was

to investigate the temporal relationship between the appearance of the cortical plate and the occurrence of the peak in the CSF protein concentration.

CSF Protein Concentration within the Fetal Rat.

As discussed above the work of Dziegielewska *et al* (1981), using the fetal rat, showed the appearance of the peak of protein concentration later in brain development than in any of the other species studied. The sampled CSF was however mostly from the *cisterna magna*. The present work, again using the fetal rat, has sampled entirely from another region of the CNS, the midbrain vesicle. In order to verify the findings from the 1981 study a separate set of samples were taken from the *cisterna magna*.

Transfer of Protein Between Blood and CSF Before the Total Protein Concentration Peak in CSF

Studying the transfer of proteins into the CSF earlier in development, at a time when there is the maximum concentration within the CSF, using permeability experiments as described above would be technically difficult. Such permeability studies require the marker to reach steady state levels between the blood and the CSF. In eutherian species the peak protein concentration occurs in the early fetal period. Experiments carried out in the fetus therefore make it necessary for the mother and fetus to be maintained under anaesthesia and in a viable condition for long periods of time. At around the time of the peak of total protein concentration (E15 for the rat and E30 for the sheep) this type of experiment may be too difficult due to the small size and fragile nature of the fetus. The small fetus, *in utero*, must be maintained in a healthy condition while steady state levels of the marker are reached and then uncontaminated samples of CSF must be removed from around the brain.

All marsupial species so far investigated do not form their cortical plate until after birth (Reynolds *et al*, 1985; Reynolds and Saunders, 1988; Saunders *et al*, 1989). One such species, *Monodelphis*, has been used in the present study to examine the transfer of proteins from the blood and into the CSF. This animal is born after a gestational period of only 2 weeks and before any cells have migrated from the ventricular zone to form the cortical plate (see section: Development of the Neocortex). This makes this species an ideal model for studying the protein concentration changes within the plasma and the CSF early in brain development. The cells of the cortical plate first appear in this species between P3-P5 (Saunders *et al*, 1989). Like all species studied, the protein concentration within the CSF was higher earlier in development and declined thereafter. There was also a peak of total protein concentration within the CSF at the time that the first neocortical plate cells are starting to differentiate (Dziegielewska *et al*, 1989). This particular study also examined the protein composition of CSF and plasma at four different ages: 1, 9, 45 days and adult. During the course of the present work, CSF and plasma samples were measured also for their total protein concentration in order to verify the results of Dziegielewska *et al* (1989) and to confirm the timing of the protein peak within the CSF during neocortical development.

Using this model two main questions can be answered: 1) Is the specific transfer mechanism, described in sheep and rat also present in the marsupial species and 2) what is the origin of proteins in the CSF at the stage of brain development when the protein concentration is increasing to a peak rather than decreasing from a peak, as investigated in previous experiments (Habgood *et al*, 1992; Dziegielewska *et al*, 1991).

In the present study different species of albumins were used as well as an albumin which had been chemically modified. The use of different species of albumins may give some insight into the selectivity of the species specific transport mechanism during development, especially during the period of peak protein concentration within the CSF. Chemical modification of albumin may

give an indication of the recognition mechanism used in transporting this protein from the blood into the CSF during this early stage of development.

Results

Total Protein Concentration in CSF

Figure 2.2 shows two camera lucida illustrations from sections through an E18 rat fetus, one sagittal, the other parasagittal but close to the midline. These drawings show the areas occupied by the CSF within this developing fetus. The ventricles, lateral (I and II), midbrain (mesencephalic) (III) and the hindbrain (IV) are clearly seen. The *cisterna magna* is also illustrated close to the IVth ventricle and separated by a single cell-thick boundary represented by a thin line. The work of Dziegielewska *et al* (1981) sampled CSF from around this area throughout their experiment using pooled samples in the youngest fetuses. A graph of their results is shown in Figure 2.3 indicating the considerable and significant difference between concentrations of protein in the plasma compared to those in the CSF, even at the very earliest stages of development.

Figure 2.4 shows photographs taken of an E16 rat fetus. Using a fine glass micropipette CSF was drawn out from the lateral ventricle. Using the same puncture site in the neocortical wall, the CSF was replaced with a solution of Indian ink. This opaque black fluid subsequently diffused throughout the ventricles and under intense illumination could be see within the ventricular system of the fetus. The skin and part of the skull of the fetus were also removed for Figure 2.4 (b) and (c) in order to observe to what extent the dye was able to leak out of the ventricles and into the exposed subarachnoid space. The pictures show that 30 minutes after the dye infusion much of it has diffused throughout the ventricular system and yet is still enclosed within it. The dorsal view, Figure 2.4 (b), shows that a small amount has diffused from the injected left lateral ventricle to the right side through what appears to be the interventricular foramen. This connecting region between the left and the right

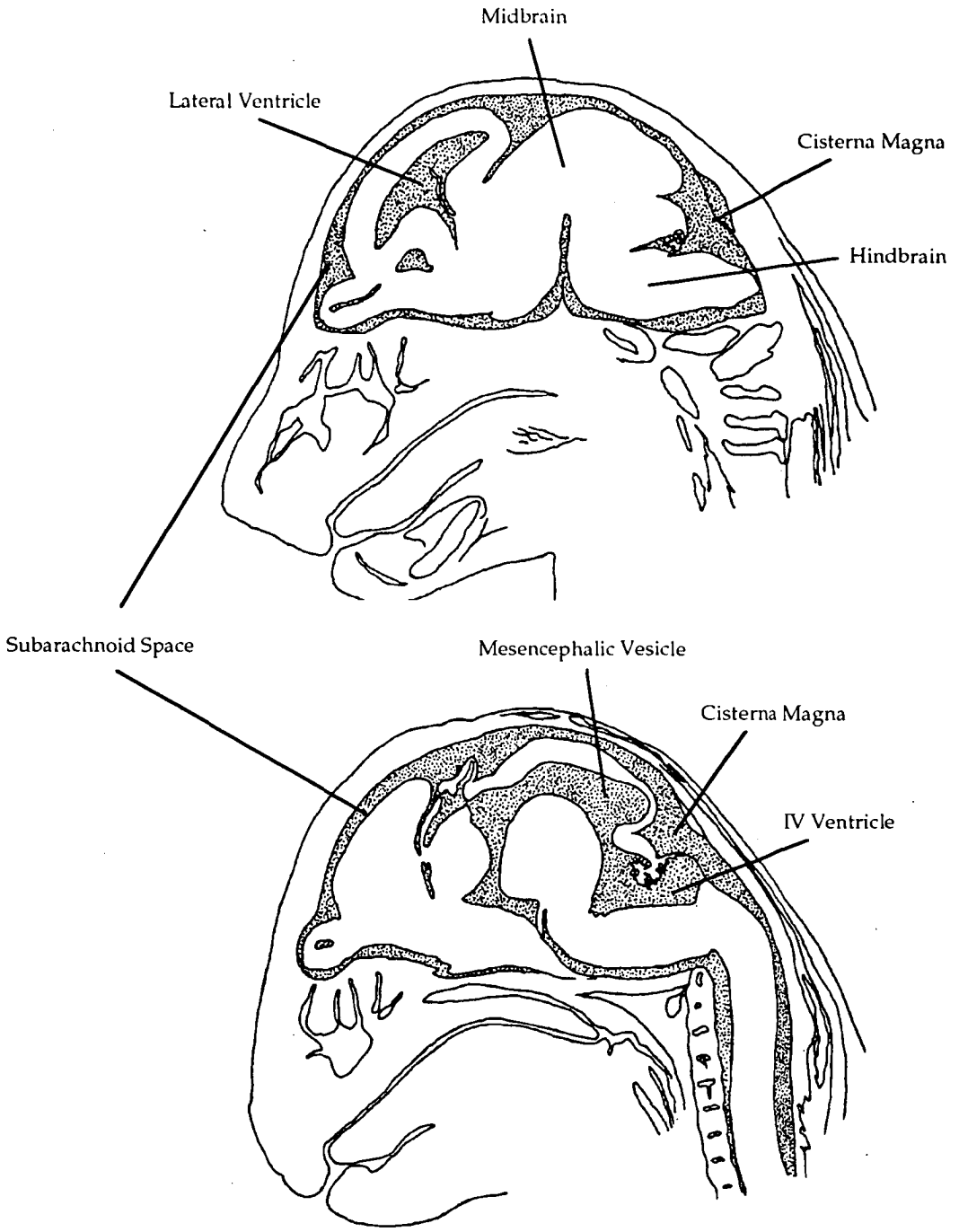


Figure 2.2; Two camera lucida illustrations of sections through an E18 rat fetus; sagittal (bottom) and parasagittal (top) showing the ventricular spaces inside the brain and subarachnoid spaces on the surface.

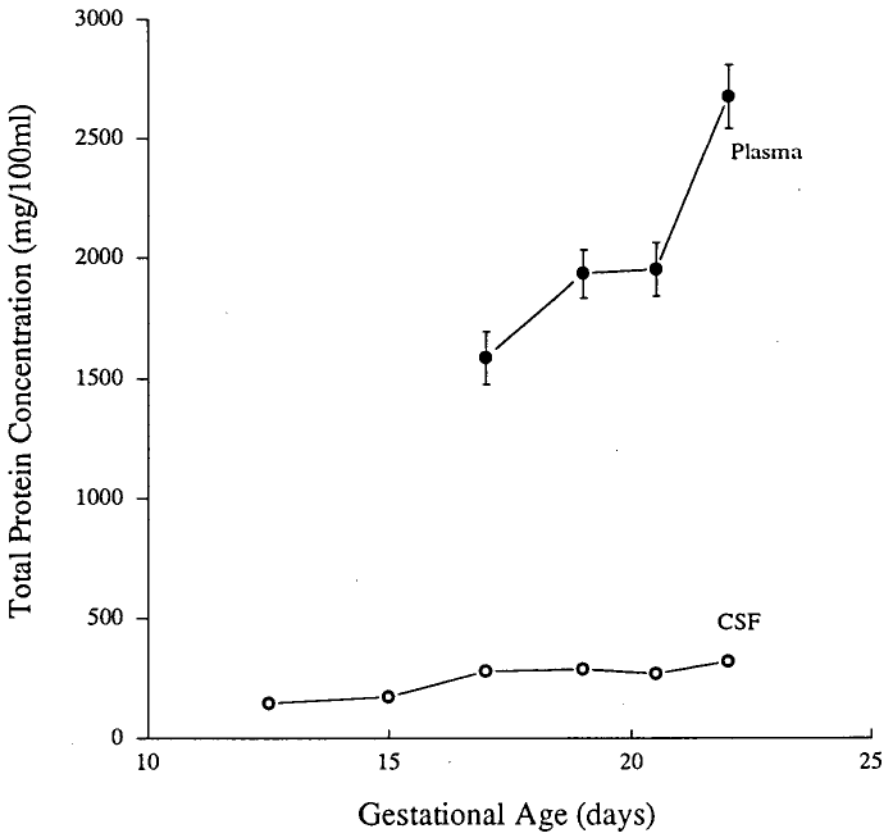


Figure 2.3; Total protein concentration (ordinate, mg/100ml) in plasma and cisternal CSF of fetal rats (abscissa, gestational age, days). Mean values of plasma and CSF shown with SE of the mean for plasma. From Dziegielewska *et al* (1981).

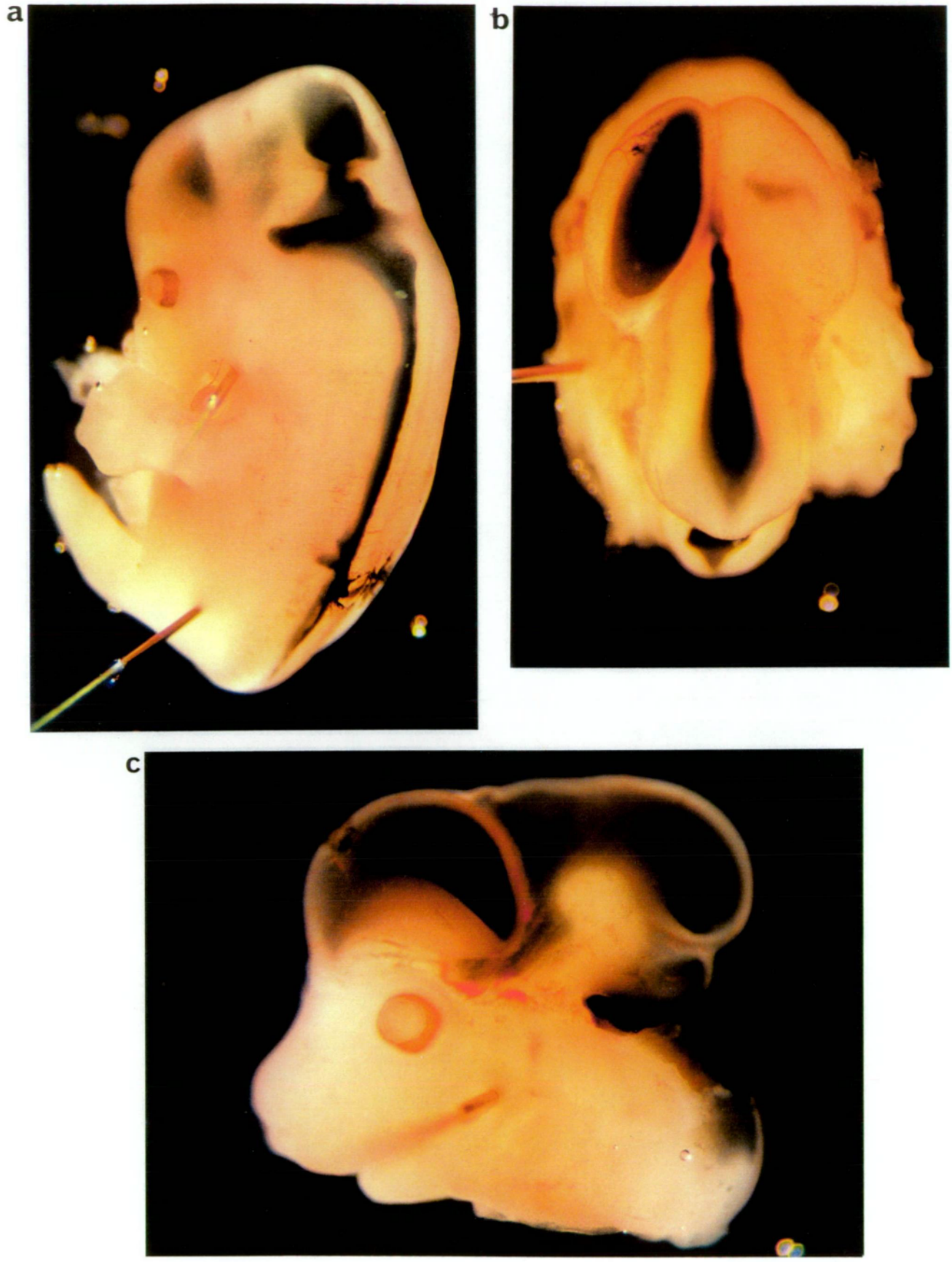


Figure 2.4; Photographs of an E16 rat fetus, 30 minutes after Indian ink was injected into the left ventricle and CSF removed from the spinal cord (**a**). The skull covering the brain was removed in order to visualise more clearly the ventricular system within the brain (**b**, horizontal; **c**, sagittal).

sides of the ventricles allows for unrestricted movement of fluid between the two. A greater amount of dye would have been expected within the ventricle opposite to the side injected due to this free access, however the opening may have been obstructed by part of the choroid plexus tissue which is situated in this region.

In the present study total protein concentration was measured, using the Bradford method (1976), in CSF from the midbrain vesicle (IIIrd ventricle). The same sampling site was used throughout the study. During sampling care was taken not to remove too much fluid and therefore cause damage to tissue within the brain, resulting in contamination of the sample.

Figure 2.5 shows the concentrations of total protein within the IIIrd ventricle at various gestational ages (E14-E20); these are referred to as "ventricular" CSF samples. Removing CSF from this region at anytime after E20 proved difficult due to a much increased thickness of brain tissue through which the micropipette had to be inserted. This made it unclear when the end of the pipette was in the ventricle. The whole ventricular system is interconnected from the first day of closure of the neural tube, therefore it cannot be assumed that a sample of fluid from the IIIrd ventricle came only from this region. It is likely that fluid was also drawn from the other ventricles although how much would depend on the quantity of fluid removed from this region. The graph also plots the results of Dziegielewska *et al* (1981) of total protein concentrations from pooled samples removed from the region of the *cisterna magna*, these are referred to as "cisternal" CSF samples.

Between 12 and 13 days gestation the concentration of cisternal and ventricular CSF protein was lower than later on (ventricular, 92 ± 20 mg/100ml; cisternal, 144 ± 20 mg/100ml). Concentrations of protein at both sites rose during the following few days. The cisternal concentration reached a peak at around the time of birth (317 ± 15 mg/100ml) whereas the peak of protein concentration within the midbrain vesicle occurred much sooner between 15 and 17 days gestation but at lower concentration (E15, 134 ± 21 mg/100ml; E17, 156

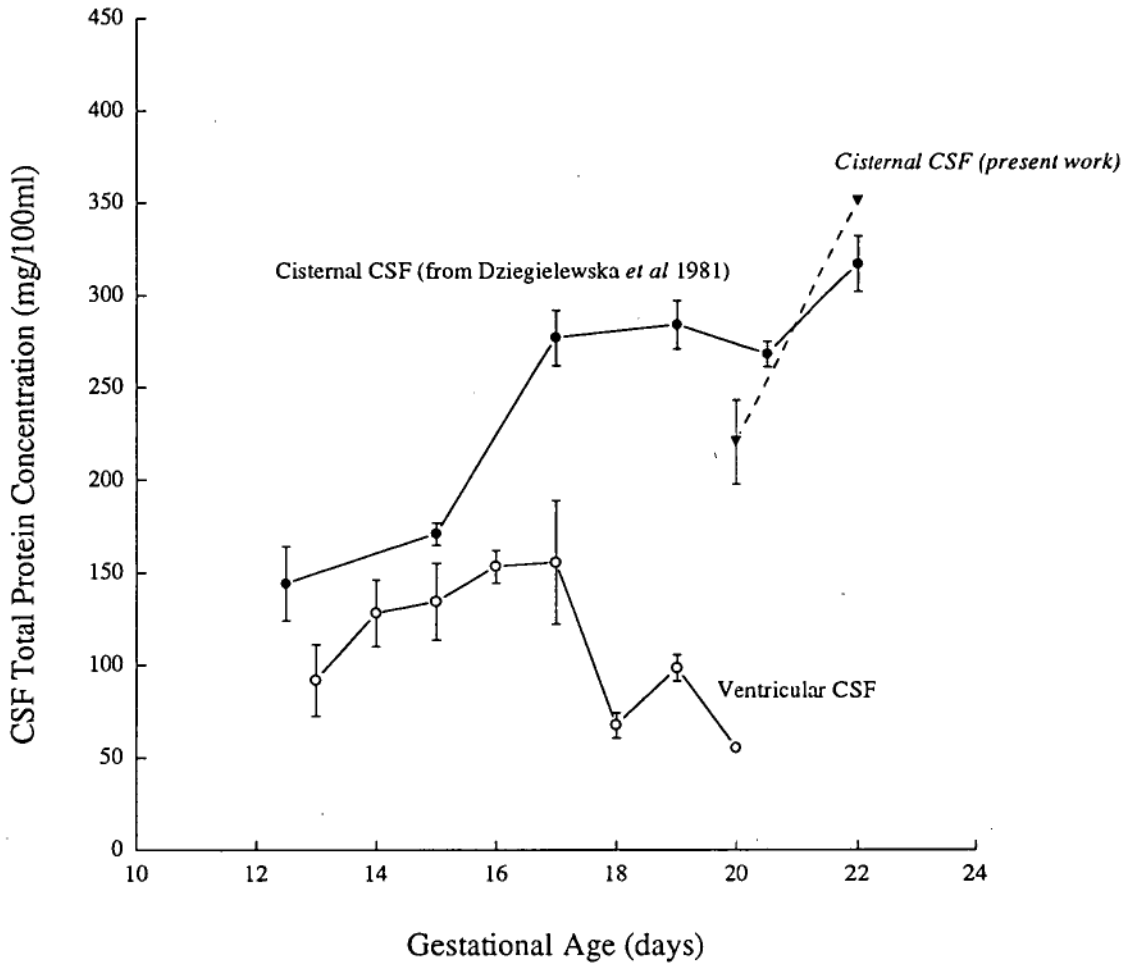


Figure 2.5; Total protein concentrations (ordinate, mg/100ml) in the CSF contained in the IIIrd and the *cisterna magna* of the fetal rat at various gestational ages (abscissa, days). Mean ± SEM. See table 2.4 for n values of the present work.

± 34 mg/100ml). The total protein level in ventricular CSF then declined sharply (E18; 67 ± 7 mg/100ml) at all the subsequent ages sampled.

In order to be sure that the difference between cisternal and ventricular CSF protein concentration was not due to a methodological difference between the present experiments and those of Dziegielewska *et al* (1981), CSF was collected from the *cisterna magna* of fetuses at two ages, 20 and 22 days gestation. The values found at both ages were much higher than those of the ventricular CSF (E20, 221 ± 23 mg/100ml; E22, 351 mg/100ml) and comparable to the measurements of Dziegielewska *et al* (1981) (E20-E21, 267 ± 7 mg/100ml; E22; 317 ± 15 mg/100ml).There is no significant difference between the two groups of E20 values; those from Dziegielewska *et al* (1981) and from the present study ($P > 0.2$).

These results therefore show significant regional differences in total protein concentration during brain development in the rat. The measurements made in 1981 are consistent with the ones in the present study when CSF from a similar region of the fetus, namely the *cisterna magna*, was used.

Concentrations of Total Protein in Cisternal and Ventricular CSF of Fetal Rats

Table 2.4

Ventricular CSF (present work)			*Cisternal CSF (Dziegielewska et al 1981)*		
Age *(days)*	CSF protein concentration *(mg/100ml)*	n	Age *(days)*	CSF protein concentration *(mg/100ml)*	n
			E12-13	144 ± 20	5
E13	92 ± 20	4			
E14	128 ± 18	6			
E15	134 ± 21	13	E15	171 ± 6	12
E16	153 ± 9	25			
E17	156 ± 34	13	E17	277 ± 15	12
E18	67 ± 7	7			
E19	99 ± 7	8	E19	284 ± 13	10
E20	55	2			
			E20-21	268 ± 7	25
			***E20**	***221 ± 23**	***4**
			E22	317 ± 15	2
			***E22**	***351**	***1**

Note; Age in days of gestation. Mean ± SEM.

**Cisternal CSF values sampled during the present work*

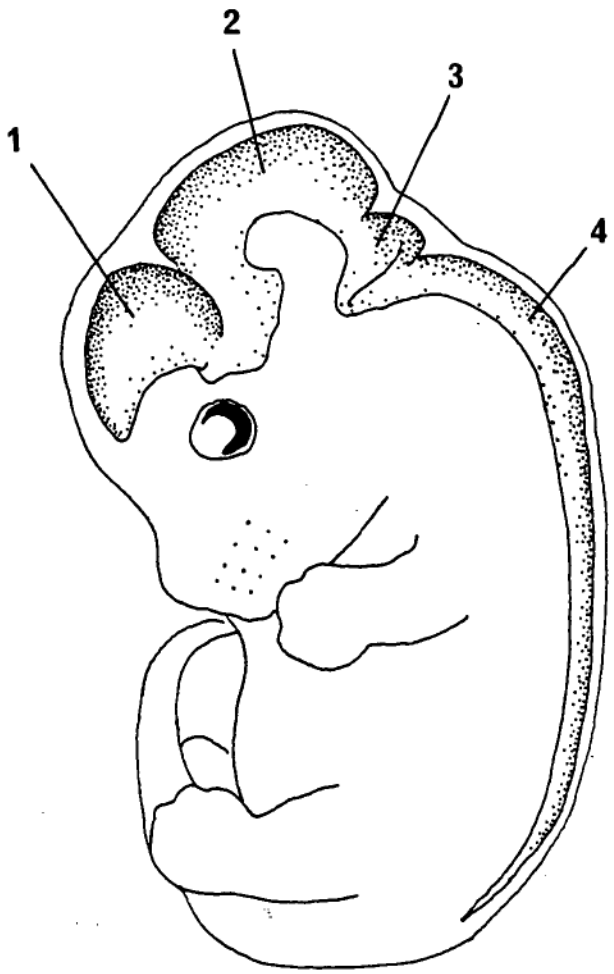


Figure 2.6; Diagrammatic representation of an E15 rat fetus. The stipled region represents the CSF space within the CNS. 1, I and II ventricle within the cerebral ventricles; 2, III ventricle within the mesencephalon; 3, IV ventricle within the rhombencephalon; 4, spinal cord.

Permeability Experiments in *Monodelphis domestica*

Estimations of Steady State

Figure 2.7 shows the concentration of bovine serum albumin (BSA) within the CSF and plasma at 4 different time intervals; 6, 11, 15 and 24 hours after IP injection into P11 *Monodelphis*. Also shown is the CSF/plasma ratio (%) at these times.

At time zero, when the IP injection was given, the levels of the marker protein within the plasma and the CSF were at zero because all of it was contained in the intraperitoneal cavity. The plasma levels then rose steadily and penetration of the marker protein into the CSF occurred. The maximum concentration attained within the CSF and the plasma was after 15 hours (plasma, 460 ± 73 mg/100ml; CSF, 3.52 ± mg/100ml). This was also the time when the CSF/plasma ratio was maximum (15 hours, 8.52 ± 0.76%). Between 15 and 24 hours the fall in the plasma and the CSF concentration is slight which is also mirrored in the very slight drop in the ratio over this 9 hour period (15 hours, 8.5% ± 0.8%; 24 hours, 7.1% ± 1.5%). However, as this difference is not significant (P>0.5) it is clear that a steady state for the injected albumin was maintained for many hours.

Figure 2.8 is a similar graph showing the results obtained in similar experiments in P24 *Monodelphis*. The CSF/plasma steady state ratio was reached sooner than in the younger animals, by 11 hours (11.9% ± 1.3%) and this value was maintained until 15 hours. The actual concentration of BSA within the plasma remained similar for the duration of the experiment. These values were obtained from individual animals using the litter based model (Habgood, 1991, see Methods) which could explain the variation in values.

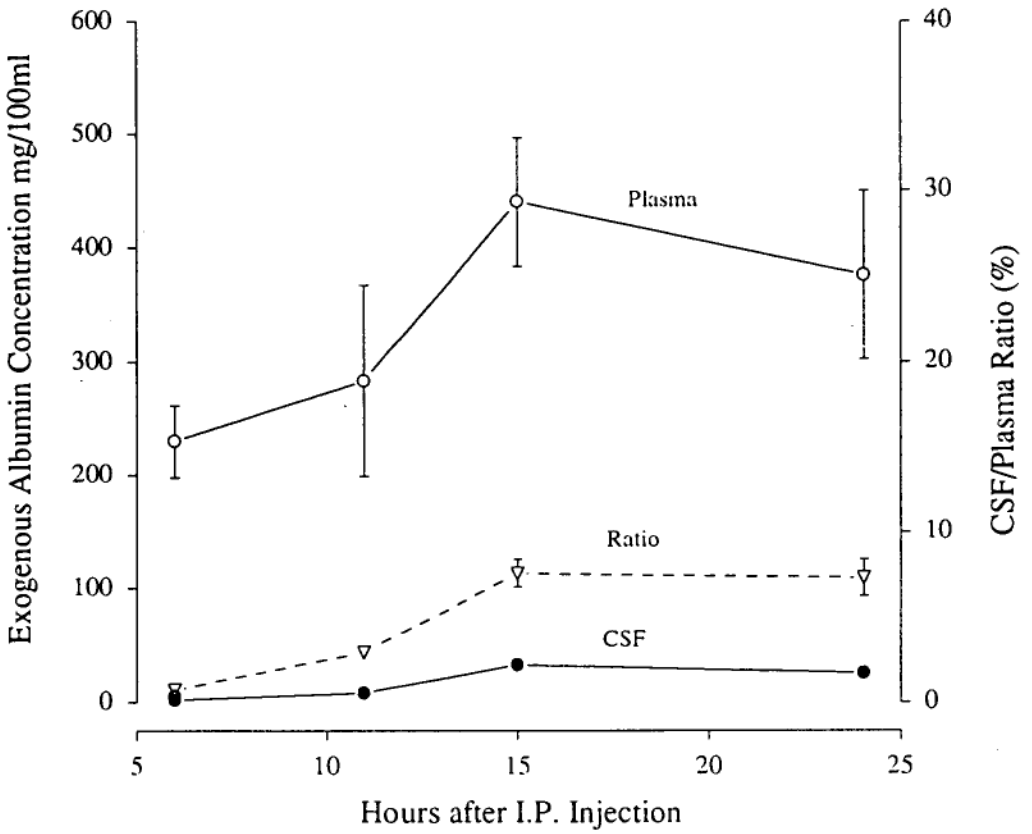


Figure 2.7; Bovine serum albumin concentration (ordinate, 100mg/ml within the plasma and CSF (solid lines): 6,11,15 and 24 hours (abscissa) after IP injection in 11 days old *Monodelphis domestica*. CSF/plasma ratio (%, dotted line). Mean values of plasma and CSF shown with SE of the mean for plasma and ratios.

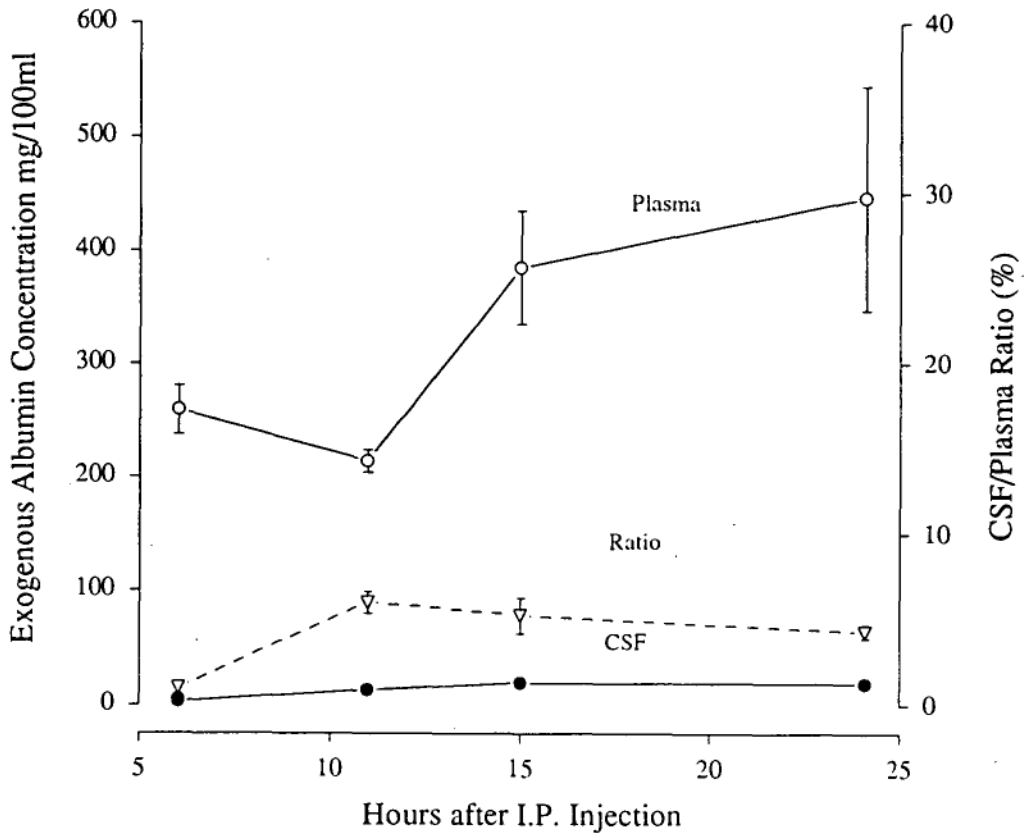


Figure 2.8; Bovine serum albumin concentration (ordinate, 100mg/ml within the plasma and CSF (solid lines): 6,11,15 and 24 hours (abscissa) after IP injection in 24 days old *Monodelphis domestica*. CSF/plasma ratio (%, dotted line). Mean values of plasma and CSF shown with SE of the mean.

From these results at both P11 and P24 in the *Monodelphis* a steady state CSF/plasma ratio would appear to have been reached by 15 hours after a single IP injection of an endogenous albumin. Therefore in all the subsequent permeability experiments in the *Monodelphis* CSF and plasma samples were collected 15 hours after an IP injection.

CSF/Plasma Ratios for *Monodelphis* Albumin

The CSF/plasma ratios for endogenous *Monodelphis* serum albumin (MSA) at different postnatal ages are illustrated in Figure 2.9 and also shown in Table 2.6. The concentrations of this endogenous protein are assumed to be at steady state, a stable concentration of the albumin being maintained in plasma without any major fluctuations over short periods of time. The CSF/plasma steady state ratios early in development were considerably higher in the younger animals than later on (P5, 46.1% ± 4.6; P32-P36, 1.7% ± 0.1). By P20-P24 there was already a significant drop in the ratio compared to the earliest age group; the ratio had fallen to around one fifth of the value at 5 days. The most prominent fall in the CSF/plasma ratio was between the 5 day and 7-8 day period, with the ratio almost halving (5 days, 46.1%; 7-8 days, 25.9%).

Steady State CSF/plasma Ratio for Exogenous Albumins

Figure 2.11 and table 2.6 show the CSF/plasma steady state ratios (%) for the exogenous albumins, human serum albumin (HSA) and bovine serum albumin (BSA) in 5 different age groups; 7-8, 10-12, 15-16, 20-24 and 32-36 days postnatal.

At all the age groups sampled, the endogenous and exogenous albumin ratios became progressively smaller with increasing age. In all animals the amount of foreign albumin injected IP. was calculated to give the same plasma concentration irrespective of the age of the animal. Therefore the lower steady

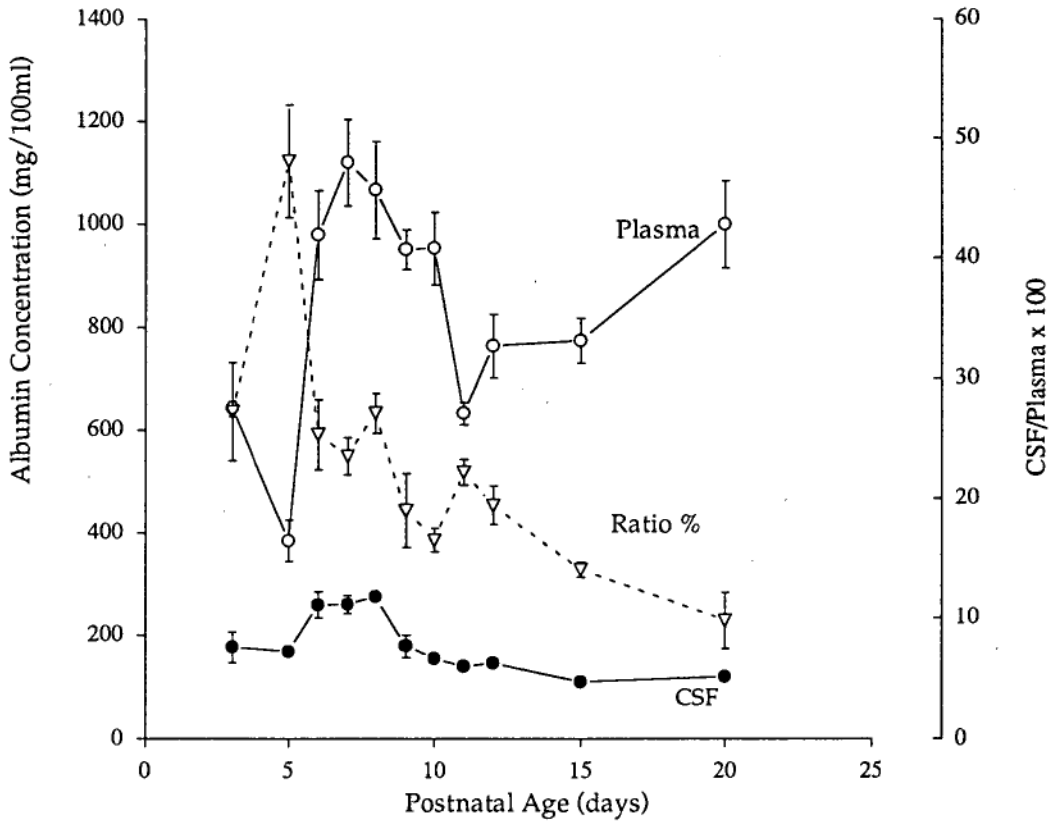


Figure 2.9; Albumin (endogenous) concentration (left ordinate, mg/100ml) in the plasma and CSF of *Monodelphis domestica* at various postnatal ages (abscissa, days). Mean values of plasma and CSF shown with SE of the mean. Also shown is the CSF/plasma ratio (right ordinate, %).

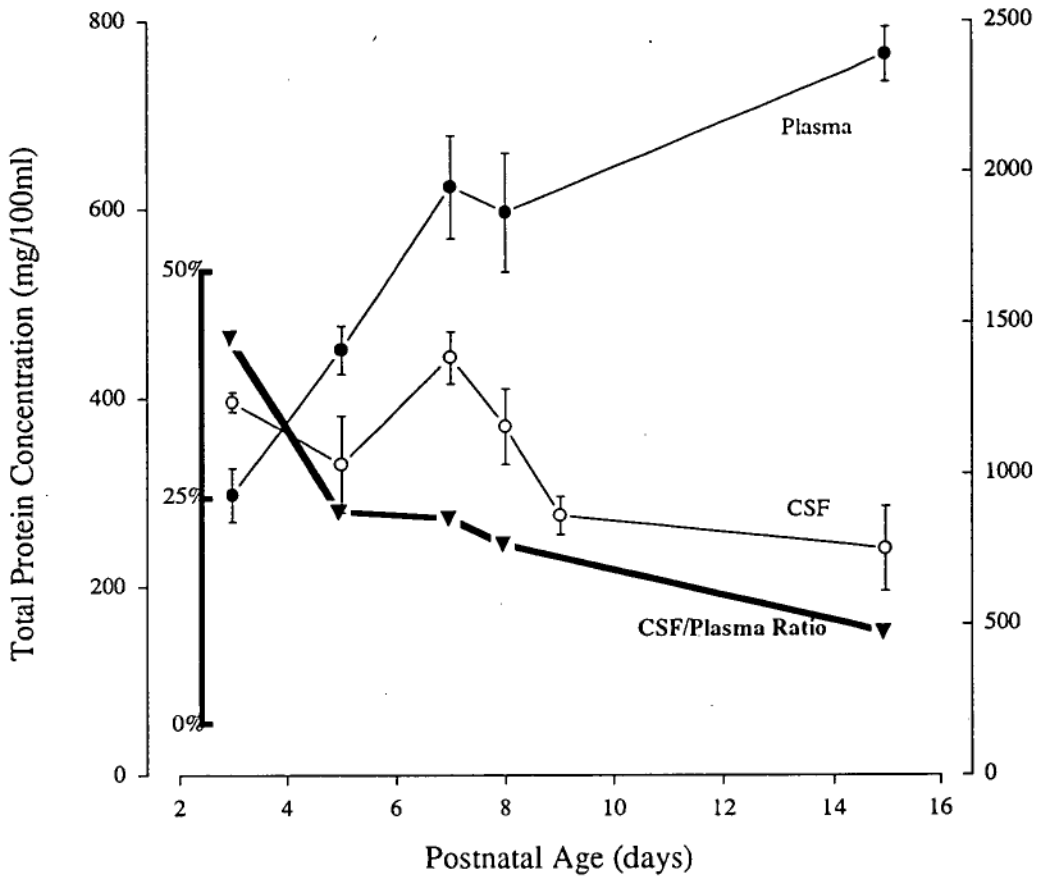


Figure 2.10: Total protein concentration (ordinate, mg/100ml) within plasma and CSF of postnatal *Monodelphis domestica* (thin lines). Ordinate: protein concentration in mg/100ml; abscissa, postnatal age in days. Mean ± SEM. CSF/plasma ratio (%, thick line).

state ratios in all the older age groups is due to lower concentration of the protein within the CSF.

When comparing the CSF/plasma ratios for HSA with those of MSA at the different ages it appears that generally there is no significant difference between the exogenous and the endogenous albumin except in the 20-24 day age group.

The other exogenous albumin used, BSA, however reaches CSF/plasma ratios which are significantly different and lower than the MSA. This is with the exception of the youngest age group (7-8 days) whose CSF/plasma ratio was not significantly different from MSA. Therefore, in this 7-8 day age group the CSF/plasma ratios for both the HSA and BSA were similar to the MSA (MSA, 25.9% ± 1.0; BSA, 22.4% ± 1.4; HSA, 25.4% ± 2.3).

In the oldest age group (32-36 days) the CSF/plasma ratios for both the BSA and the HSA were higher than the MSA. This higher CSF/plasma ratio was probably due to changes in the sink effect at the older ages. The increased drainage of CSF at the older ages could cause differences between these and the younger animals.

In general, HSA appears to have similar steady state CSF/plasma ratios to the endogenous albumin levels and therefore both proteins appear to penetrate into the CSF to a similar extent. This is in contrast to BSA which shows a significantly lower permeability level into CSF except in the earliest age group studied (P7-P8).

Steady State CSF/Plasma Ratio for Iodinated Albumin

Iodinated albumin (^{125}I HSA) provided an alternative method for measuring the CSF/plasma concentration ratios in otherwise similar permeability experiments. The results (described above) from using the immunological method of measuring albumin concentrations can therefore be compared with

those from using radioactive counting. The iodinated albumin was injected into animals in 3 different age groups; 7-8, 10-12 and 20-24. In the two later age groups the ratios of ^{125}I labelled HSA were not significantly different from those of the unlabelled HSA. In the earliest age group there appeared to be a difference between the labelled and non-labelled HSA although the number of animals injected with the radioactively labelled albumin was small (n = 3). This was too few for statistical analysis.

Steady State CSF/plasma Ratio for Modified Exogenous Albumin

The modified albumin, succinylated bovine serum albumin (succ-BSA) was used in 3 groups of animals; 7-8, 10-12 and 15-16 days of age. This was to study the possible effect that chemical modification could have on permeability of the protein into the CSF. In all three groups the CSF/plasma ratio was significantly lower than the *Monodelphis* albumin levels ($P<0.001$). Like other albumin species, both exogenous and endogenous, the ratio for succinylated BSA in the early age was much higher than later (7-8 days succ-BSA, 17.2% ± 4.1).

Postnatal Age (days)	MSA	BSA	HSA	Succ-BSA	I^{125}-HSA
5	46.1 ± 4.6 (14)	—	—	—	—
7 - 8	25.9 ± 1.0 (18)	22.4 ± 1.4 (18) n.s.	25.4 ± 2.3 (12) n.s.	18.3 ± 1.3 (7) P<0.001	17.2 ± 4.1 (3)*
10 - 12	18.3 ± 1.0 (18)	11.5 ± 0.9 (16) P<0.005	15.1 ± 1.2 (9) n.s.	3.1 ± 0.4 (6) P<0.001	15.1 ± 1.1 (5) n.s.
15 - 16	10.8 ± 0.6 (28)	6.6 ± 0.4 (6) P<0.001	11.0 ± 1.2 (13) n.s.	5.84 ± 0.2 (7) P<0.001	—
20 - 24	8.1 ± 0.5 (6)	4.3 ± 0.4 (5) P<0.001	6.6 ± 0.4 (11) P<0.01	—	6.8 ± 0.2 (11) P<0.01
32 - 36	1.65 ± 0.1 (12)	2.6 ± 0.3 (8) P<0.005	2.8 ± 0.3 (3) P<0.05	—	—

Table 2.6; Steady-state CSF/plasma ratios for various species of albumins (MSA, monodelphis serum albumin; HSA, human serum albumin; BSA, bovine serum albumin and modified albumins; I^{125} HSA, iodinated human serum albumin and Succ-BSA, succinylated bovine serum albumin) mean and S.E.M. shown with "n" in brackets. MSA ratios are the naturally occurring steady state CSF/plasma ratios, all others ratios were measured 15-20 hours after I.P. injection. P values indicate significant difference between the exogenous albumin species and MSA. * Too few values for statistical analysis.

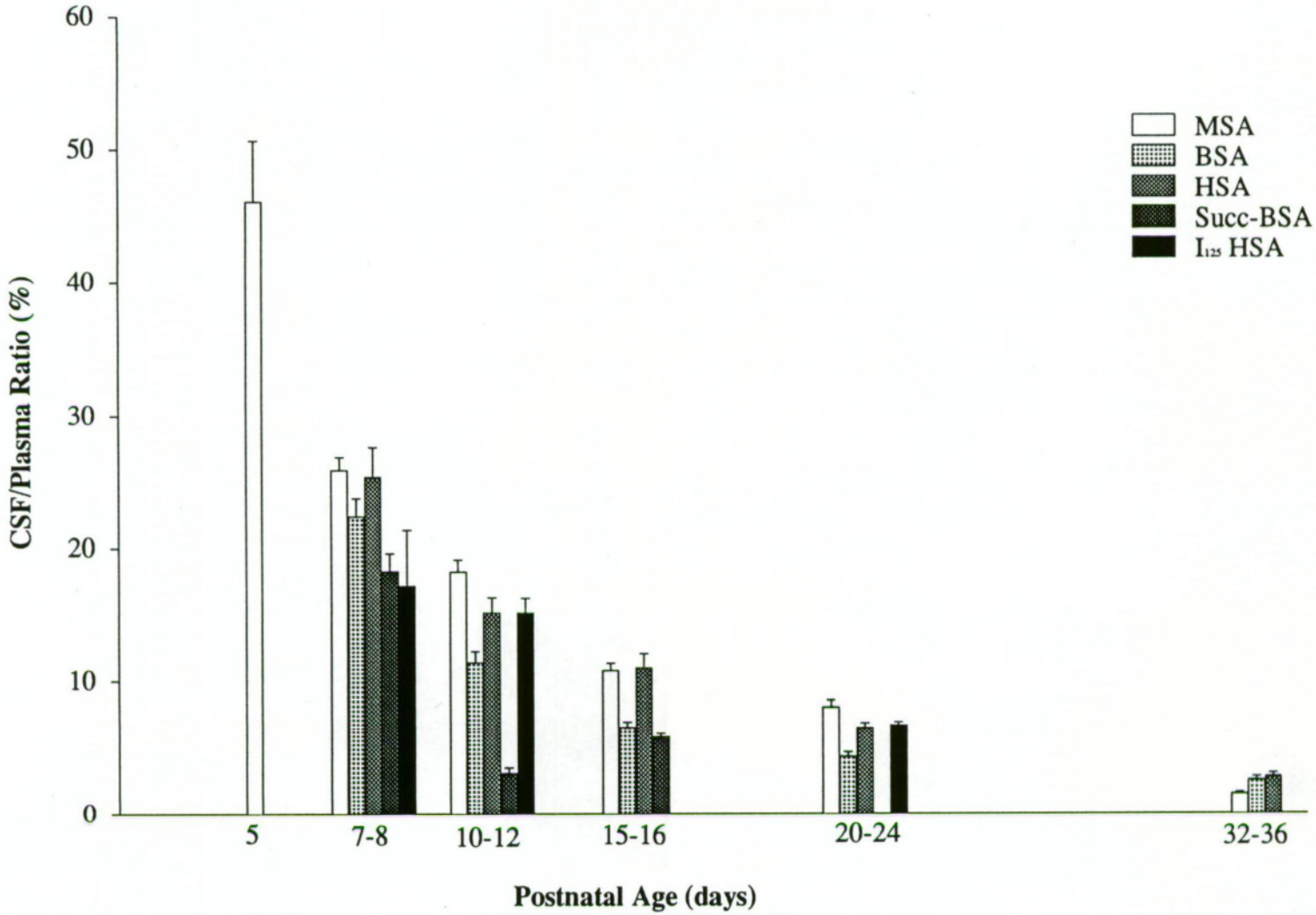


Figure 2.11; CSF/plasma ratios (%) for endogenous and exogenous albumin sampled 15 hours after IP injection in *Monodelphis domestica* at various age groups. Error bars show SEM. See table 2.6 for n values and significant differences.

Discussion

CSF Protein Concentrations within the Ventricles of the Fetal Rat

In the present study, the total concentration of proteins in CSF within the midbrain vesicle (IIIrd ventricle, in the adult) was measured. This is a large and prominent space in the very young fetuses from which fluid is more easily removed than from the lateral ventricle. Measuring the total protein concentration from this ventricle would be expected to give a developmental profile of total protein concentration changes occurring in the ventricular CSF compared to the study of Dziegielewska *et al* (1981) which estimated protein concentrations in CSF from the *cisterna magna*. Figure 2.3 illustrates the results from Dziegielewska *et al* (1981) and shows the striking difference between protein concentrations of the CSF in the *cisterna magna* and those in the plasma at this early stage of development. It provides a suitable illustration for the term "barrier", even in the very young animals. At this very early stage the brain and the spinal cord are undergoing rapid and large changes and yet throughout their growth, the fluid environment which surrounds their internal and external surfaces contains only a fraction of the protein concentration which is in the plasma.

In the present study a peak of protein concentration was found earlier, (E15-E17), than in the previous study of the rat fetus, at around the time which is consistent with other species. In the present studies the protein concentration was measured within the midbrain vesicle, whereas the studies of Dziegielewska *et al* (1981) measured it from the region of the *cisterna magna*. Cisternal samples were also removed in the present study in order to verify the total protein concentration values of Dziegielewska *et al* (1981) and these were found to be similar. Sampling CSF from the *cisterna magna* of younger fetuses (E15-E17) was technically difficult and not easy to determine from exactly which region of

the CSF spaces the CSF had been sampled. These two CSF spaces in the older fetuses are more defined and larger, making it easier for significant volumes of CSF to be removed. It was far too difficult to sample CSF from the lateral ventricles at all ages. It was for this reason that the midbrain vesicle was used. This site is closer to the lateral ventricle than the *cisterna magna*. Therefore, the peak of protein concentration in the ventricular CSF in the rat fetus occurred at E15-E16, which is also the time at which the cortical plate first appears. This corresponds to the association between the peak of CSF protein concentration and early cortical plate formation, that was described in the Introduction, for several species including the fetal sheep (Cavanagh *et al*, 1983; Dziegielewska *et al*, 1991, see Table 2.2 and Figure 2.1) or fetal pig (Cavanagh and Møllgård, 1985). The results from the present study also show that there are regional differences in the protein concentration in the fetal rat CSF, as has been described before for other species (see Introduction).

The large and significant regional differences in protein concentrations between the fluid removed from the ventricles and the fluid removed from a region on the outer surface could be a reflection of little or no communication between the two. The outer surface is the subarachnoid space in which the *cisterna magna* is situated. It is from this region that the *cisternal samples* were removed. The level of communication between the ventricles inside the brain and the subarachnoid space on the outside was first investigated fully by Weed (1917, reviewed by Davson *et al*, 1987).

A simple "Weed" type of experiment in which Indian ink dye was injected into the ventricular system of the E16 fetus (figure 2.4) does not show any obvious pathway to the brain's outer surface.

Early in development, the ventricles originally form as dilatations of the anterior end of the closed neural tube and are isolated from the external surface of the CNS. The fourth ventricle is separated from the *cisterna magna* by the neuroependymal lining of the neural tube often referred to as the rhombencephalic roof of the fourth ventricle. This barrier between the inside and

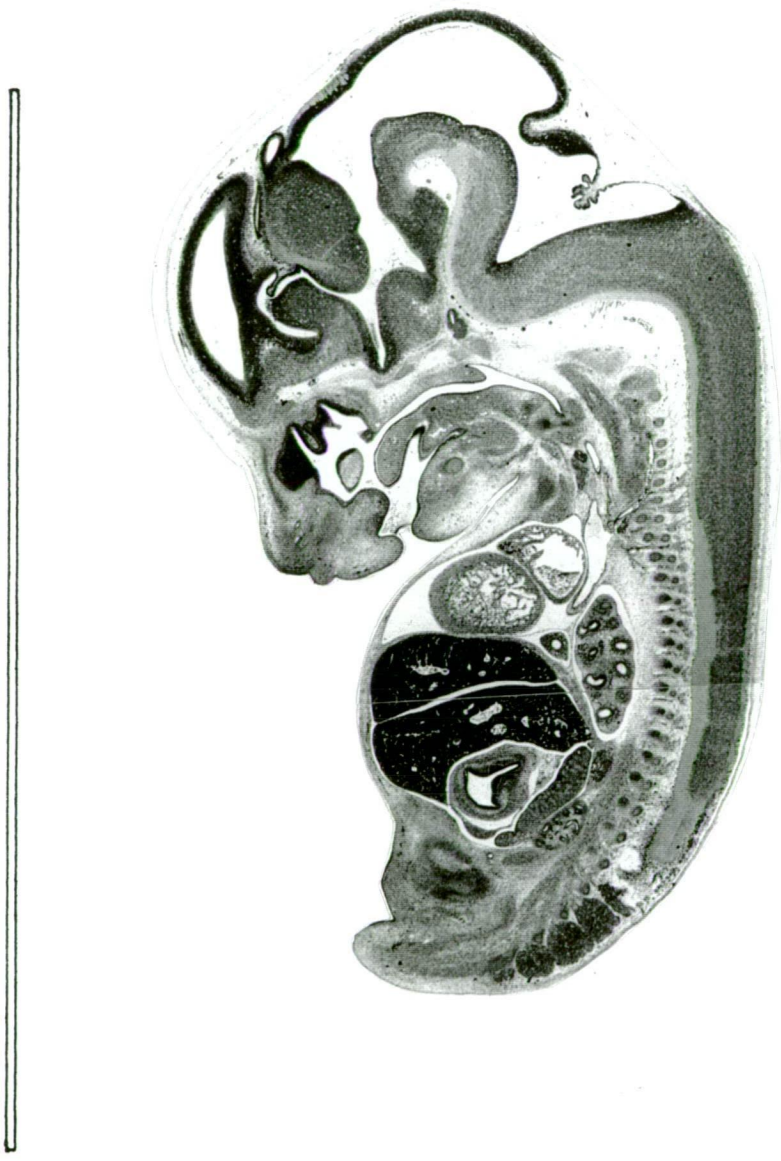
the outside of the brain becomes less obvious in the later stages until eventually opens up with the formation of the foramina of Luschka (Jones, 1980), two lateral openings of the IVth ventricle. Immediately before this opening, Weed (1917) found that this single layer of cells was permeable to intraventricularly injected substances: Prussian blue reagents (these consisted of an isotonic mixture of potassium ferrocyanide and ferric ammonium citrate which are later acidified, after fixation to form the blue colour). Similar results were later obtained by Jones (1980) and Jones and Sellars (1982a, b) who showed, under the electron microscope, the presence of pores situated in the rhombencephalic roof and demonstrated later in development the ability of CSF to pass through this apparent barrier, before the opening of the foramina of Luschka. By injecting dextran, molecular weight 150K, into either the lateral or the mesencephalic vesicle (midbrain vesicle) of the fetal rat Jones and Sellars (1982b) found that after E17 this large molecule was able to pass from the ventricles into the subarachnoid space. This is at a time before the ventricles had opened up to the outside of the CNS by the formation of any significant openings or foramina. It was not clear from the above work what effect, if any, the dextran injection had on the pressure within the ventricles and if this could have caused any disruption to the roof of the rhombencephalon. Dextran is a large molecule (MW, 150K), much larger than most proteins (albumin MW, 60K), and this result would suggest that diffusion of proteins from the ventricles to the subarachnoid space is possible.

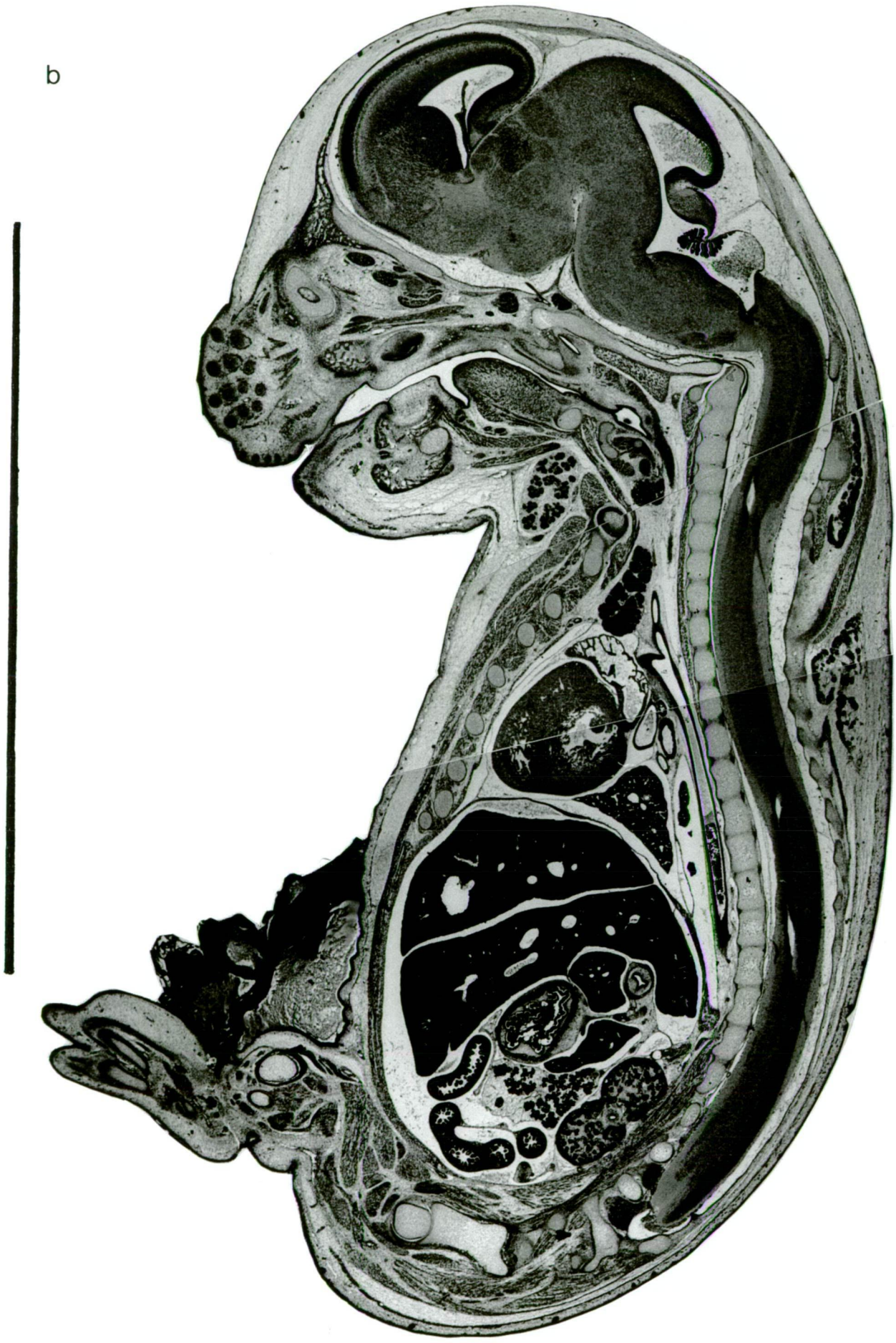
Figure 2.2 shows two camera lucida representations of an E18 fetus showing the ventricular space and the subarachnoid space with the separation between the two. What is not clear is to what extent the inside of the CNS communicates with the outer surface of the brain and spinal cord. Figure 2.12 shows two para sagittal sections through two rat fetuses; one E15 and the other E18. The cisternal and subarachnoid region surrounding the brain at E18 are well defined compared to the E15 fetus which has larger ventricles but a narrow and indistinct subarachnoid region. There does not appear to be any obvious connection between the inside and outside of the brain at either age. As

Figure 2.12

Photomicrographs of parasagittal sections through rat fetuses stained with H and E. (a), E15; (b), E18. Notice the large area of the midbrain vesicle in (a). Scale bar = 10mm.

a




b

mentioned above, the studies of Jones and Sellars (1982a, b) did show the movement of dextran into the subarachnoid space from the ventricles at E17; however the dextran was injected into the ventricles and the disruption this may have caused was not considered.

From the present studies in the rat, there is a peak of protein concentration within the CSF of the ventricles between E15 and E17. This occurs earlier than the peak measured in the cisternal region (Dziegielewska *et al* 1981; see Figure, 2.5). Later in development the ventricular and subarachnoid spaces are continuous with one another, however the development of communication between the inside and the outside appears to occur gradually (Jones, 1980): firstly with the opening of small pores in the IVth ventricle which may allow diffusion of small molecules between the two compartments, and later with larger openings through which the bulk flow of CSF can occur. Differences in CSF production in various regions throughout the ventricular system and subarachnoid space may cause regional differences in concentration of substances. This also highlights the need for caution when considering the permeability of the blood-CSF barrier. The choroid plexuses, the site of CSF production and the exchange interface for plasma proteins, are situated in all regions of the ventricular system. The composition of a CSF sample drawn from a single site in the ventricular system is therefore more representative of the barrier in that specific region than of the system as a whole. This does however depend on the volume of CSF withdrawn from the region sampled. Even in the adult there is a difference in the concentration of protein between lateral ventricle (22.0 ± 7.8 mg/100ml) and *cisterna magna* (28.2 ± 5.2 mg/100ml) as was shown in the sheep (Dziegielewska *et al*, 1991). So the presence of a protein concentration difference does not necessarily mean the compartments are separate.

Albumin in the Developing Choroid Plexuses

Much of the previous work investigating the origins of proteins in the CSF has been done at the stages of brain development which are after the peak of protein within the CSF (Cavanagh *et al*, 1983; Habgood *et al*, 1992). Dziegielewska *et al* (1991) visualised the exogenous marker proteins (foreign albumins) using immunocytochemistry. They compared the distribution of endogenous sheep albumin with that of the injected human serum albumin within the tissues of the brain. From their results it is clear that the route of entry of protein into the CSF is via cells of the choroid plexus which stained for both the sheep and human albumins. The presence of a transcellular route for protein into the CSF was suggested by Saunders (1977). An ultrastructural basis for this route was proposed by Møllgård and Saunders (1977).

The choroid plexuses, situated in the lateral, third and IVth ventricles, secrete CSF from a single layer of epithelial cells which cover a network of blood capillaries. By E16 in the rat the morphological appearance of the choroid plexus is similar to that in the adult (Chamberlain *et al* 1973). The choroid plexus forms from precursor cells which line the cerebral ventricles. The epithelial cells of the choroid plexus are the interface between the blood and the CSF, the blood/CSF barrier. The intercellular movement of proteins from the blood and into the CSF from the earliest stages of choroid plexus development is restricted by tight junctions present between the forming cells (Saunders, 1992).

As development of the choroid plexus progresses, the rate of the CSF secretion increases along with the overall ventricular volume, as the brain grows. What is not clear is how these are related functionally. Increased CSF secretion and reabsorption means that there is a greater general turnover of the fluid. Estimation of these changes during development have been made (Johanson and Woodbury, 1974; Bass and Lundborg, 1973).

Blood/CSF Barrier Protein Permeability in *Monodelphis Domestica*

For these permeability studies the IP injection volume was not more than 10% of the *Monodelphis*' own estimated blood volume. The concentrations of the injected solutions were prepared so as to be iso-osmotic in terms of the protein concentration (colloid osmotic pressure). Thus disruption of blood vessels caused by either an increase of the blood volume or due to its concentration having an effect on the osmotic pressure was avoided.

The original idea that the "barriers" protecting the brain at an early stage of development are immature and more "leaky" (Behnsen, 1927; Birge *et al*, 1974; Stastny, 1974; Adinolfi, 1986), allowing a greater penetration of substances from the blood, has been proved to be incorrect in the case of protein permeability (Saunders, 1977, 1992; Dziegielewska and Saunders, 1988) but is still widely believed by many (eg. Risau *et al*, 1986; Ganong, 1993). However, in more recent years a number of experiments have focussed on the idea of specific transfer mechanisms between blood and CSF which operate only during early stages of development. By using *Monodelphis* as a model for permeability experiments the present study has examined this mechanism in not only a species distantly related to previously studied species, but also at the time of brain development which is much earlier and covering a wider spectrum of developmental stages.

The peak of total protein concentration within the CSF in *Monodelphis domestica* occurs shortly after the time the cortical plate begins to appear (Dziegielewska *et al*, 1989) and this was also confirmed in the present study. Both events occur postnatally, between P5 and P6. However, the CSF/plasma concentration ratio for total protein declines steadily with an increase in age. As in the studies of Dziegielewska *et al* (1986a) using the tammar wallaby, the CSF/plasma concentration ratios for the endogenous albumin in *Monodelphis* declined steadily throughout this time. This is a reflection of the steady decline in the total protein concentrations within the CSF along with a rise in the plasma levels with increasing age. This confirms the work of Dziegielewska *et al*

(1989) who made estimates of the CSF/plasma ratios of total protein and of some individual proteins at 4 different ages; 1, 9, 45 days postnatal and adult in the *Monodelphis*. Transferrin, α_2-macroglobulin, α_1-antitrypsin as well as albumin were estimated and all 4 proteins showed a decline in their CSF/plasma ratios along with increasing age. The present results are similar to those from another marsupial species the tammar wallaby, *Macropus eugenii*. (Dziegielewska *et al* 1986a) in which a peak of CSF protein concentration occurs at P15 (see table, 2.2). The present studies confirm the previous results of Dziegielewska *et al* (1989) as well as showing the profile of albumin concentrations within the plasma and the CSF which also shows a peak of this protein within the CSF at the time of cortical plate appearance.

Previous studies have not investigated the permeability of the blood-CSF barrier to proteins so early in development, ie. before the peak of protein concentration in the CSF. In the present work, it was possible using *Monodelphis*, to investigate the permeability of this barrier before the peak as well as after.

Dziegielewska *et al* (1991), showed that the HSA CSF/plasma concentration ratios were the lowest attained of all the exogenous albumins in fetal sheep. They showed a correlation between the steady state ratios for this albumin and other smaller inert molecules, such as sucrose and inulin, and their molecular sizes. Therefore the penetration of these molecules, including the HSA, into the CSF from the blood was dependant on the molecular size. The other albumin markers (BSA, GSA) that were used in this study attained a much greater penetration into the CSF and showed no significant correlation between the CSF and plasma concentrations. It was suggested that these molecules were transported into the CSF by a species specific mechanism that may be a receptor mediated mechanism present on the epithelial cells of the choroid plexus. This mechanism seems to be precise enough for the exact structure of the albumin molecule to be important (Dziegielewska *et al*, 1991).

In the present studies, the earliest ages studied, P7-P8, showed CSF/plasma concentration ratios for exogenous albumins which were similar to the endogenous albumin ratios. At this age there was no significant difference between the exogenous albumin ratios (HSA and BSA) and that for MSA. Only the chemically modified albumin, succinylated BSA, showed a CSF/plasma ratio which was significantly less than MSA (approximately 30% less). Nevertheless the striking features of the albumin results at this age are the very high CSF/plasma ratio for all albumins studied and their numerical similarity. This reduction of the CSF/plasma ratio due to chemical modification was studied and shown by Habgood *et al* (1992) in newborn rats and argued that it provided evidence of two mechanisms of entry into the CSF. The first component is by passive diffusion and the second by a selective transfer mechanism. The chemical modification was suggested to cause a disruption of the species specific transfer mechanism's ability to discriminate. Therefore it is likely that most of the succinylated BSA present in the CSF would be entering by passive diffusion. If the succinylated BSA is entering the CSF purely by passive diffusion then the high ratio (18.3% ± 1.3) obtained suggests that much of the ratio for the other albumins (MSA, HSA and BSA) can be accounted for by passive transfer. The lack of difference in the ratios for the three unmodified albumins may have been because at this age any contribution from specific transfer is small. By P10 -12 the succinylated albumin ratio (indication of passive transfer) had fallen to about 15 % of its value at P7-8. In contrast the ratio for all unmodified albumins did not decline nearly so much by P10-12 (30% for MSA, 40% for HSA and 50% BSA) but the decline was greater for BSA and at this age there was a significant difference between BSA on the one hand and MSA and HSA on the other. These results could be accounted for by the "switching on" of an albumin transfer mechanism from plasma to CSF between P7-8 and P10-12. The mechanism does not discriminate between MSA and HSA but does transfer less BSA. As described in the Introduction specific differences in albumin transfer have been described previously in fetal sheep (Dziegielewska *et al,* 1991) and

newborn rats (Habgood *et al*, 1992). However this is the first study in which the developmental onset of such a transfer mechanism has been described.

In the permeability experiments presented above the CSF/plasma ratio drops with an increase in the age of the animals. There is a greater CSF "sink effect" in older animals (see section, 2.5). If the rate of CSF turnover is increasing along with age then the CSF/plasma ratios will decrease but there would not be a difference between the different albumins.

After 7-8 days of age there were significant differences between the CSF/plasma ratios (%) of individual exogenous albumins. If the barrier between the blood and the CSF acts as a simple filter mechanism or sieve then this would tend to distinguish between different molecules on the basis of molecular size and charge. Habgood *et al* (1992), using young postnatal rats, showed that BSA reached the same CSF/plasma ratio irrespective of the charge on the molecule. This was achieved by modifying the albumin with succinylation, carboxymethylation and lactosylation. The overall molecular charge of the modified albumins was then determined indirectly by comparing their electrophoretic mobilities. The results showed that the transfer mechanism is not dependent on the charge of the molecule alone since chemical modification reduced the level of transfer irrespective of which charge had been placed on the molecule.

At all ages the CSF/plasma ratios for the unmodified, exogenous albumins had values which were much greater than 50% of the endogenous albumin ratio. In some cases the ratios were not significantly different from the MSA. This has also been found in other studies using different species: sheep (Dziegielewska *et al*, 1991), rat (Habgood *et al*, 1992). If some albumin was made *in situ* within the CNS and the *in situ* produced albumin contributed a larger proportion of that found in the CSF then the CSF/plasma ratios for foreign albumins at steady state would be dramatically different to the animals endogenous albumin. In fact, there was no significant difference between the HSA, except in the 20-24 day age group, and the endogenous protein indicating

that *in situ* synthesis is unlikely to play a significant role in the increased concentrations of protein within the CSF early in development.

The ability to distinguish between and transport different species of albumin appears to only occur for a particular period of time in development. The oldest animals studied in the present work were between 32 and 36 days postnatal. The CSF/plasma concentration ratios for different albumins at these ages have been shown in the one age group (32-36). These ratios are markedly less than all the ratios at the younger ages and there is no significant difference between the HSA and BSA ratios indicating no discrimination between the two. However, both of these albumins had significantly larger ratios than the MSA. The BSA and MSA values were from the same P32 day animals and the HSA values from different animals at P36. It seems unlikely that these exogenous albumins were transferred into the CSF in preference to the MSA. At these older ages the clearance of the exogenous albumins from the plasma and the CSF may have changed dramatically, due to increased turnover of the CSF and increased function of the liver. This may have changed the absolute concentrations of exogenous albumins in the plasma or CSF so that steady state conditions no longer existed at the time of sampling (15 hours). This would make interpretation of this result difficult.

The present study has not compared any of the differences in molecular structure which may exist between different species of albumin but it has shown that most of the albumin within the CSF of the marsupial species *Monodelphis domestica* is derived from the plasma. In this species during the time of the peak protein concentration within the CSF only a small proportion of the albumin is derived from the plasma by a species specific transport mechanism; the bulk appears to enter by diffusion. During this early period the transfer mechanism does not appear to be able to distinguish between albumins from different species possibly because transfer is mainly due to diffusion. Discrimination of different albumins occurs immediately after the peak of total protein concentration in the CSF. Exactly how this mechanism is able to facilitate the transfer as well as

discriminate between different albumins is not clear. This may become more apparent when more is known about the molecular structure of the receptors for these proteins, especially within the developing CNS.

The only other study which examined protein transfer across the blood/CSF barrier in a marsupial species used the wallaby species *Macropus eugenii* (Dziegielewska *et al*, 1988). This study found that there was a similarity between the HSA ratios and those of wallaby albumin. However, only HSA was used as an exogenous albumin, therefore it is not clear if there was a species specific transfer mechanism present. By contrast, the E60 sheep fetus had a CSF/plasma steady state ratio for HSA of less than 50% of that of the sheep albumin. However in the wallaby, and now the *Monodelphis*, the HSA ratio approached the endogenous levels. Dziegielewska *et al* (1988) discuss the correlation of their results in the wallaby with the immunological cross reactivity between albumins. Cross reactivity between HSA and SSA is weak whereas that between wallaby and HSA is strong. The results from the present study fit with this correlation. In their further study in 1991, Dziegielewska *et al* constructed lipophilicity plots (Habgood, 1990) of 3 albumins, HSA, BSA and SSA, based on the available data of their amino acid sequences (HSA, Dugaiczyk *et al*, 1982; BSA, Brown, 1975; SSA, Brown *et al*, 1989). These plots compared the lipophilicity of each albumin molecule along their respective amino acid chains. Based on this data BSA and SSA were more closely matched than HSA.

Permeability of Iodinated Albumin

^{125}I-HSA reached similar steady state CSF/plasma ratios as that of unlabelled HSA in two of the age groups: 20-24 days and 10-12 days; both of these measurements, HSA and ^{125}I-HSA were made by completely different methods and provided a very similar result. In the 7-8 day age group statistical analysis would have been unreliable as only 3 values were obtained, and all were from a 7 days litter. Dziegielewska *et al* (1980b) used iodinated sheep albumin in

the fetal sheep (E60) and found that these reached the same steady state ratios as the endogenous protein. However in their later study (1991), which measured the penetration of proteins into different compartments of the fetal sheep brain Dziegielewska *et al* found marked differences between the labelled and unlabelled albumin (^{125}I-labelled sheep serum albumin (SSA) steady state ratio in *cisterna magna*: 3.9% ± 0.8%; SSA steady state ratio in the same region: 15.7% ± 1.3%). The labelled SSA reached similar levels to the HSA which they suggest enters the CSF by passive diffusion, therefore iodination of the endogenous albumin is sufficient to stop the transfer mechanism from recognising the molecule as SSA. In other studies it was found that iodination of fetuin and of α-fetoprotein considerably reduced the permeability of these proteins in E60 fetal sheep (Dr. K. M. Dziegielewska, personal communication). Rosenfeld *et al*, (1993) reported that iodination of the molecule, brain derived neurotrophic factor (BDNF), from the family of trophic factors which includes nerve growth factor (NGF), did not cause any changes to the protein folding but they suggested probable alteration in the local conformation around the site of labelling. This study also found that iodination produces various derivatives with different modifications which affected the binding of the protein to its receptor and subsequent internalisation.

During the present studies no check was made to assess how much of the albumin molecule was labelled after the iodination process and the possible structural changes that may have occurred. However, despite this the CSF/plasma ratio for the labelled HSA was not significantly different from the unlabelled HSA at both 10-12 days and 20-24 days postnatal. At 10-12 days, immediately after the peak of total protein concentration within the CSF, both HSA values were similar and not significantly different from the MSA. In the later age group both of the HSA values were significantly different from the MSA indicating that both albumins were discriminated against at this age. Therefore, comparison of steady state ratios between labelled and unlabelled proteins of the same species, using the postnatal *Monodelphis*, indicates that both species of proteins may be recognised in the same way. Iodination of the HSA

did not appear to have disrupted the ability of the species specific transfer mechanism to recognise the HSA molecule in the present experiments in *Monodelphis*, but given the results from other studies, it clearly may be a problem in some circumstances.

Functional Significance

The present study has examined the specific transfer of albumin in the young of the marsupial species *Monodelphis domestica* at a time before, during and after the peak of total protein in the CSF. During this period it has been shown that the barrier (blood-CSF) undergoes changes which alter accessibility of the CSF to albumin from the plasma. The significance is that the developing CNS is "protected" from the nonspecific entry of proteins into the CSF from the circulating blood. During this time there are specific mechanisms operating which are supplying the CSF with proteins from the plasma for a certain period during development. Although proteins have access to the CSF at a specific stage during development, this does not mean they then have access to the extracellular space surrounding the cells of the brain itself. This is because of the presence of the CSF-brain barrier, membrane specialisations that link adjacent cells in the neuroependyma (ventricular zone) of the immature brain (Fossan *et al*, 1985; Møllgård *et al*, 1986).

The functional significance of a greater penetration of protein into the CSF is not clear. One possibility is a correlation between the level of angiogenesis and the time for which this transfer mechanism operates; as a greater network of capillaries suppling the brain is formed, then the mechanism suppling large carrier proteins (ie. albumin) from the blood to the CSF is down regulated. This is also at a time when there is greater cell division and migration occurring within the CNS and demand for nutrients is high.

A high concentration of protein within the CSF during this early stage of brain development may have an effect on the surroundings due to colloid osmotic pressure. This may be essential for proliferation and growth of cells within the CNS. Specific transfer of albumin into the CSF may simply be a means of maintaining these osmotic forces.

These present studies have only investigated a species specific transfer mechanism for the protein albumin. This protein is abundant, both in adult and fetal life, in the plasma and provides an easily measured "marker". The plasma is made up of a *milieu* of proteins with a variety of different functions such as: carrier molecules, immunoglobulins, growth promoters, enzymes, hormones. Many of these are essential for normal growth and development. It would seem unlikely that a closed and tight barrier at the blood-CSF interface early in development denies access to all proteins circulating in the blood. A more realistic idea is a selective barrier which throughout development is able to provide only those proteins for the CSF which are necessary for the development of the CNS.

Chapter Three

In Vitro Studies of Neocortical Development

Introduction

Background to Culturing Nerve Tissue/Cells

Studying the growth and development of cells in culture conditions, isolated from the other tissues of the body, has for many years been an important tool available to the researcher. Tissue culture was first started by Wilhelm Roux (1893) and Gustav Born (1895) (reviewed by Jacobsen, 1992) but the technique was not used to study the nervous system until a few years later when in 1907 Ross Harrison observed the outgrowth of small processes from explants of frog neural tube. In comparison to today's techniques the method was simple but effective; it used a drop of clotted lymph on a slide in which the piece of tissue was cultured (Harrison, 1907).

Since these earliest experiments, the use of tissue culture to study certain aspects of developmental neurobiology has created a large and multifaceted field. The tissues are now handled in various ways and maintained in culture in a variety of different media. Growing and developing cells within the nervous system have been studied using three main *in vitro* techniques:

1) Dissociated cell culture, in which a piece of tissue is first removed from a specific region and the cells dissociated mechanically or enzymatically before culturing (Cavanaugh, 1955).

2) Tissue explant culture, in which a region of the nervous system is removed and maintained in culture (Murray, 1971).

3) Whole embryo culture, in which the whole embryo is isolated and maintained in the culture medium (eg. New, 1978)

During the period of culture the tissue or cells can either be plated onto a variety of specially created surface coatings or suspended in a nutrient fluid medium.

These techniques all have advantages as well as disadvantages. Cells dissociated from the tissue in which they normally reside are more accessible for immediate, minute by minute, observations of their behaviour during growth and differentiation. However, separated and isolated from their normal cellular environment, cells in culture may not display the characteristics normally shown *in vivo*. The larger the piece of tissue in culture the less accessible are individual cells for studying their changes and growth which may occur *in vitro*. In all cases however the supply of nutrients and substrates necessary to sustain normal growth and development is quite different from the situation *in vivo*, due to the lack of a blood supply.

Selecting the most suitable medium which will provide the best environment for cells to behave as they would *in vivo* has been the subject of much research in the past. Early studies tried simplifying the medium by using such solutions as sodium chloride (Lewis, 1912) and also providing a medium which would normally be present *in vivo* such as adult CSF (Martinovich, 1931). However, the survival time of central nervous system tissue in culture has been shown to be greatly increased when serum components were provided in the culture medium (Fischbach and Nelson, 1977). This may be because serum proteins, normally present in the fetal CSF, are supplied to the cells. These serum components are supplied to the medium in many forms: fetal to adult sera from a variety of sources; cow, horse, pig etc. These are usually added as whole sera to the basic nutrient media in a variety of concentrations. The basic nutrient media to which these supplements are added are numerous, varying in such ingredients as ionic concentration, buffering capacity, glucose concentration, etc. Therefore, the combinations of different media with sera added is large, for example: Romijn *et al* (1984) (2.6% albumin with transferrin in R12), Oorschot and Jones (1986) (20% FCS in Dulbecco's modified Eagle medium), Kriegstein *et al* (1987) (serum free medium, transferrin added), Götz

and Bolz (1992) (25% horse serum in 50% Eagles Medium and 25% Hank's balanced salts). These are common sera and media combinations used to maintain nerve cells in culture.

The use of a whole serum during tissue culture does however have disadvantages. The sera can vary between batches thus causing differences in neuronal survival and ability to maintain the tissues in culture. The sera are biochemically undefined providing a vast *milieu* of different proteins and growth factors, some unknown, in a variety of concentrations in the medium. The isolation of essential serum components may reveal factors critical for neuronal survival, this has been suggested in work done by Kaufman and Barrett (1983). In more recent years much work has been done to develop defined media for the use of culturing nerve cells and tissues. Much of this work however was based on adding known concentrations of proteins, such as albumin and transferrin (Romijn *et al*, 1988), which are normally present in relatively high concentrations in plasma. Using a medium that is defined enhances the reproducibility of experiments and simplifies the interpretation of data (Kumar, 1983). Maintaining nerve cells in any environment which is not only similar to that which is present *in vivo* but also provides cells with similar access to proteins in the culture medium as they would normally experience means that interpretation is made easier to relate to the *in vivo* situation. Nevertheless a difficulty in using such a technique which provides known concentration of a single protein is, that proteins such as albumin are known carrier proteins in the plasma, binding a number of other molecules (eg. hormones). Albumin itself may not have any effect on the survival of cells in culture but any of the factors which bind to it, may. Therefore, albumin as a component of a culture medium may not be as defined as initially thought. It is common however in serum free culture to add certain molecular components which have been found to be important. These include hormones such as insulin, hydrocortisone, growth factors such as epidermal growth factor and fibroblast growth factor, and some vitamins (see Laerum *et al*, 1985).

The presence of serum in the nutrient medium not only promotes the survival of nerve cells in culture but also affects their differentiation. Raff *et al,* (1984) showed that in the presence of serum, a glial progenitor cell differentiated into an astrocyte (Type II). In the absence of serum this progenitor cell developed into an oligodendrocyte. Thus two cells with distinctly different functions in the central nervous system were formed from the same precursor and only because of the changes in the chemical environment to which they were exposed.

The period of time for which cells in tissue culture can be maintained viable is dependent on the size of the tissue and distance through which oxygen has to diffuse. The rate of molecular diffusion is dependent on the square of the distance. It is for this reason that whole embryo culture can only proceed for a short period of time.

Tissue culture techniques have been used to study a wide variety of problems in the development of the central nervous system. One area has been to examine the interaction of cells formed in the developing neocortex (see Introduction, Neocortical Development) (eg. Edmondsen and Hatten, 1987; Caeser *et al,* 1989; Hatten, 1990; Bolz *et al,* 1990; Bolz *et al,* 1992; Wolburg and Bolz, 1991). Using dispersed cells the movement of neurons along radial glial fibres *in vitro* has been examined (Edmondsen and Hatten, 1987; Hatten, 1990; Hatten and Mason, 1990) as well as the neurochemical and morphological appearance of these neurons in slice cultures (Caeser *et al*, 1989; Bolz *et al*, 1990; Wolburg and Bolz, 1991; Götz and Bolz, 1992; Novak and Bolz, 1993). In all these studies the embryonic or postnatal tissue has been either sliced, or the cells dissociated and exposed to varying levels of a variety of sera so that cell survival is continued for as long as possible. The formulae of the different media used were empirically established as the ones which were capable of maintaining the cells for the longest period of time *in vitro*. How the composition of the culture medium compared to what the cells would normally be exposed to *in vivo* was not considered.

One of the problems when studying the development and maturation of any one specific area of the neocortex *in vitro* is, that the chosen region *in vivo,* during its development, has a high degree of cell to cell interaction with other areas. This interaction increases along with development as an increasing number of afferent inputs arrive in the neocortex to synapse with cells which have migrated to their final positions. At the same time efferent axons leave the neocortex to interact and form synapses with other areas of the CNS. Therefore maintaining *in vitro* a small piece of tissue which has or will have a complete neuronal circuit, and not be affected by the limits of diffusion, is difficult.

One technique which addresses this problem is a system of coculturing, whereby an explant of early postnatal neocortex is juxtaposed to a region of brain tissue that forms either afferent or efferent connections with the cortex *in vivo* (Yamamoto *et al,* 1989; Bolz *et al,* 1990; Bolz *et al,* 1992; Yamamoto *et al,* 1992). This system uses thin pieces of tissue so that diffusion of nutrients to cells does not limit the time of survival. During this time cells appear to migrate within the neuronal tissue and axons grow and are able to communicate with cells of the other region in which they are cocultured. Coculturing a region of thalamus (lateral geniculate nucleus) with visual cortex from P0 rats Bolz *et al* (1990, 1992) showed that thalamocortical axons terminated in their appropriate cortical target layer. This was found to occur irrespective of whether the axons entered from the white matter side of the slice or the pial surface. Molnár and Blakemore (1991) showed a lack of regional specificity for connections formed between thalamus and cortex in culture. Axons sent out by the thalamus invaded slices of cultured cortex irrespective of where the slice originated. In further work using a similar technique, cortical slices from the fetal and postnatal rat showed migration of cells to more superficial regions of the slice; these preparations were maintained in culture for many days while this migration was occurring (Gotz and Bolz, 1992). A substantial migration of cells from the ventricular zone out to the more superficial region of the neocortex was shown to occur between E18/E19 and P3 *in vivo* . From their data more than 90% of all the BrdU (bromodeoxyuridine) labelled cells were in the outer 1/3 of the

neocortical wall after 7 days *in vivo*. After 8-22 days *in vitro* a E18/19 slice of neocortex showed that prelabelled nuclei had migrated to every region of the neocortex although less than 25% of all the labelled cells had reached the outer 1/3 of the cortical wall, unlike the situation *in vivo*. This outer 1/3 region contains the cortical plate.

Each of these techniques therefore shows that particular aspects of cortical development can be studied in culture although the level of migration and axonal growth is very much reduced compared to *in vivo*. During the early part of cortical development *in vivo* (initial formation of the cortical plate), ie. between E15 and E22 in the rat, there is a large amount of cell proliferation. Whether the same level of cell proliferation continues to a similar extent *in vitro* is not made clear in these previous studies.

Another aspect of these *in vitro* studies, which does not appear to have been considered, is how closely the culture medium compares with CSF *in vivo* at a corresponding stage of brain development. The protein concentration of the CSF early in development is high compared to the concentration in adult, (see Chapter One). Previous studies have shown that the cells of the developing brain have very limited access to the CSF; this is in contrast to the adult, where the CSF is able to diffuse freely from the ventricles into the extracellular space (Saunders, 1992). This is due to the CSF-brain barrier which has been shown to be present only during the earlier stages of neocortical development. The high concentration of protein within the CSF may not be experienced by cells in the neocortex when the CSF-brain barrier is present. Therefore, the chemical composition of the extracellular fluid which surrounds the cells of the neocortex is unclear, due to the architecture of the developing brain, ie. cells of the neocortex are exposed to a high level of protein only at the interfaces of the ventricular zone and the pial layer. This is in contrast to dispersed cells or slices in which cells have access to protein in culture medium on all sides.

Isolated Entire CNS *In Vitro*

Nicholls *et al* (1990) described a new and innovative approach to research certain aspects of CNS development by removing an entire central nervous system and maintaining it in culture for up to 4 days. The animal species used was the marsupial *Monodelphis domestica* (Nicholls *et al*, 1990). The authors used postnatal animals between the ages of 1 and 4 days and after careful dissection, the CNS was maintained in Kreb's fluid for up to 4 days. Throughout this period compound action potentials elicited at one end of the spinal cord could be recorded at the other. These *in vitro* preparations showed continued cell division and nuclear movements, in both neocortex and spinal cord, whilst in culture, as well as spontaneous rhythmical respiratory activity and reflex responses to electrical stimulation (Nicholls *et al*, 1990; Stewart *et al*, 1991; Zou *et al*, 1991). Further experiments, in the same species using this model, studied the development of connections by axons growing through injured spinal cord at around the same age (Woodward *et al*, 1993). All these experiments concentrated on the spinal cord and hindbrain, examining both the electrophysiology and cellular morphology of these regions *in vitro*. During this time in culture it was shown that the cellular environment appeared to be maintained *in vitro* and that neuronal processes continued growing and forming synaptic connections (Woodward *et al*, 1993). The advantages of using a preparation such as this are that it provides a means for studying in real time the effects of different substances present in the culture medium, on a CNS behaving, it appears, as it would normally *in vivo*.

During the course of these early studies, using postnatal *Monodelphis domestica*, the conditions in which the preparations were maintained were further modified by using a basal Eagle's medium which was continuously bubbled with 5% CO_2 in oxygen. The medium did not contain L-glutamine but had a small amount of fetal calf serum (FCS; 0.2%) as well as antibiotic gentamycin ($0.1 mg.ml^{-1}$) added. The contents of this basal culture medium are listed in the Methods and do not include any serum or serum proteins. The only serum is provided by the FCS added at the time of culturing.

The results from these earlier studies and the evidence that fibre growth and cell proliferation continue in culture conditions seem to suggest that the environment in which the preparation is maintained may be similar to that *in vivo*. It has been shown that cells not only remain viable but continue to divide and send out axons with or without the presence of serum or serum proteins. The model has been used in a variety of ways to examine the development of neuronal circuits in the spinal cord and hindbrain.

Saunders *et al* (1992a) used the E15-E16 fetal rat isolated CNS to show that the recovery of the conduction and growth of axons through the crush was not just a marsupial phenomenon. These rat fetuses were at approximately the same stage of development as the *Monodelphis domestica*, used in the earlier studies, in terms of the neocortical development (Saunders *et al*, 1989; Saunders *et al*, 1992a). The level of development within the spinal cord in these two species would also appear to be the same. Between E15 and E16 in the fetal rat cells in the dorsal neocortex differentiate into the cortical plate (see Introduction). This occurs in the *Monodelphis* between 3 and 8 days of age and compared to the rat takes place over a much longer period of time (Saunders *et al*, 1989). A significant difference between the two models, rat and *Monodelphis*, was the temperature at which the recovery of conduction through the spinal cord occurred. In the *Monodelphis* recovery of conduction occurred at 24°C whereas in the rat it only occurred when the temperature was raised to 29°C (Saunders *et al*, 1992a). This may be a reflection of different *in vivo* body temperatures of the two different species at this stage in development. The *Monodelphis* has a lower body temperature than the rat (Fadem *et al*, 1982) of around 34°C. The rat is also *in utero* whilst the opossum is *post partum* and firmly attached to the mother's teat during the first few weeks outside the uterus. Thus the body temperature of the neonatal *Monodelphis* may be even lower than would be produced from the already lower body temperature of the adult. These differences between the two species may explain the difference in temperatures at which recovery of conduction occurs.

The Present Work

The present study has looked at the changes which occur in the neocortex of the whole CNS of the fetal rat which was isolated and maintained in culture. The entire central nervous system was isolated from the E15 rat fetus and maintained under sterile conditions for a period of up to 48 hours. During this period of time the changes which occur *in vitro* have been studied and compared with those which occur *in vivo*. These include the changes in the general morphology, proliferation, migration and differentiation of cells. A comparison is also made between the *in vitro* and *in vivo* neocortex and the distribution of proteins penetrating into the neocortex from either the medium or the CSF.

This model is examined for its use as a means to study neocortical development *in vitro*, during the period of time in which the cortical plate begins to form *in vivo*. It is assessed both in terms of the cellular environment within the neocortex as well as the protein environment. By removing the entire CNS from the fetus disruption to the CSF/brain barrier is limited. This has been studied under the light and the electron microscope before and after periods in culture.

The culture medium used throughout this study is basal Eagle's medium, the same formula which was used in the study by Saunders *et al* (1992a). The amount of fetal calf serum added to this medium has been adjusted to a total protein concentration that would normally be present within the CSF *in vivo* at this age. The effect on the changes which occur within the neocortex in culture in the presence of this serum is also studied, as well as the access of certain serum proteins into the isolated CNS.

Results

Light Microscopical Appearance

Coronal sections through the isolated, cultured fetal rat neocortex were examined under the light microscope after different times in culture. The fetal rat *in utero* develops rapidly with dramatic changes occurring from day to day (see Figure 2.2). This meant that throughout this study the comparison of morphological changes which had occurred *in vitro* between cultured preparations from different mothers could only be considered valid if the "starting point" of each CNS was known although the female rats were time mated (see Methods). In other words, it had to be established that fetuses from each uterus were at exactly the same stage of development before being placed into culture. This was initially checked using the "age/crown rump length" graph of Butler and Juurlink (1987). However this was considered as only an estimate before a more accurate timing could be calculated by examining the level of development of the neocortical wall under the light microscope and by comparing this with the data of Bayer and Altman (1991) relating age and stage of neocortical development. In most cases comparisons between preparations that had been cultured for different time intervals in different conditions used fetuses from the same litter which ensured that all fetuses were of the same age (runts were excluded).

The first study of the cultured fetal rat brain, from E15 to E16, involved comparison of dimensional changes which occurred during the first 24 hours *in vitro* with those which occurred *in utero*. Figures 3.1(a) - (d) show camera lucida outlines of coronal sections through four brains. The interventricular foramen was chosen as the reference point for each set of diagrams, a region in the brain where there is communication between the two lateral cerebral ventricles throughout development. The sections were taken from brains which had been

serially sectioned; care was taken to ensure that every coronal section was mounted on a slide. This made it possible to select sections from the brains that are directly comparable to the same section from another brain as all sections were the same distance from the interventricular foramen. All sections from each brain were cut at the same thickness (3μm). Figure 3.1(a) shows a control brain fixed and sectioned at time 0 hour (ie. without any culture period). Figure 3.1(b) and (c) were from brains which had been cultured for a total of 24 hours, in 0% FCS and 10% FCS respectively. Figure 3.1(d) was from an E16 fetus *in vivo* to compare growth *in vitro* with that *in vivo* over the 24 h period of the study.

A total of 53 preparations were cultured for this period of 24 hours at 29°C. Thirty five of these were in 10% FCS and the remainder in serum free medium. All these preparations were then processed for paraffin histology and examined. Illustrations and micrographs were taken from preparations that are considered to be typical examples.

The difference between the two *in vivo* preparations, E15 and E16 was striking; 24 hours produced a large difference in the overall dimensions of the forebrain. At E16 the neocortical wall in all regions was thicker when compared with those of the E15 preparation. The overall dimensions were also larger, with the lateral ventricles showing a large increase in volume. When comparing the coronal sections from the two cultured preparations E15 + 24h *in vitro*, the two brains appeared to be larger than those of the E15 "starting point"; the neocortical wall had increased in thickness although this was not reflected by an increase in the overall size of the preparation (width or depth).

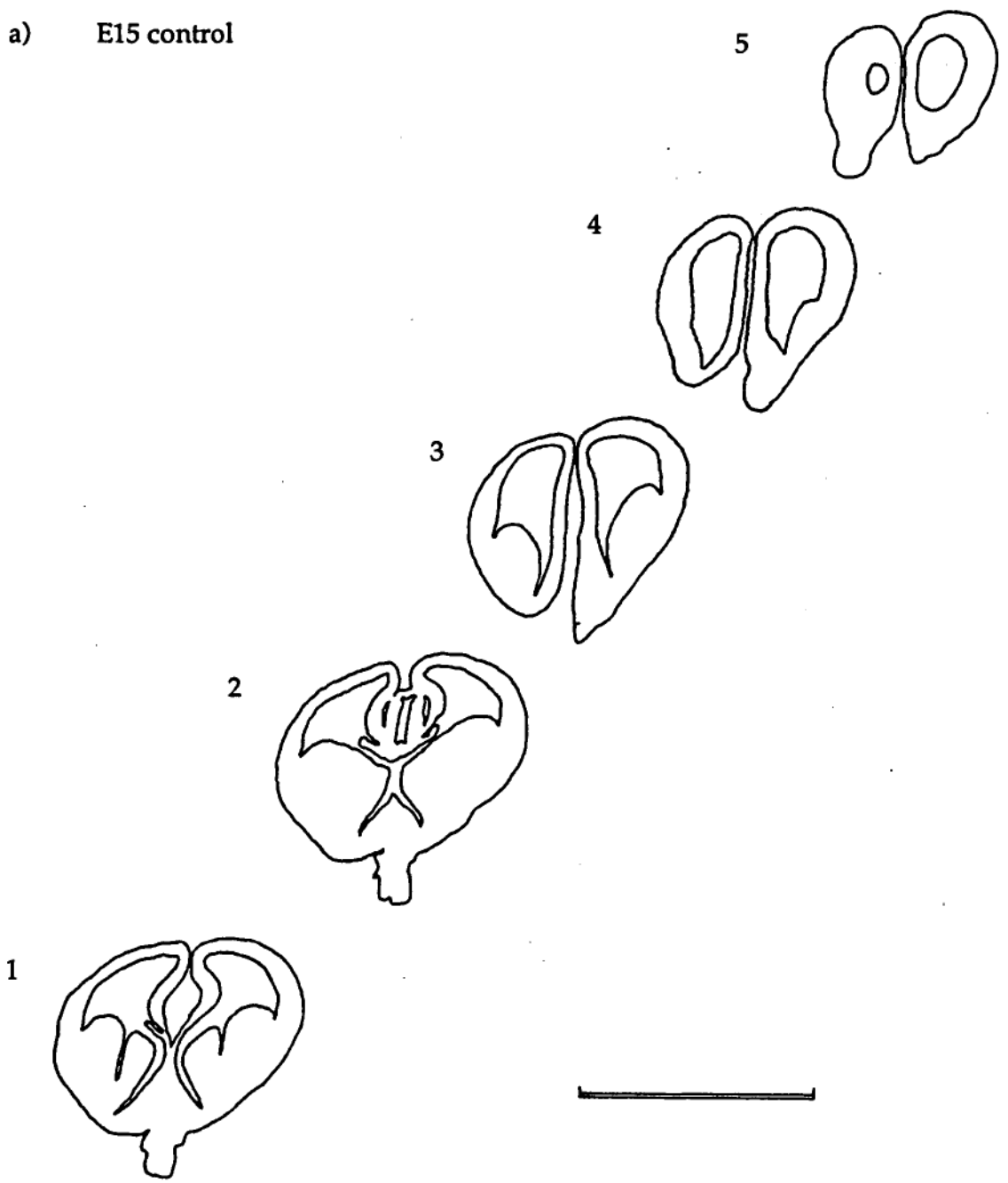
Comparison between the two cultured preparations with the two *in vivo* controls (E15 and E16), showed the E15 neocortex after being cultured in 10% FCS had a greater thickening of the neocortical wall after 24 hours in culture than the preparation in 0%FCS. This is clearly seen by the extent to which the ventricles have narrowed with a decrease in their volume. As all sections were taken at equal intervals from the interventricular foramen it would appear that not

Figure 3.1

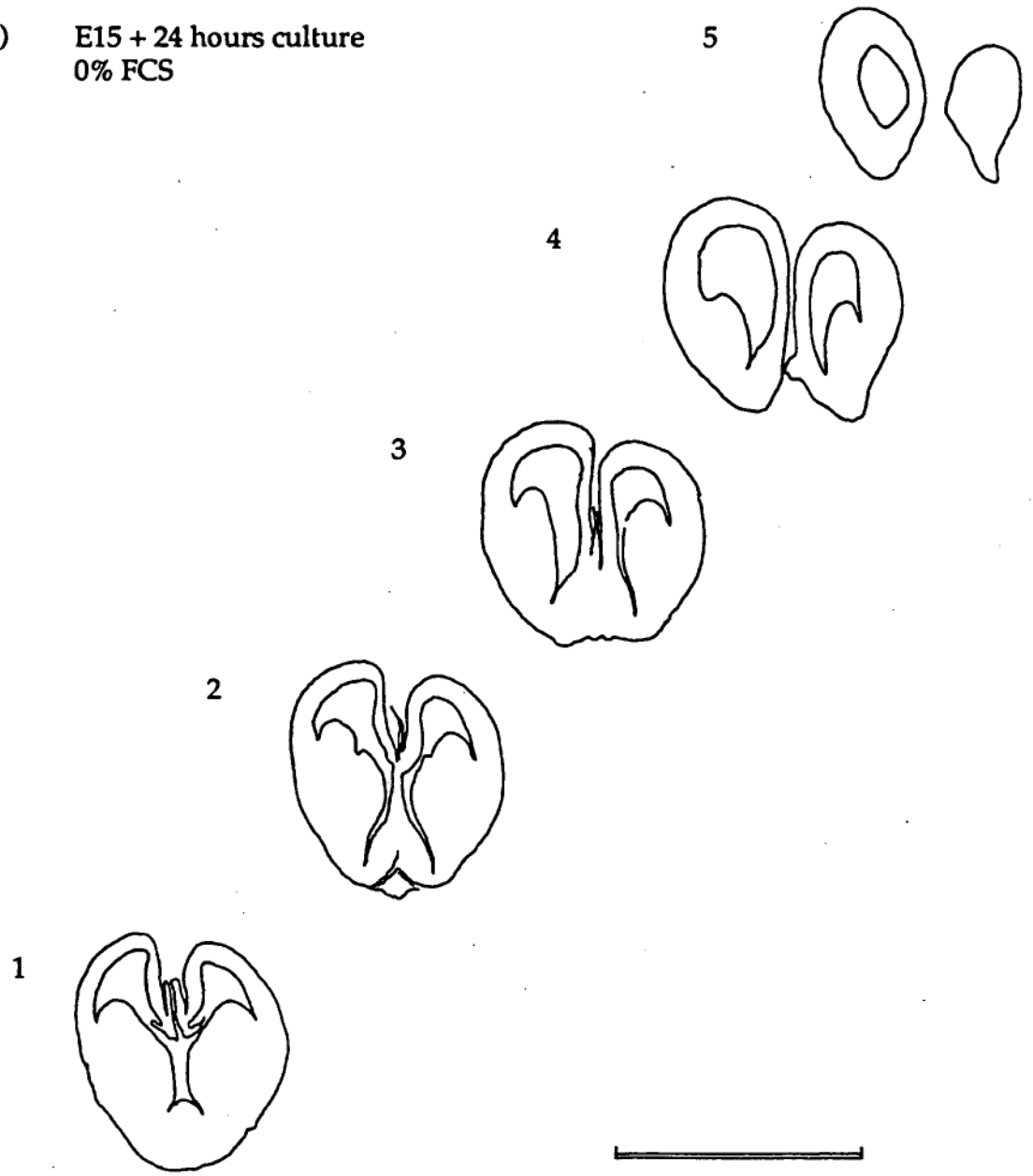
Camera lucida illustrations of serial sections through control (a, E15 non-cultured; d; E16, non-cultured) and cultured (b, E15 cultured for 24 hours, 0% FCS; c, E15 cultured for 24 hours in 10% FCS) brains. The first illustration from each preparation (labelled 1) was chosen as the section through similar regions of the interventricular foramen. Each subsequent section (2, 3, 4 and 5) was approximately 0.3 mm rostral to the previous one. Scale bar = 2 mm.

Note the increased thickening of the neocortical wall in the preparation cultured in the presence of 10% FCS compared to the preparation from the protein free medium.

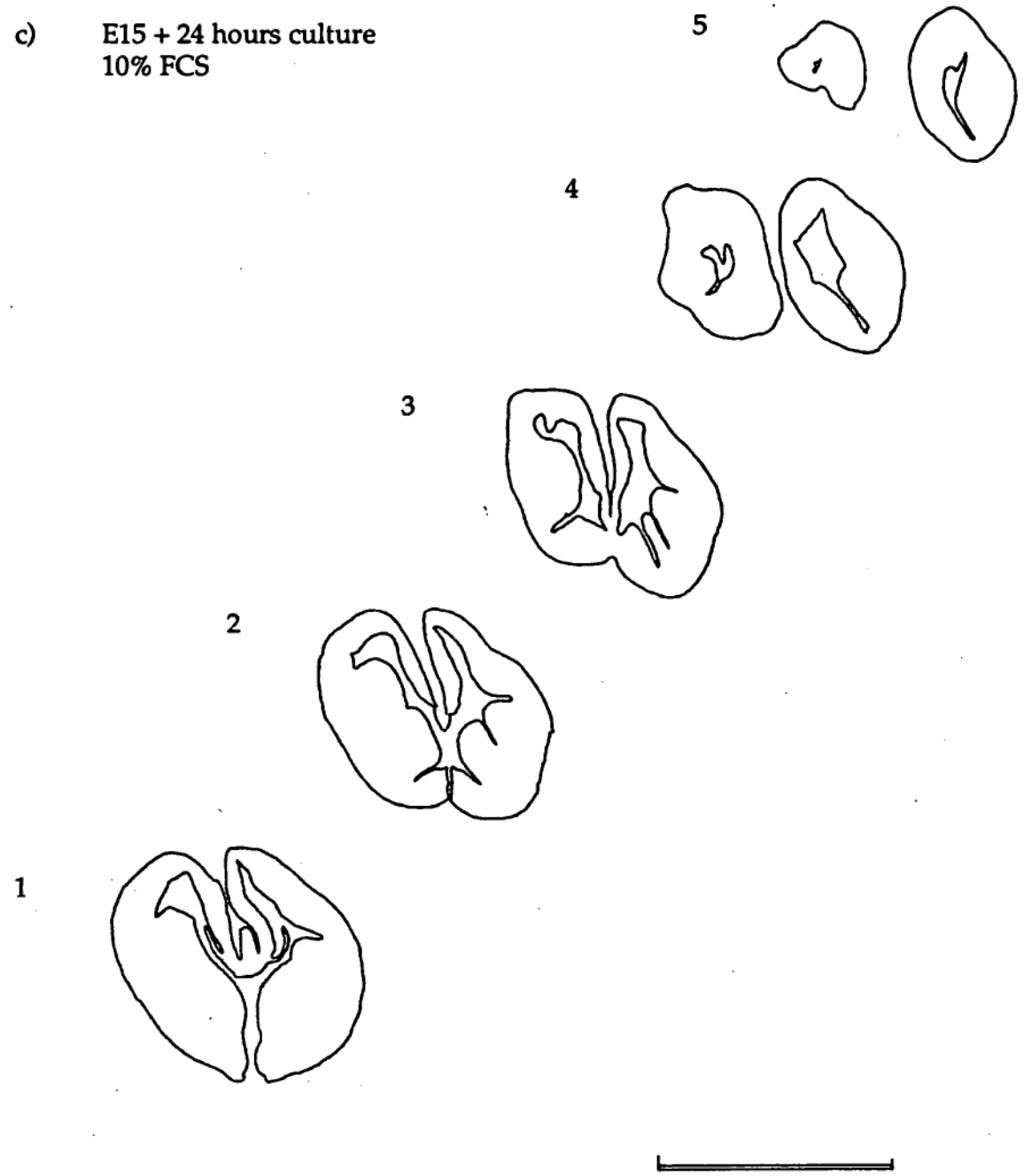
a) E15 control



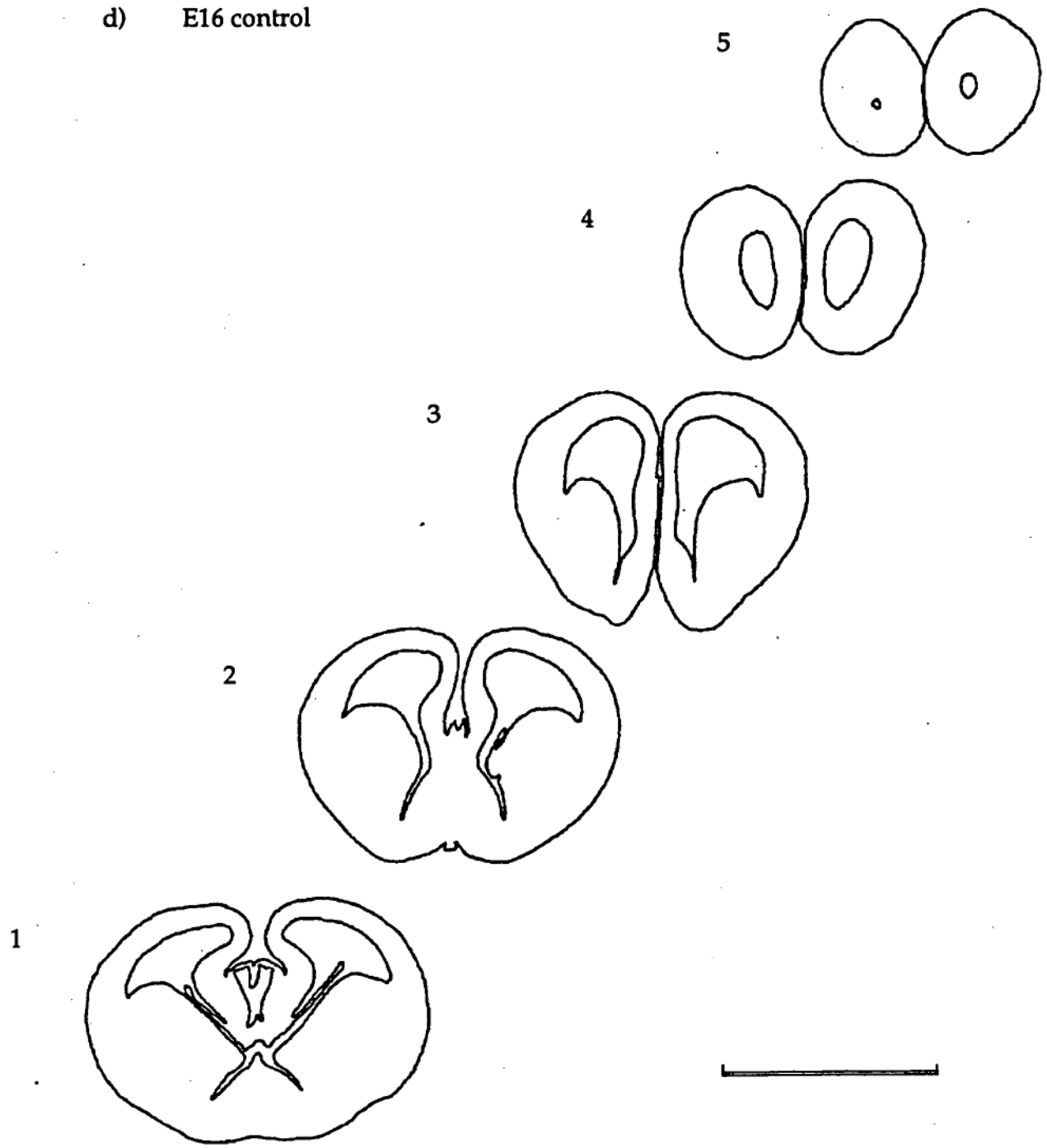
b) E15 + 24 hours culture
0% FCS



c) E15 + 24 hours culture
10% FCS



d) E16 control



only has the volume of tissue increased after 24 hours in culture but the level of increase is greater in the medium containing 10% FCS (See Figure 3.1(c)).

Morphological Appearance of the E15 Neocortex

Figure 3.2(a) and 3.3(a) shows both high and low power micrographs of coronal sections of E15 rat brain. The sections were stained with haematoxylin/ eosin (H and E) to show both the nuclei and the cytoplasm. The morphology and changes occurring in the more ventral regions, ie. primordium of the hippocampus, were not considered. Only the more dorsal region of the cerebral vesicle and changes occurring within it were studied.

The thinnest part of the neocortical wall is the dorsomedial region that becomes gradually thicker ventrolaterally. A major component of the neocortex is the germinal layer, or ventricular zone, which is darkly stained and surrounds the large lateral ventricles (see Figure 3.2a). At low magnification, differentiating cells outside the ventricular zone, forming different layers of the neocortex, are discernible only towards the lateral edge of the brain. The most striking of these features are the first cells of the cortical plate forming a very thin, darkly stained layer only in the most lateral part of the neocortex. Between the cortical plate and the ventricular zone is an apparently uniformly stained region containing the intermediate zone and subventricular zones, these two layers are only distinguishable from one another in the more ventrolateral region. More medially the neocortex comprises of just two regions: the densely stained ventricular zone and the lighter stained primordial plexiform layer.

Under higher magnification (Figure 3.3a) the structure of the individual cells was more obvious. Dividing cells (mitotic figures) were in evidence in the ventricular zone next to the ventricular surface. The condensed nuclear material appeared as very darkly stained, irregular shaped structures, smaller than other nuclei in the region, and did not appear to have a nuclear membrane.

The darkly stained nuclei of the ventricular zone, which are not dividing, all have a radial orientation perpendicular to the ventricular surface. This gives the impression of nuclei streaming away from, or towards the ventricles. The outer edge of this zone is well defined as cells of the primordial plexiform layer are less densely packed giving an overall lighter stain; their orientation appears to be random. Within this region, at this time in development, a few larger cells are present close to the pial surface; these appear to be Cajal Retzius cells (Bayer and Altman, 1992; Dziegielewska *et al,* 1993). The thickness of the neocortex increases ventrolaterally along with an increase in the number of cells in the cortical plate.

Morphological Appearance of the E16 Neocortex

Between E15 and E16 *in vivo* there was a marked change in the appearance of the neocortex. This is shown in Figure 3.2(b) and 3.3(b), both high and low power light micrographs of coronal sections taken from a similar region as those of the E15 forebrain (illustrated in Figures 3.2a and 3.3a). The ventricular zone has the same appearance 24 hours on, but the differentiating layers present only in the lateral aspect at E15 have now spread dorsomedially, increasing the thickness of the neocortex. By E16 the primordial plexiform layer has been replaced by the insertion of cells within this layer to form the cortical plate below the marginal zone and immediately above a cell sparse region which forms the subplate. In between this region and the ventricular zone are the intermediate and subventricular zones. This subventricular zone has a slightly less dense staining than the ventricular zone. The early cortical plate is recognised in the low power micrograph (Figure 3.2b) as a thin line, darkly stained, which lies towards the outside of the neocortex, immediately below the marginal zone.

A noticeable difference between the neocortex at E15 and that at E16 was the appearance of a few mitotic figures outside the ventricular zone and in the

subventricular zone. One mitotic figure not near the ventricular surface is indicated in Figure 3.3b. This was the earliest age that dividing cells were seen away from the ventricular surface. The number of mitotic figures in this different region was much less than at the ventricular surface.

Morphological Appearance of the Cultured Neocortex

Figures 3.2 (c and d) and 3.3 (c and d) show both low and high power micrographs from two CNS preparations from the E15 rats cultured for 24 hours at 29°C in 0% and 10% FCS. In both preparations the general tissue preservation is good. Both brains have maintained their overall structure and form and neither preparation shows any significant sign of tissue degradation within the neocortex. Both cultured preparations appear to have a much thicker neocortical wall than the E15 control preparations and both lateral ventricles in each preparation have shrunk in volume considerably. No obvious cortical plate is present in either preparation (cf. E16 *in vivo* control, Figure 3.3b). However there does appear to be a large increase in the neocortical wall thickness reflecting a general increase in the CNS tissue volume, especially in 10% FCS.

The two low power micrographs show that the ventricular zone appears to have increased in thickness. In both preparations this darkly stained region of the ventricular zone is considerably thicker than the E15 control. The preparation cultured in 10% FCS showed a larger increase in this region although it is not clear where the division between ventricular and subventricular zone occurs from these micrographs. The increase is greater than that of the *in vivo* preparations. The whole region, ventricular and subventricular zone had also increased in thickness in 0% FCS although to a lesser extent.

In the high power micrographs (Figures 3.3c and d) the continued survival of cells in both preparations appeared to be good, with a few pyknotic figures, small rounded nuclei, densely stained indicating cell death. In the densely stained ventricular zone the nuclei of both cultured preparations appeared

the same. They seem to have retained their radial arrangement lying perpendicular to the ventricular surface. In these preparations the boundaries between the germinal, ventricular zone and the subventricular zone were ill-defined. The overall density of cells and staining of nuclei were similar in both regions, however at higher magnification there appear to be morphological differences between these two regions. Cells and nuclei in the subventricular zone have a more rounded appearance and their positioning appears to be more disordered (cf. ventricular zone).

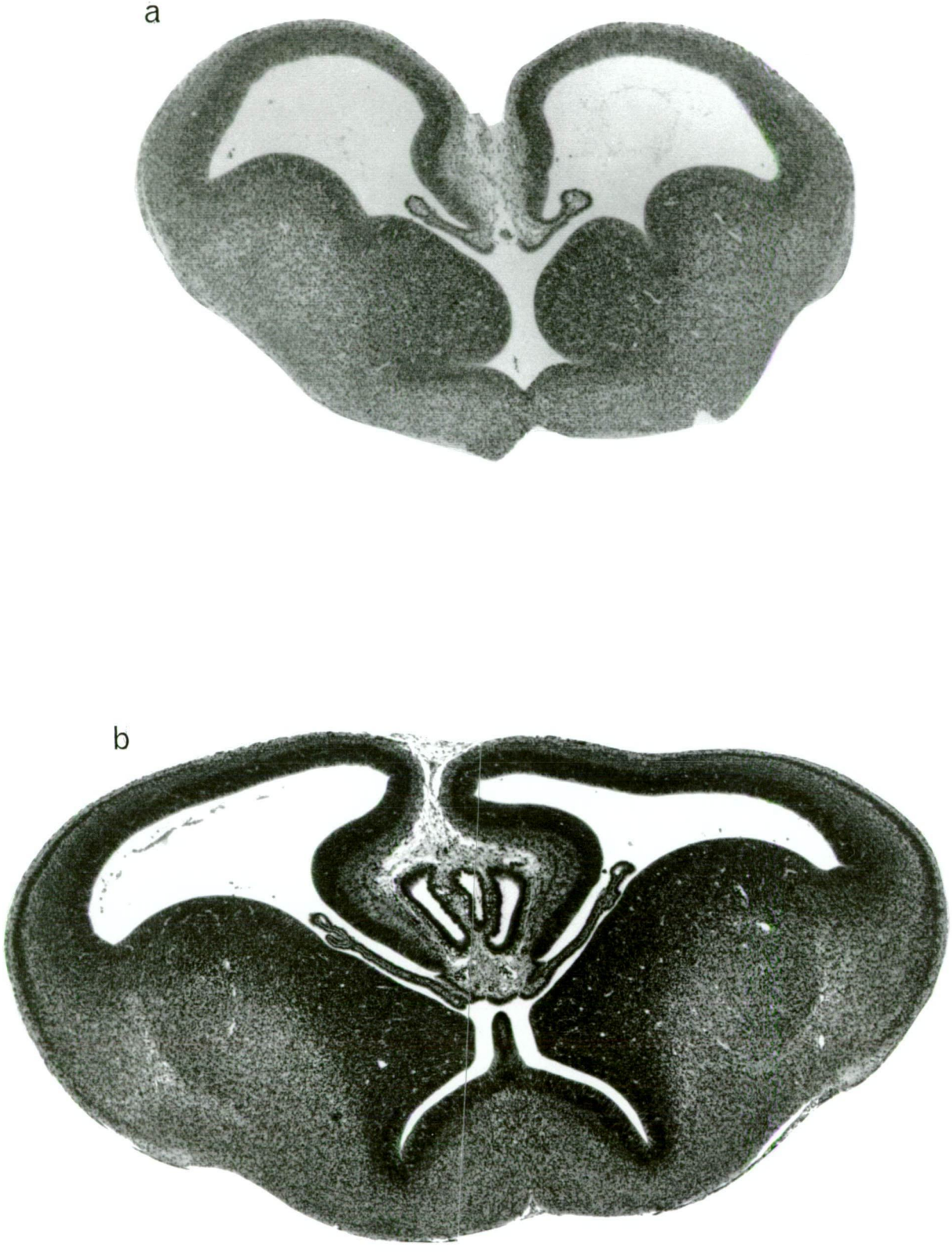
The neocortical walls of both preparations appeared to have increased their volume with an increase in the number of cells formed whilst *in vitro*. This increased growth is more noticeable in the presence of protein within the culture medium (10% FCS). A clear and striking sign of increased cell proliferation within the cultured preparations was the large numbers of mitotic figures at the ventricular zone. These were in greater abundance in the presence of 10% FCS. These cells, which were in the process of dividing, have densely stained nuclear proteins surrounded by a lightly stained cytoplasm and appear stacked up on one another in certain regions of the ventricular surface. *In vivo* these dividing cells appeared only to divide when situated next to the ventricular surface.

Generally, preparations cultured in the presence of protein were found to have groups of cells which had "bulged" out from the germinal zone and into the ventricular space. These outgrowths of cells were not seen *in vivo* or in preparations cultured without protein (Figure 3.2d).

The general morphology of the isolated CNS which has been cultured for a period of 24 hours shows that the cells within the neocortex continued to proliferate, thickening the neocortical wall to form a deeper subventricular zone. This result led on to the next set of experiments to determine from which region of the neocortex cells had come from. Also the differences between the CNS which has proliferated in culture and that which has remained *in utero* were investigated.

Figure 3.2

Coronal sections (5μm, H and E) through 4 fetal rat brains: (a), E15 immediately after dissection; (b), E16 immediately after dissection; (c), after 24 hours in culture in serum free medium; (d), after 24 hours in culture with 10% FCS. Note the markedly thicker cortical wall in the experimental preparation cultured with 10% FCS as well as the overcrowding of cells in the ventricular and subventricular region. The ventricular spaces within the lateral ventricles in the control brains (a) and (b) (E15 and E16) are much larger than those of the cultured preparations (c) and (d). Scale bar = 1mm.


a
b

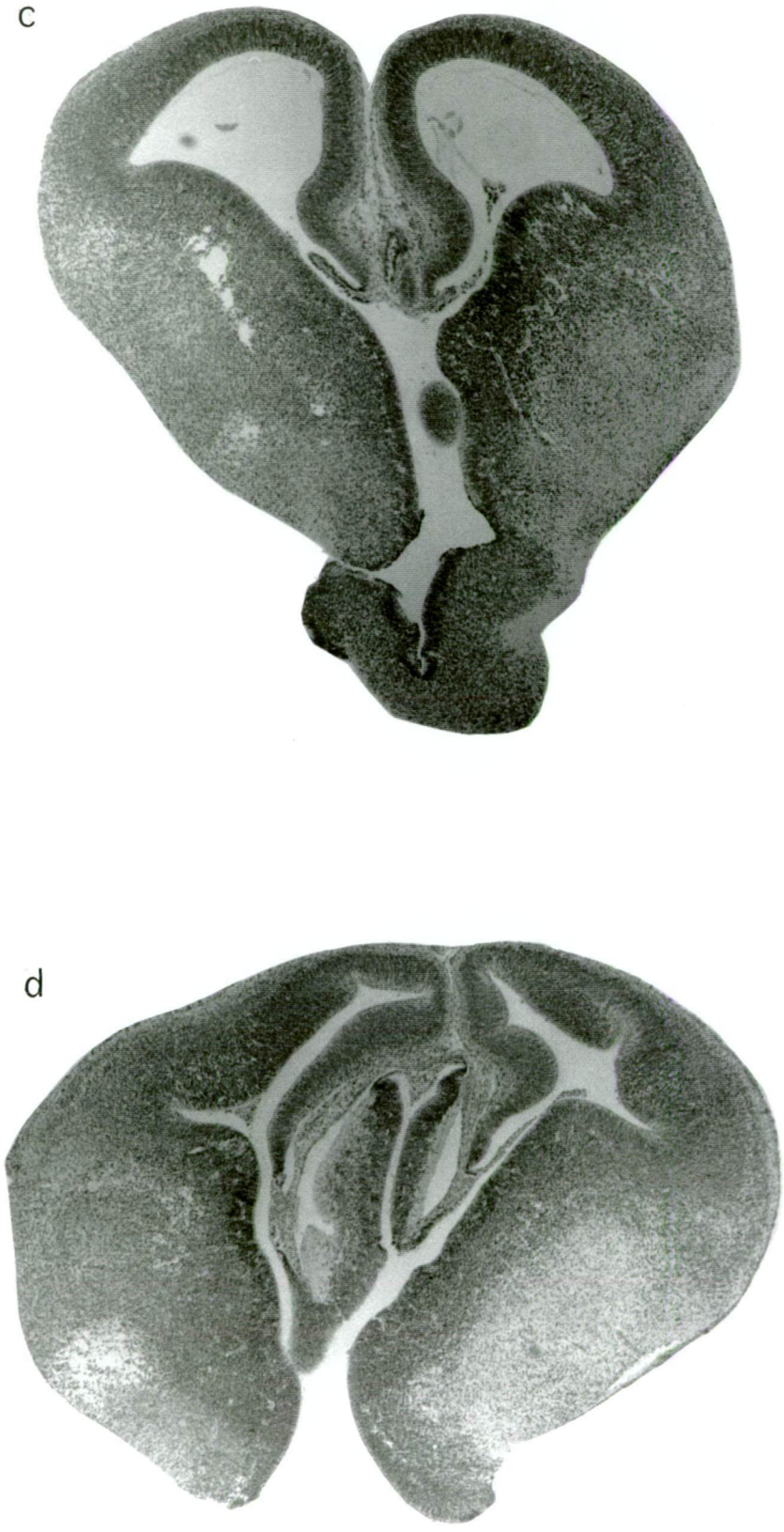
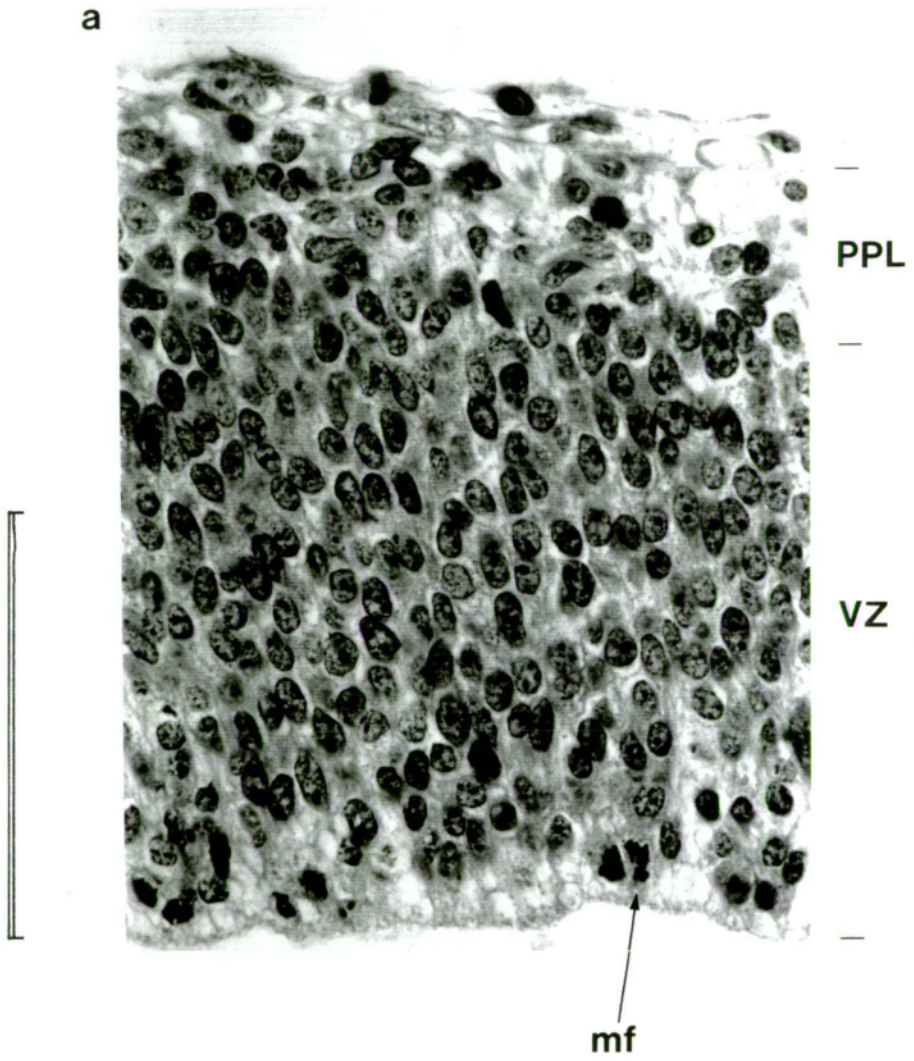

c
d

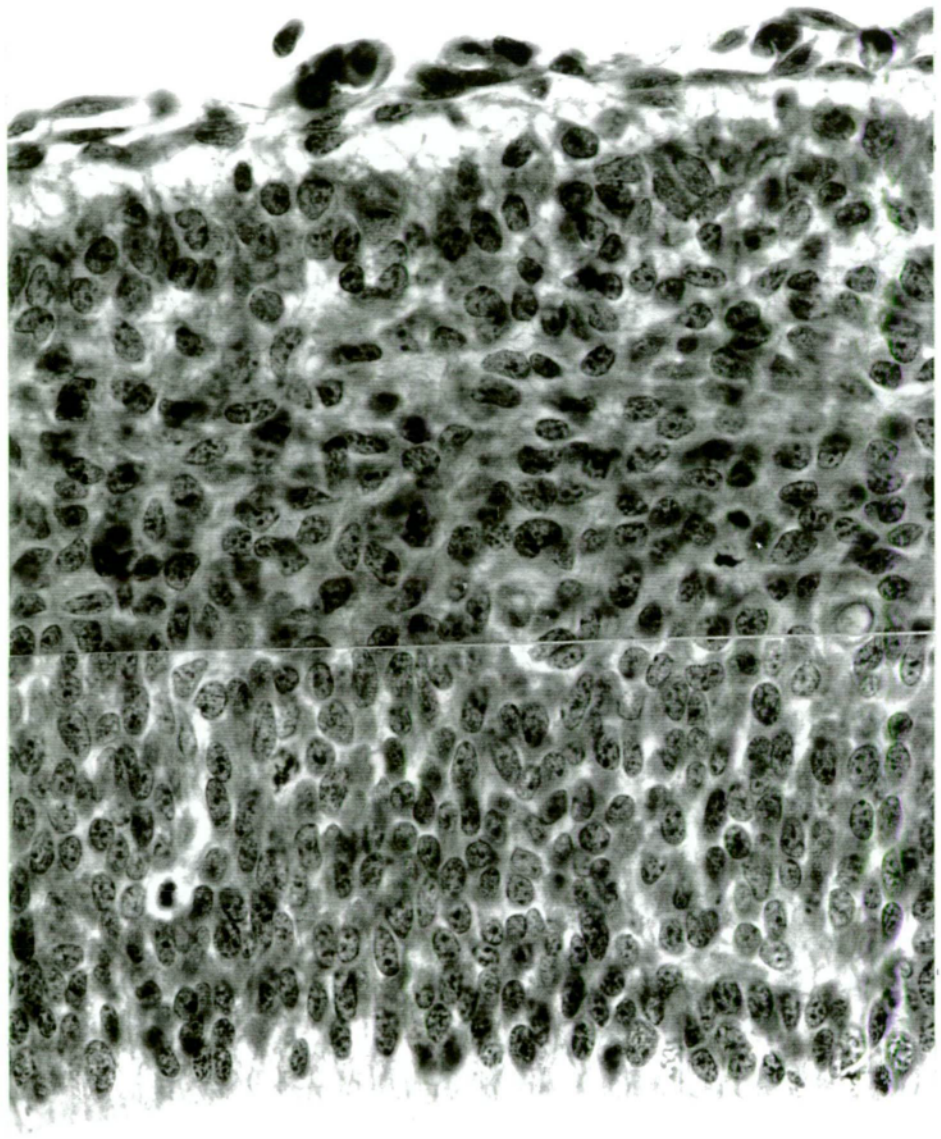
Figure 3.3

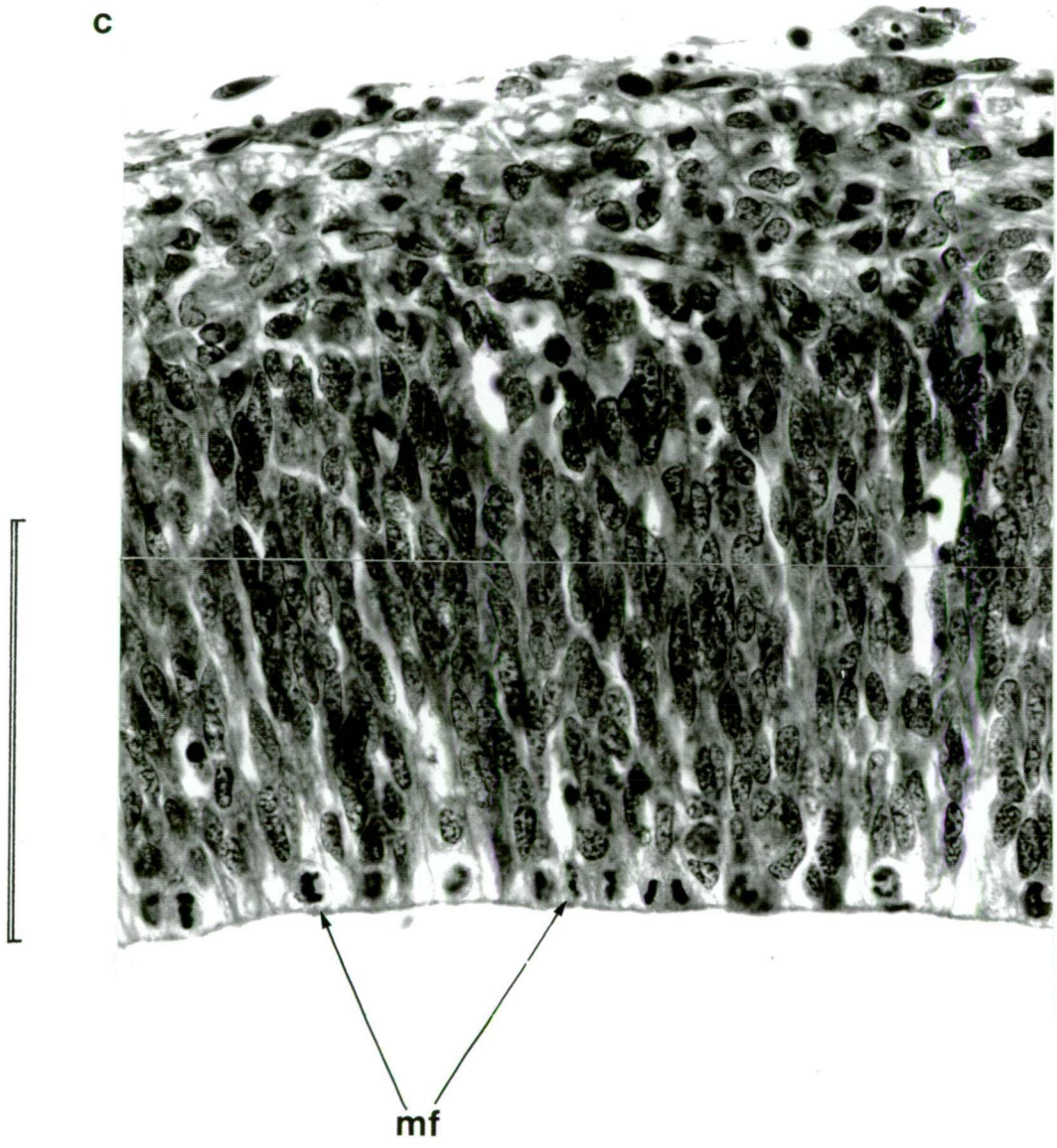
High power micrographs (H and E stained) of the dorsal neocortex coronally sectioned from both control and experimental CNS preparations shown in Figure 3.2. (a), E15 control; (b), E16 control; (c), 24 hours culture serum free medium; (d), 24 hours culture 10% FCS. All preparations were cultured at 29°C. The different zones of the neocortex are marked in (a) and (b): VZ, ventricular zone; SZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, marginal zone and PPL, primordial plexiform layer. Micrographs were taken with a X100 objective lens using an "Edge" R400 (Edge Scientific Instruments, USA, 3D light microscope). Scale bar = 100μm.

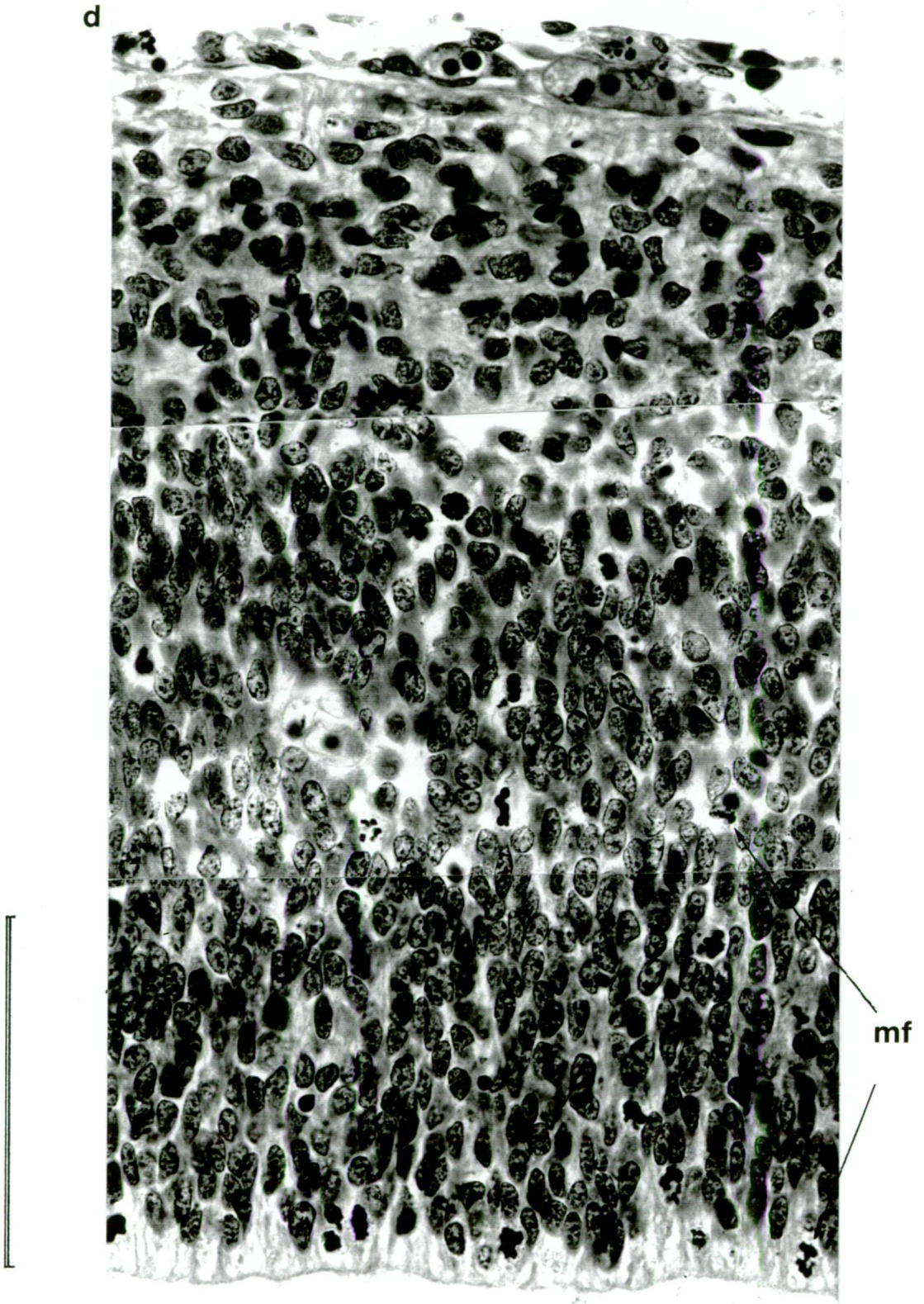
Note the large increase in neocortical wall thickness in (d) and the mitotic figures (mf) at the ventricular surface as well as in other areas. These dividing cells lie more superficially in the subventricular zone and the intermediate zone. Both cultured preparations do not appear to have a cortical plate although this may be more dispersed in (d).


a
PPL
VZ
mf

b




c
mf


d
mf

It was clear from the results so far that in culture there was a proliferation of cells in the neocortex which was increased in the presence of 10% FCS; this concentration was similar to the concentration of protein within the CSF of the fetal rat *in vivo* at this stage of development (E15-E16). This level of protein was therefore used for all subsequent culture experiments.

Labelling of Dividing Cells in the CNS

The following set of results was obtained from experiments undertaken to answer two questions:

i) What level of nucleic cycling was still occurring in the ventricular zone *in vitro* ?

ii) What was the fate of cells once division had occurred?

The questions were answered by exposing different CNS preparations to the thymidine analogue, BrdU, for different periods of time. The preparations were exposed to BrdU (20μM), for 30 minutes, immediately after isolation from the fetus. This experiment was carried out at two different temperatures (29°C and 33°C) in order to determine what effect a raised temperature would have on the proliferation of cells within the neocortex in culture.

Labelling of Cells with BrdU during Culture

Figure 3.4 (a, b, c and x, y, z) shows light micrographs, coronally sectioned, of the neocortex from 6 different cultured preparations. Micrographs (a, (b) and (c) are of preparations cultured at 29°C and (x), (y) and (z) from preparations cultured at 33°C. All preparations were exposed to BrdU for 30 minutes immediately after removal from the fetus and before being placed in culture for 3 different periods of time at each temperature. Preparations at 29°C

were cultured for a total of 3 hours, (a); 13 hours, (b) and 40 hours, (c). In total 4 preparations were cultured at each time interval and processed for paraffin histology, typical examples are described.

All micrographs show that nuclei within the ventricular zone had taken up BrdU at both temperatures. There appeared to be an increase in both the thickness of the neocortical wall as well as the number of stained nuclei within the ventricular zone.

After 3 hours in culture at 29°C (including 30 minutes in BrdU) the cortex showed a discrete band of stained nuclei lying towards the outer edge of the ventricular zone (Figure 3.4a). It also appears that a few stained nuclei were lying outside the germinal layer in the subventricular zone. During this period of time in culture there did not appear to have been much change in the overall appearance of the neocortical wall.

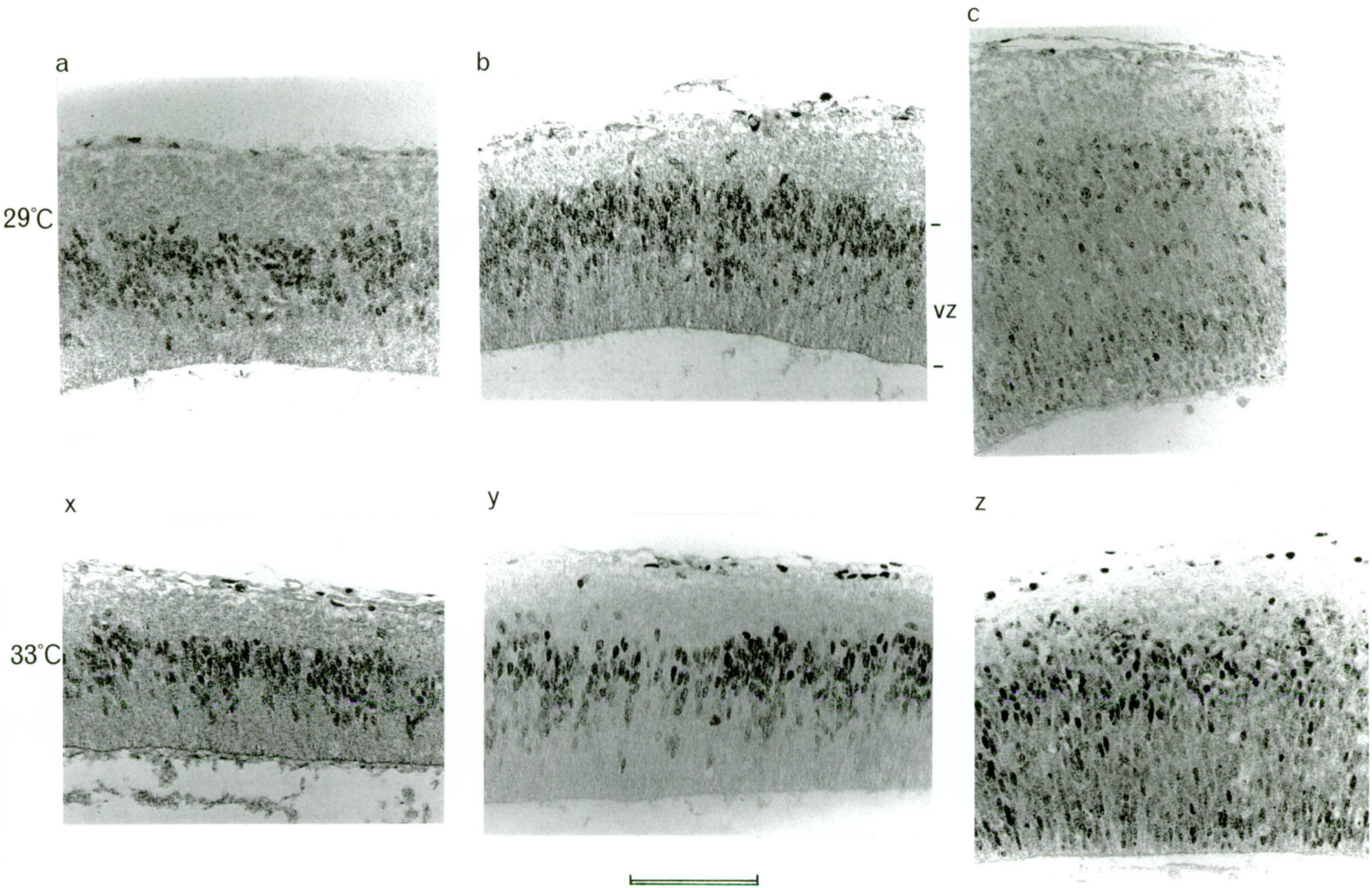
After 13 hours it appears that the neocortex has become slightly thicker although the main increase in thickness appears to be an increase in the thickness of the ventricular zone (Figure 3.4b). Light microscopy of the tissue shows tightly packed, densely stained nuclei. Their radial alignment, perpendicular to the ventricular layer, can be seen. The band of BrdU stained nuclei appears to have widened with a large number of stained nuclei now spread further across the widened ventricular zone. There appears to be a gradient of cells stained for BrdU in the ventricular zone, the stronger stained nuclei were further way from the ventricular surface than the weaker ones.

The remaining time interval illustrated was after 40 hours (Figures 3.4c). This shows a further increase in the neocortical wall thickness and large increase in the area covered by BrdU labelled nuclei in the ventricular zone. After 40 hours in culture the now much thicker wall and ventricular zone appear to have BrdU labelled nuclei close to the ventricular surface as well as labelled nuclei which appear to have remained in the more superficial part of the ventricular

Figure 3.4

Photomicrographs of coronal sections of the dorsal neocortex from 2 sets of preparations (pial surface on the top). All preparations were cultured for 30 minutes in 10% FCS, containing 20μM BrdU and then for different periods of time at 2 different temperatures, in order to study the effects of temperature on the changes occurring in the neocortex: (a), (b) and (c) at 29°C, (x), (y) and (z) at 33°C. (a), 2.5 hours; (b), 13 hours; (c), 24 hours. (x), 3 hours; (y), 13 hours; (z), 40 hours. Scale bar = 100μm.

These micrographs were taken with a X40 objective lens using an "Edge" R400 (Edge Scientific Instruments, USA) 3D light microscope. Nuclei containing the BrdU label are darkly stained and clearly visible in all preparations within the ventricular zone (VZ). Note the position of the darkly stained nuclei close to the ventricular surface after 24 hours in culture.


a
b
c
29°C
vz
x
y
z
33°C

zone. These nuclei do not appear to have moved away from this position since the start of culture.

During culture BrdU labelled nuclei or cells do not appear to have left the ventricular zone although their position within this area has changed. The spread of labelled nuclei towards the ventricles appears to increase along with an increase in the period of culture. There does however appear to be an increase in the depth of the region outside the ventricular zone.

In order to investigate the effect of temperature the whole experiment was repeated at 33°C. Figure 3.4 (x), (y) and (z) shows three micrographs of the neocortex from preparations cultured for 3 different time intervals. After 3 hours the comparison between the raised temperature of 33°C and 29°C shows little difference. At the time intervals shown the labelled nuclei are at approximately equivalent positions. After only 24 hours at 33°C these nuclei are at the same position as those after 40 hours at 29°C. Therefore the movement of these nuclei occurred faster at the slightly higher temperature. The overall amount of cell proliferation also did not appear to have increased at the slightly higher temperature, although the movement of nuclei and cells within the neocortex whilst in culture appeared to occur sooner.

In summary therefore, it appeared that the movement of cells and nuclei which occurred in the neocortex whilst in culture happened at a faster rate at the higher temperature. This seemed apparent when looking at the BrdU labelled neocortex: after 24 hours at 33°C and after 40 hours at 29°C, the position of the labelled nuclei was similar. This decrease in the time for migrating nuclei to reach the ventricular surface before there was a dramatic decline in tissue preservation was also increased in the presence of protein (10% FCS) in the culture medium. All subsequent experiments were therefore carried out at 33°C, a temperature closer to that *in vivo* (37°C). Using a marker such as BrdU, it did not seem that a raised temperature increased the proliferation or development of cells within the neocortex. However, it is not clear whether or not labelled cells

in the CNS were given enough time in culture to show marked changes in the state of neocortical development. By 24 hours in culture a few mitotic figures were seen first outside the VZ (see Figure 3.2d). These may equate to the well described secondary mitotic zone seen *in vivo* in the rat and also known as the subventricular zone. Preparations which had been cultured for the longest periods of time did not show a significant number of BrdU positive nuclei outside what appears to be the ventricular and subventricular zones.

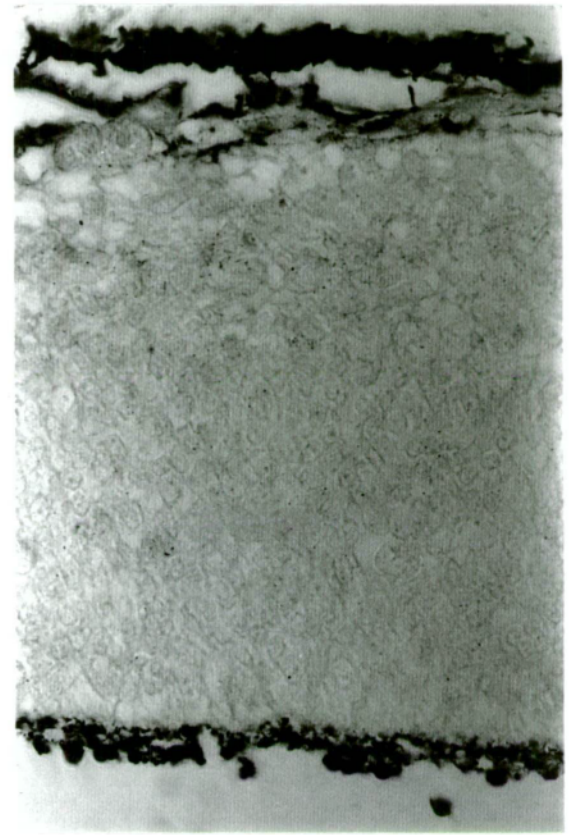
Figure 3.5

High power micrographs (silver enhanced immunogold staining for bovine albumin and bovine fetuin; see Methods) of the dorsal neocortex sectioned coronally from 3 preparations cultured for: (a), 3 hours; (b), 13 hours; (c, 1 and 2), 24 hours. All preparations were cultured at 33°C. The ventricular zone (VZ) and primordial plexiform layer (PPL) are indicated in Figure (a) and all figures have the same orientation: pial surface at the top. Micrographs were taken with a X100 objective lens using an "Edge" R400 (Edge Scientific Instruments, USA, 3D light microscope). Scale bar = 100μm.

Bovine albumin staining is confined to pia arachnoid/mesenchyme on outer surface and on the inner ventricular surface at 3 hours. Subsequently there was a small uptake of albumin into a few ventricular zone cells (especially after 13 hours) and in a few dying cells (at 24 hours). In contrast, fetuin uptake into ventricular zone cells was slightly apparent at 3 hours and by 13 hours was prominent, especially in cells deeper into the ventricular zone. There was also some uptake of fetuin into a few cells in the marginal zone. By 24 hours fetuin positive cells have migrated into the outer zones of the neocortex. This outer region corresponds to the cortical plate and intermediate zone in the brain *in vivo*.

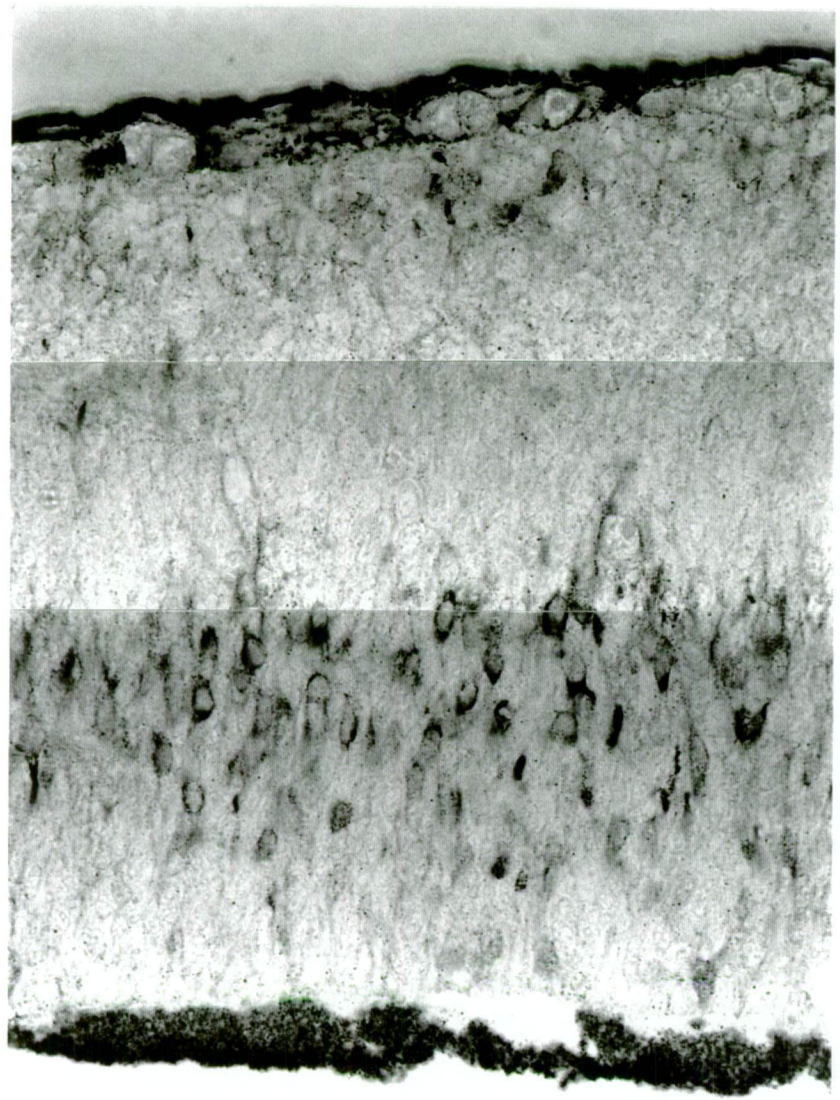
a

fetuin

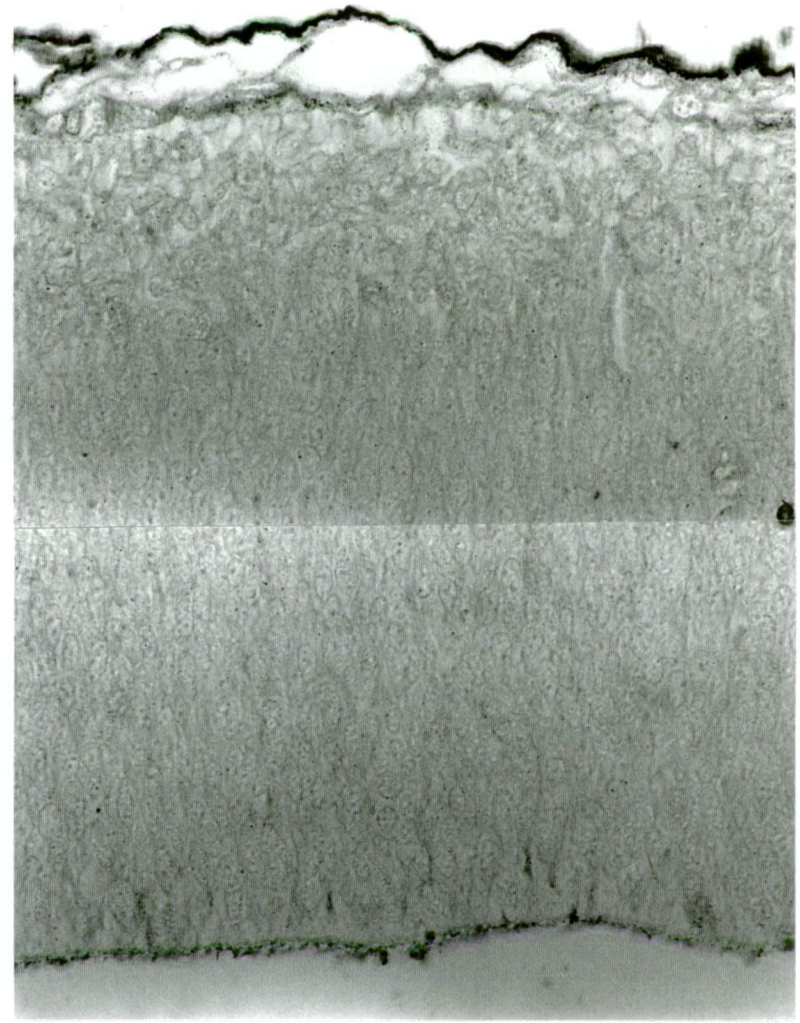


albumin

b

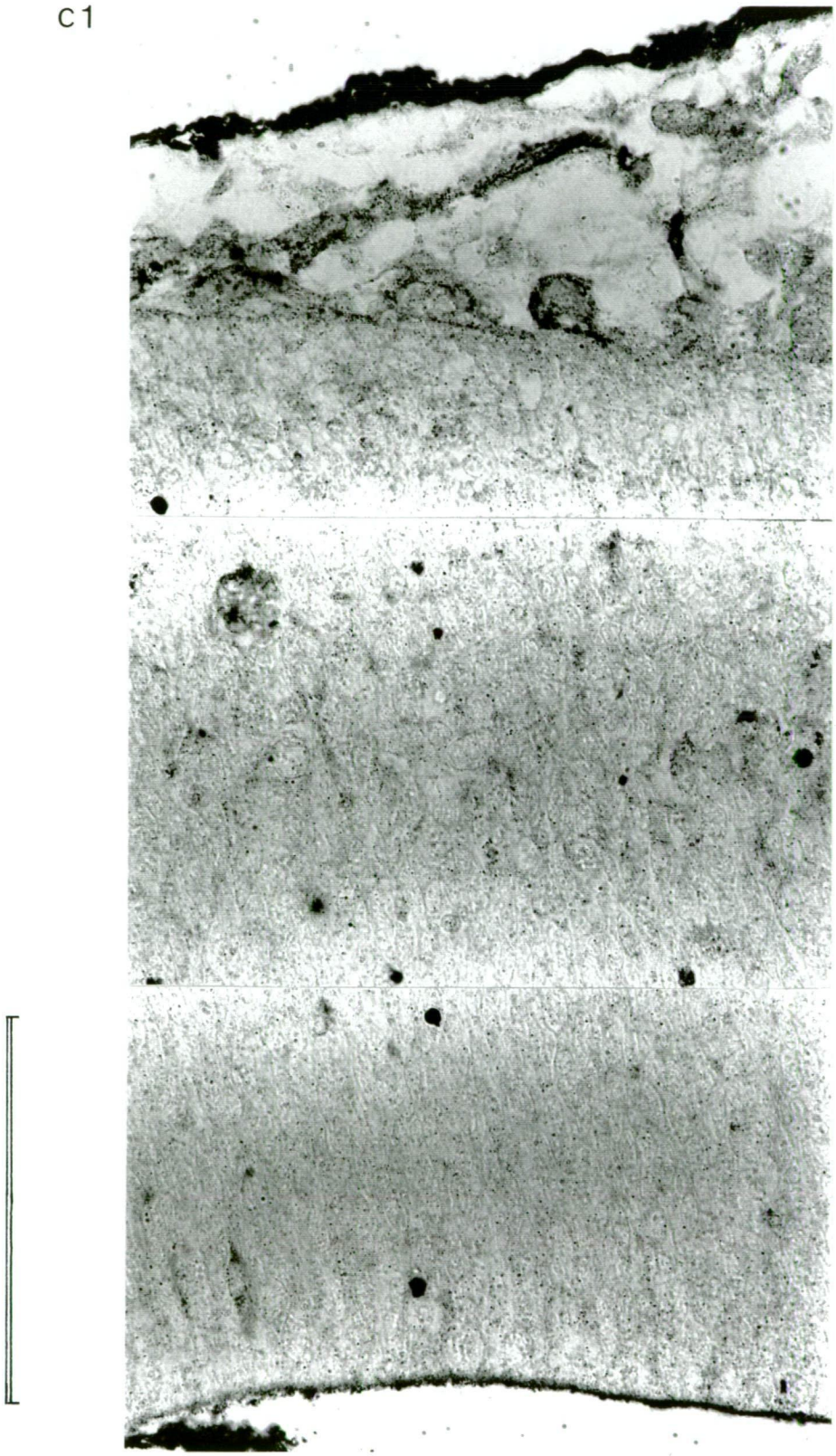


fetuin



albumin

C1



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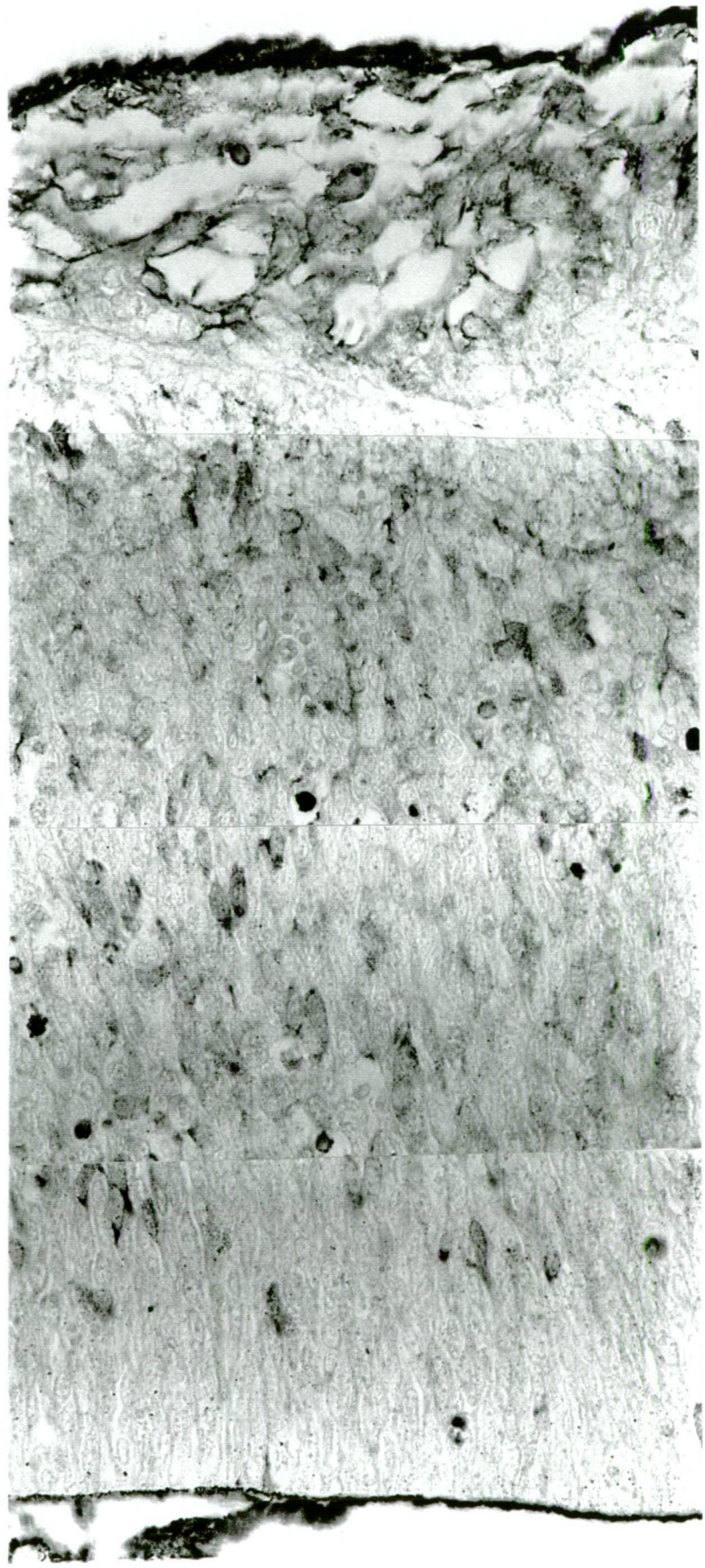
c 2



fetuin

Exogenous CSF Protein Staining within the Neocortex

Figure 3.5 (a, b and c) shows coronal sections through the dorsal neocortex of brains from the same litter of fetuses cultured for 3 different periods of time, in 10% FCS at 33°C. Adjacent pairs of sections from each brain have been stained for either bovine serum albumin or bovine fetuin, both present in the culture medium.

All micrographs show positive staining for both proteins within the ventricle and on the outer (pial) surface of the brain. There does not appear to be any staining for the bovine albumin within the neocortex at any stage during the time in culture. This is in contrast to the bovine fetuin staining which appears to be present in the neocortex at all stages *in vitro* with the exception of the 3 hour time interval. After 13 hours in culture, bovine fetuin is present within cells of the ventricular zone. This staining seems only to be within the cells and processes lying perpendicular to the ventricular surface. There does not appear to be any extracellular staining. There also appears to be a small amount of staining for the protein within the more superficial region of the neocortex towards the pial surface. After a further 11 hours in culture (24 hours, Figure 3.5c) the thickness of the whole neocortex (including the ventricular zone) appears to have increased with a number of specific cells stained for fetuin. There is also a number of cells stained for the protein in the more superficial region of the neocortex beyond the subventricular zone and close to the pial surface. These cells are lying in a region of the cortical plate although the exact morphology of these cells is difficult to determine. Throughout the neocortex the staining does not appear to be extracellular but associated with either the cells or processes.

Therefore after 24 hours in culture, 10% FCS at 33°C, it appeared that there was a specific uptake of fetuin by certain cells within the neocortex. This uptake increased as the period of time in culture increased.

In Vivo Staining for Exogenous and Endogenous Proteins

Figures 3.6 and 3.7 show a series of micrographs of the neocortex from an E15 and E16 rat fetus, sectioned coronally. These fetuses were injected with a solution of bovine fetuin, into the lateral ventricle and left for 30 minutes before being removed from the uterus and fixed. Figures 3.5 (a and b) and 3.6 (a and b) are pairs of micrographs from the E15 and E16 neocortices which compares the immunocytochemical staining for rat albumin and rat fetuin. The two pairs of micrographs are therefore showing the endogenous protein within the neocortex.

At E15 both micrographs show positive staining for both proteins in the ventricle and on the outer surface, within blood vessels on the pial surface. These micrographs were counterstained with toluidine blue (see Methods) which enables the cell structure to be seen in the neocortex. There is no positive staining for either of the proteins within the neocortex.

At E16 (Figure 3.7a and b) the staining for albumin and fetuin endogenous proteins appears to be similar to the E15 micrographs. The proteins are present both within the blood vessels on the pial surface and within the ventricles. Like the neocortex at E15 there does not appear to be a significant amount of staining for the endogenous proteins within the neocortex.

Figure 3.6 (d) and 3.7 (d) shows the immunocytochemical staining for bovine fetuin in sections from the same brains as shown stained for rat fetuin and rat albumin. Both micrographs show the presence of the protein both on the outside of the brain as well as in the ventricular space. Figure 3.6 (d) shows red blood cells present in the subarachnoid space of the E16 brain indicating a small amount of damage possibly caused when the bovine fetuin was injected. The staining for bovine fetuin within the neocortex, at E15, appears to be only associated with cells, there does not appear to be any extracellular staining. There are a small number of cells within the ventricular zone which have specifically taken up the protein.

Figure 3.6

Micrographs of coronal sections through the neocortex of E15 rat into which a solution of bovine fetuin was injected into the ventricle and the subarachnoid space. (a), (b) and (d) are stained immunocytochemically; (a), rat albumin; (b), rat fetuin and (d), bovine fetuin. For comparison (c), *in situ* hybridisation for rat fetuin. Micrographs were taken with a X40 objective lens using an "Edge" R400 (Edge Scientific Instruments, USA) 3D light microscope. Scale bar = 100μm.

Note the presence of rat albumin and fetuin ((a) and (b) respectively) in the blood vessels at the pial surface and within the ventricle. Bovine fetuin is also present within the ventricle, (d) and in the subarachnoid space. Within the dorsal neocortex there does not appear to be any rat fetuin or rat albumin, however a few cells are stained positively for bovine fetuin. There is a very low level of signal for rat fetuin mRNA in the primordial plexiform layer (PPL), (c).

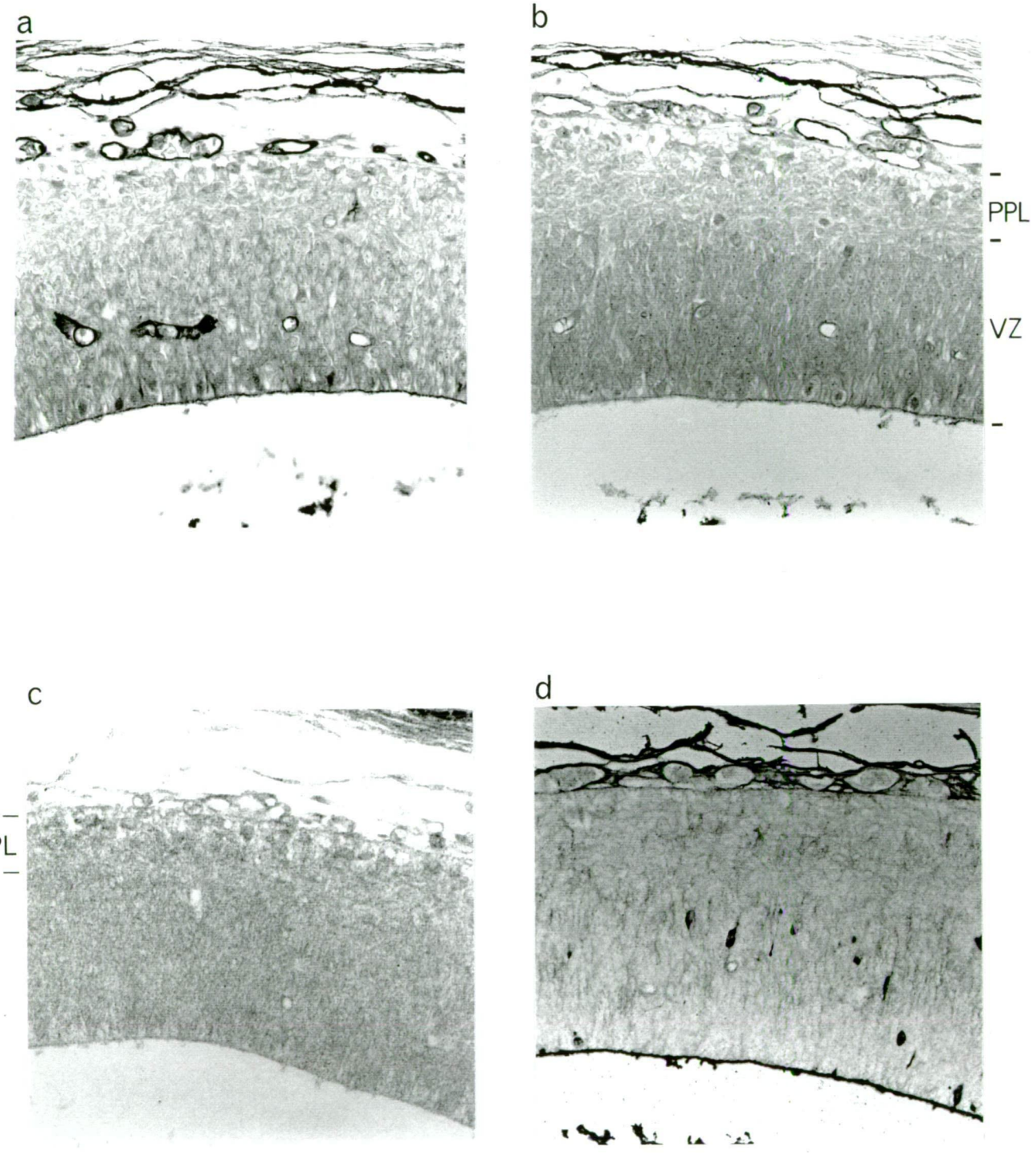
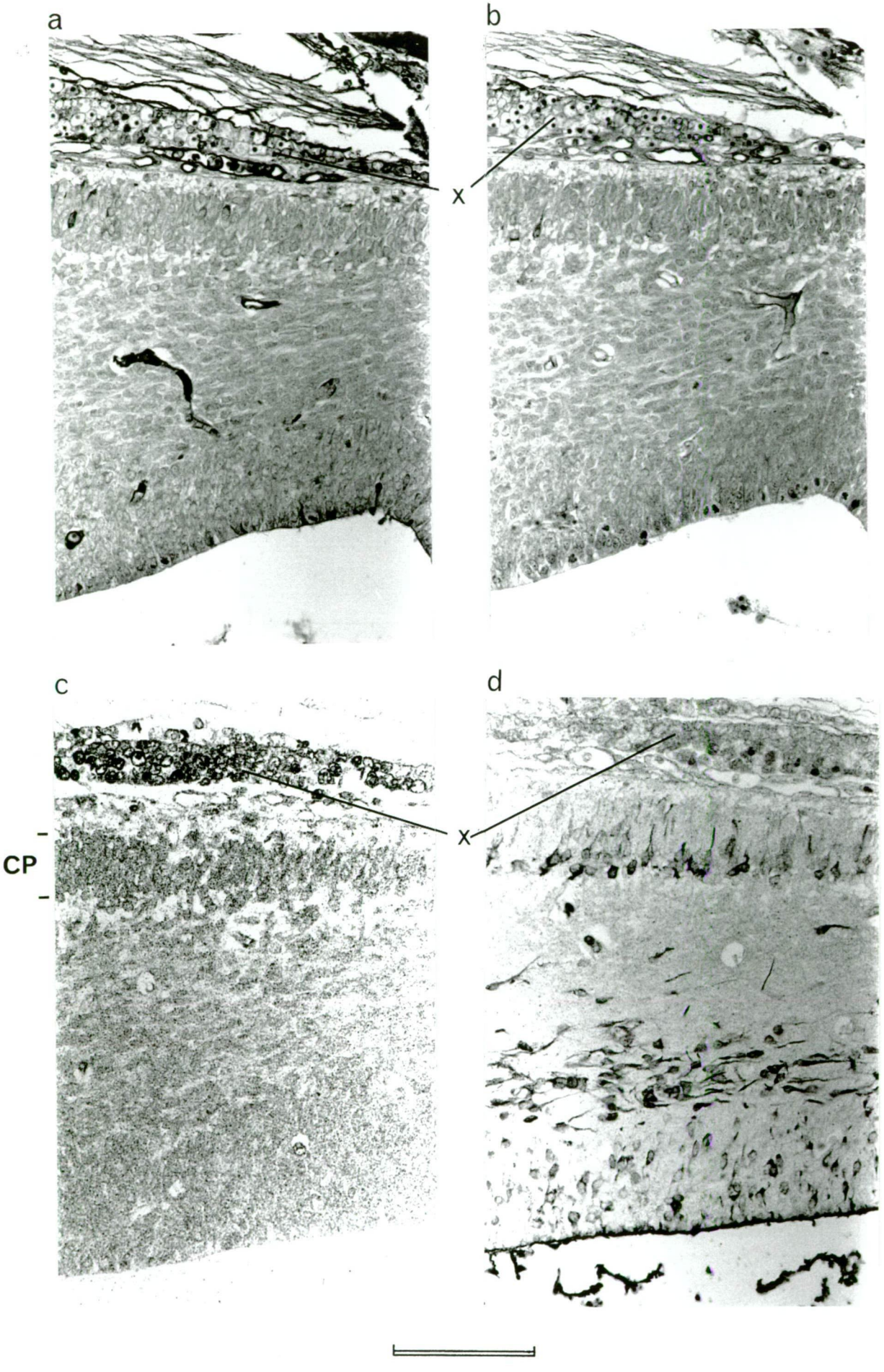

a
b
PPL
VZ
c
PPL
d

Figure 3.7

Micrographs of coronal sections through the neocortex of E16 rat into which a solution of bovine fetuin was injected into the ventricle and the sub arachnoid space. (a), (b) and (d) are stained immunocytochemically; (a), rat albumin; (b), rat fetuin and (d), bovine fetuin. For comparison (c) was stained for rat mRNA (*in situ* hybridisation). Micrographs were taken with a X40 objective lens using an "Edge" R400 (Edge Scientific Instruments, USA) 3D light microscope. Scale bar = 100μm.

Note the presence of rat albumin and rat fetuin ((a) and (b) respectively) in the blood vessels at the pial surface and within the ventricle. The injected, exogenous, bovine fetuin is also present within the ventricle, (d) and in the subarachnoid space. Injection of the bovine fetuin caused blood contamination (X) of the CSF in the subarachnoid space, probably due to the rupturing of blood vessels on the pial surface. Within the neocortex there appears to be a very limited amount of staining for the rat fetuin and rat albumin. However there is clear staining for bovine fetuin within specific cells of the ventricular and subventricular zones and in cells of the subplate. There is an increased level of rat fetuin mRNA signal, (c) in the region of the cortical plate, indicating synthesis of the protein within these cells.


a
b
x
c
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d
x

At E16 however there is a marked difference in the staining within the neocortex for the exogenous, bovine fetuin compared to the E15 neocortex. Again the protein is present on both sides of the ventricle but there has been specific uptake of the protein into cells of the ventricular, subventricular zones and the subplate region. Like the staining at E15 the bovine fetuin appears to be associated with the cells and is not present extracellularly.

Figures 3.6 (c) and 3.7 (c) show the *in situ* hybridisation for rat fetuin. These sections were from the same brains as the other 3 micrographs in each figure and were stained using the methods of Dziegielewska *et al* (1993) by Dr K.M. Dziegielewska. Between E15 and E16 the cortical plate has begun to form and this population of cells shows a raised level of signal for the rat fetuin mRNA. This level of signal is in contrast to the immunocytochemical stain for the protein in the same area of neocortex. Rat fetuin does not appear to be present within these cells. This discrepancy can be explained by either a difference in sensitivity of the detection mechanism used to visualise rat fetuin or, more likely by a time lag between when mRNA for fetuin first appears and when the protein is present.

In summary; when bovine fetuin was injected into the lateral ventricle and the subarachnoid space of the cerebral vesicle, specific cells within the ventricular zone, subventricular zone and cortical plate take up the protein. Endogenous proteins (albumin and fetuin) were not detected by immunocytochemistry within these populations of cells although it appeared that the synthesis of fetuin was taking place in the cells of the early cortical plate.

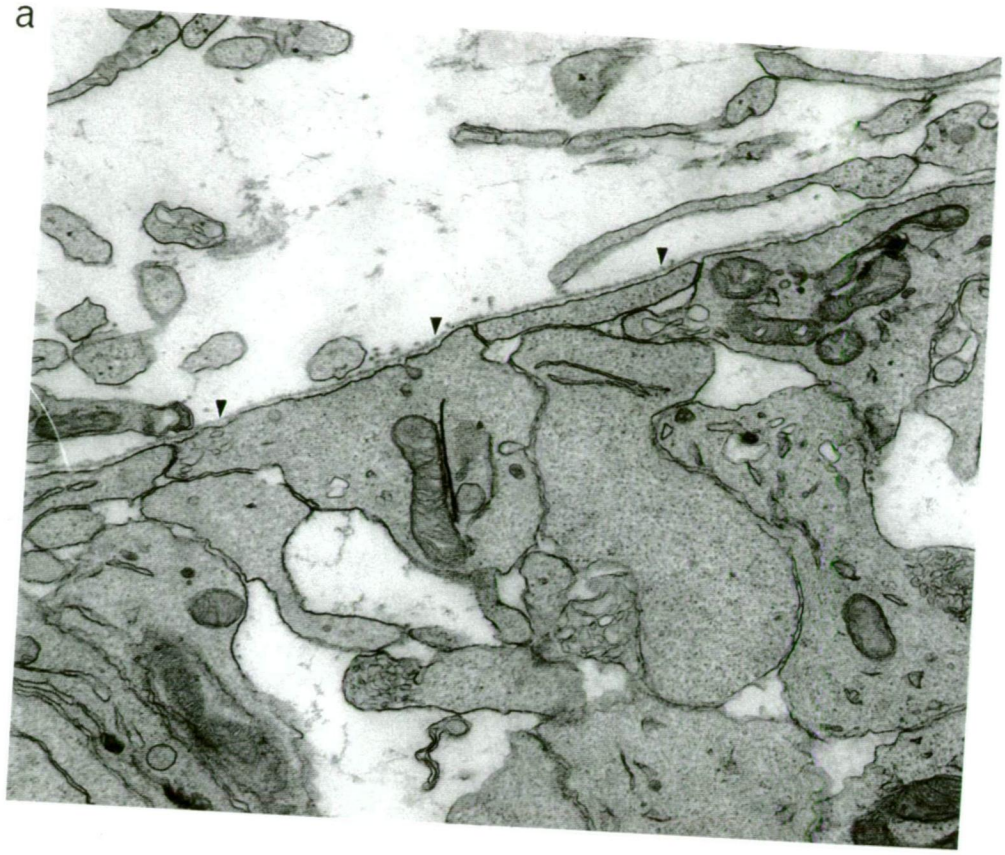
Electron Microscopical Appearance of the Ventricular and Pial Surfaces

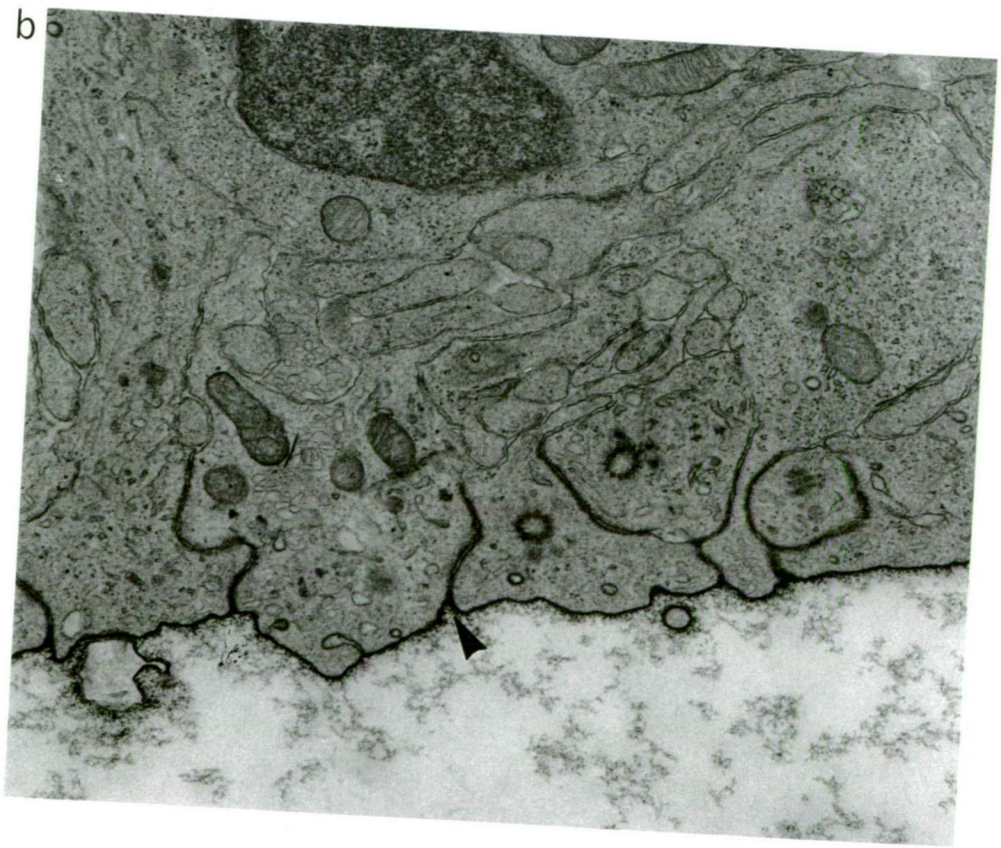
Figure 3.8 shows electron micrographs from the neocortex of two preparations. One preparation was cultured for 24 hours at 33°C in medium 10%

Figure 3.8

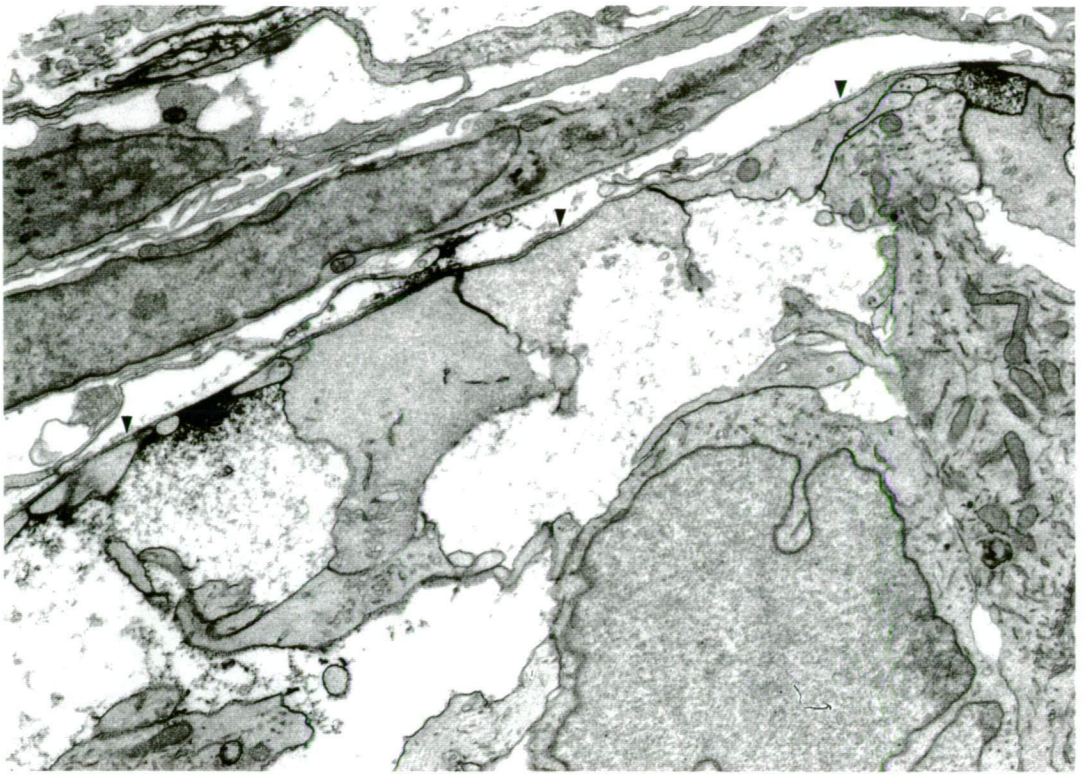
Thin section electron micrographs of cross sections through the pial surface (a and x) and the ventricular surface (b and y) from; (a) and (b), E15 control neocortex, no culture and; (x) and (y), E15 + 24 hours in culture, 33°C, 10% FCS. Scale bars = 1μm.

Note the basement membrane (small arrow head) on the pial surface of both the *in vivo* and *in vitro* preparations and the close apposition of cells immediately below forming junctions (a and x). At the ventricular surface characteristic "strap junctions" (large arrow head) in both preparations connect neuroependymal cells at the CSF-brain and medium-brain interface (b and y respectively).

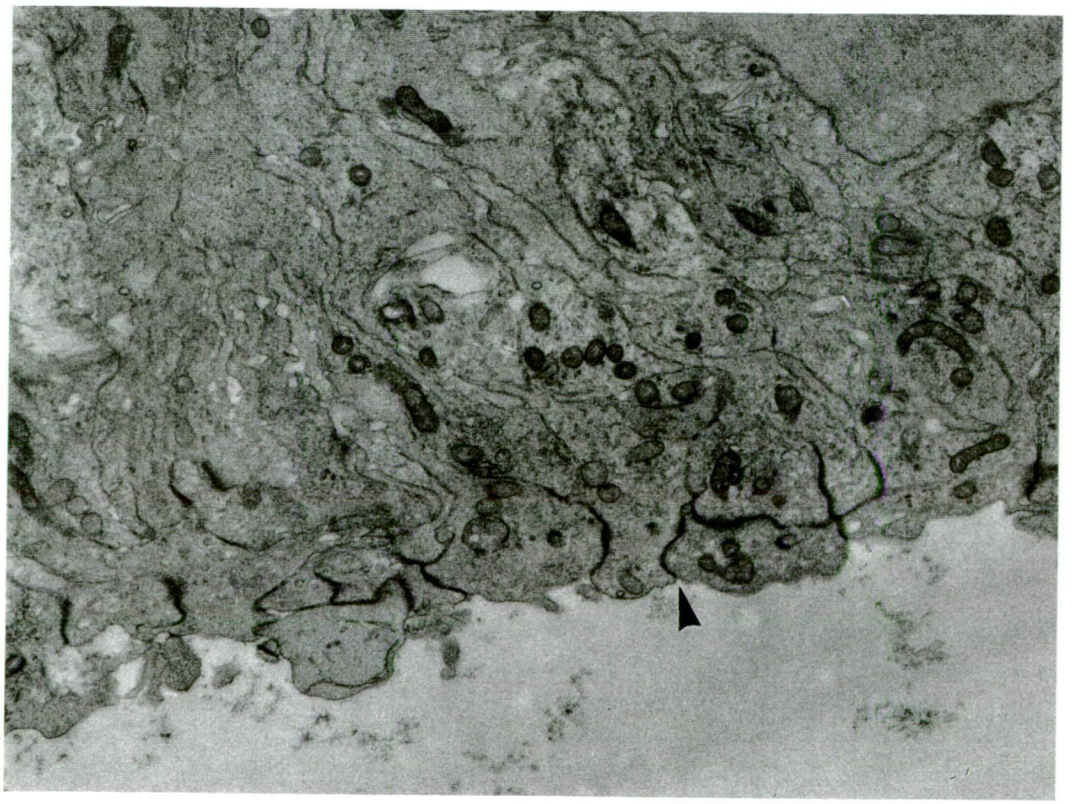

a


b

x



y



FCS, the other was a control, E15 preparation which was uncultured. The ventricular surface and the pial surface from each preparation are shown.

At the pial surface of the control and cultured preparation the basement membrane (indicated) is shown to be intact and continuous. Beneath this membrane there appears to be close apposition and junction formation between cellular elements presumed to be glial end feet. These junctions, at the pial surface, form a barrier between the extracellular space inside the neocortex and the fluid on the outside.

At the ventricular surfaces of the control and cultured preparations the characteristic strap junctions appeared to be both tight and continuous across the entire region of dorsal neocortex sectioned. The junctions were present at the most luminal part of the neuroependyma and are similar in both preparations. They also exhibit the same "tortuous" configuration as shown by Møllgård *et al* (1987) in the fetal sheep.

Analysis of CSF and the Culture Medium

The above results have examined changes in morphology as well as presence of certain plasma proteins within the neocortex before and after periods in culture. With some preparations, samples of fluid were also withdrawn from inside the preparation, the ventricles, and from the surrounding culture medium. These samples were measured for their total protein concentration and analysed using crossed immunoelectrophoresis (see Methods). These methods were able to show the protein concentration as well as the composition of the culture medium inside the ventricles and on the outside of the isolated CNS before and after 24 hours *in vitro*.

Protein Concentration (mg/100ml)	Before Culture	After 24 Hours in Culture
Ventricular Fluid	134 ± 21 (13)	158 ± 13 (10)
Culture Medium	300*	247 ± 22 (4)

Table 3.1 Total protein concentrations of fluid from within the E15 ventricles before and after 24 hours in culture. Also shown is the concentration of the culture medium on the outside of the preparation at the same time. * A single batch of FCS was used for all experiments. The protein value given is the mean of multiple samples.

During the 24 hours in culture the medium was continuously bubbled at an approximate rate of 3 bubbles per second. Over a few hours this caused the culture medium to foam therefore lowering the protein concentration in the remaining fluid. This is shown by the large decrease in the total protein concentration over the whole 24 hours from 300 mg/100ml to 247 mg/100ml ± 22. The variation in the medium protein concentration after 24 hours was probably due to the variation in the rates of bubbling causing different amounts of foaming within the flask.

Interestingly, Figure 3.2 (d) shows a connection in the base of the brain between the ventricles and the outside of the brain. This indicates that free diffusion is possible between the CSF inside the ventricles and the medium on the outside in these preparations. Yet measurements of the total protein concentration of fluid removed from these regions show that the fluid in the ventricles was at a lower concentration than that on the outside. In fact, the concentration of ventricular fluid after culture was very similar to the CSF before culturing began (ventricular CSF from E15 rat fetus, 134 ± 21 mg/100ml; ventricular fluid from E15 rat fetus after 24 hours in culture, 158 ± 13 mg/100ml).

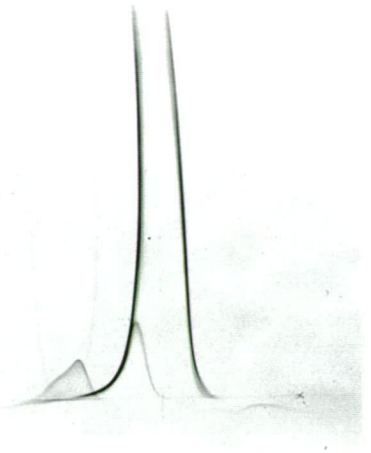
Introduction) but during dissection there seems to be an inevitable but small amount of tissue damage which enables an increased communication of fluid between these two compartments. The areas most susceptible to damage are the regions of the optic chiasm, below the developing midbrain and also the thin layer of cells covering the IVth ventricle. Total protein measurements show that after 24 hours of culture the concentration of protein within the fluid contained in the ventricles is only marginally greater than that which is found in the ventricles *in vivo* at the same age (E16, approximately 150 mg/100ml, see Chapter 2). It is likely that the CSF has diffused out of the ventricles and been replaced mostly by the culture medium for the period in culture. Therefore cells at both the ventricular surface and the pial surface are exposed to the proteins within the culture medium. The protein concentration of this medium is also very similar to that which would normally be found *in vivo* at this developmental stage. Few studies in the past have paid much attention to providing a medium concentration which resembles that of the CSF. Ames and Nesbett (1981) described a medium which resembled CSF as being better for culturing rabbit retinal cells than the more conventional recipes. Despite what appears to be a general leak of the CSF out of the ventricles and movement of culture fluid to take its place, the overall structure of the preparation appears to be well maintained with no evidence of further damage occurring during culture. Prior to these experiments it was not clear whether proteins or other substances in the medium were accessible to cells within the neocortex of isolated CNS preparations, given the presence of effective barriers to protein on the inner and outer surface (see Introduction) *in vivo*. The immunocytochemical and EM studies show that these barriers remain intact *in vitro* for at least 24 hours. However it is clear from the protein immunocytochemistry studies fetuin, at least can be taken up by cells, both at the ventricular zone interface with CSF and by cells in the cortical plate.

Figure 3.9

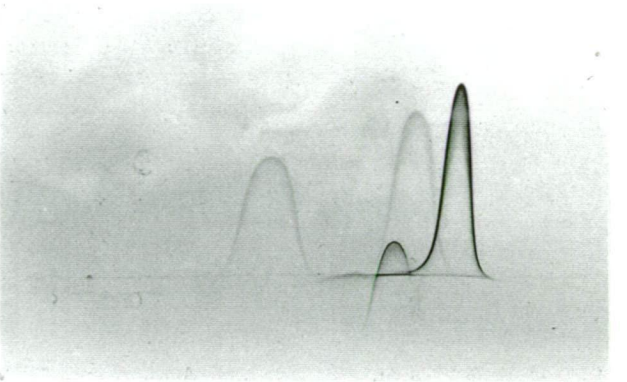
Crossed immunoelectrophoretic plates containing antibodies to fetal calf serum, (a) and (b) and antibodies to rat plasma (c) and (d). (a), 10% FCS; (b), ventricular fluid after 24 hours in culture; (c), CSF from the ventricle before culture and (d), ventricular fluid after culture.

Note the abundance of fetal calf serum proteins within the ventricles after 24 hours in culture. There is a marked decrease in the amount of rat's own proteins within the CSF after the culture period. Plates (c) and (d) contained the same concentration of antibody and the same volume of sample was added. The most prominent peak in each plate is albumin.

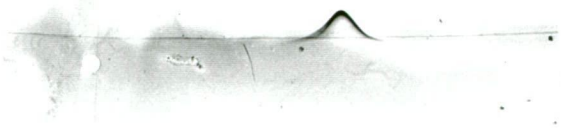
a



b



c



d

Figure 3.9 shows four crossed immunoelectrophoretic plates from four samples: two were from samples of ventricular fluid, one plate contained antibodies against rat plasma proteins, the other those against fetal calf serum. The third plate shows fetal rat ventricular CSF run against anti rat plasma antibodies and the remainder showing culture medium (10% FCS) run against antibodies to fetal calf serum. The concentrations of the antibodies used and the amount of sample applied to the plate was varied so that the protein peaks fitted onto the entire gel. Quantitative analysis of individual proteins between different plates would not be possible due to these variations. These plates show the individual proteins in the sample applied as a series of peaks. It is clear that although the protein concentration within the ventricles before and after culture is very similar most of the rat proteins present within the CSF before culturing have been replaced by the fetal calf serum proteins after 24 hours. There is only a slight trace of the rat proteins within the ventricles after this time.

Discussion

Nicholls *et al* (1990) showed that the entire CNS isolated and maintained in culture showed remarkable tissue preservation within the spinal cord. Whilst in culture, it was also shown that axons continued to grow and make synaptic contacts. Nuclei within the germinal region appeared to continue DNA replication. During this period of time however, it did not appear that there was a marked increase in the number of cells within the regions studied. The protein composition of the medium was not considered in these studies.

The present work has concentrated only on the changes which have occurred in the neocortex of the isolated CNS and results have shown that after the entire CNS has been isolated and maintained in basal Eagle's medium there was a continued proliferation of cells. This proliferation is more striking in the presence of FCS (10%) and was accelerated at 33°C. The germinal layer, or ventricular zone, within the cerebral neocortex contains cells which continued dividing as they would normally *in vivo*. When comparing the level of proliferation and cellular organisation which had occurred *in vivo* after 24 hours from E15 with that which occurred after 24 hours *in vitro*, it is clear that *in vivo* the brain shows greater morphological changes. The most noticeable event which has occurred within the neocortical wall over this period of time *in vivo* is the differentiation of cells in the region of the cortical plate. This has appeared across the entire dorsal and lateral region. *In vitro* this does not appear to have occurred in preparations cultured with or without protein. However during this time there has been a large increase in the number of cells making up the neocortical wall in the presence of FCS, some of which were in the zones outside the ventricular zone itself.

This cellular proliferation in the presence of FCS is a similar result to that obtained by Coon and Sinback (1982) who used specific regions of the CNS: cerebral hemispheres, spinal cord and regions from which the cerebellum would

have arisen. The neuroblasts were dissociated and distributed into Petri dishes before being cultured in a medium containing hormones and growth factors with either 0.5% FCS or 5% FCS. They found that cells in the higher protein medium divided at a faster rate in the first two days of culture. However, this high serum medium inhibited the development of compact monolayer regions, or masked them. The authors conclude that they also got qualitatively different results in the presence of a high serum concentration.

Specific changes made to the media have been shown, in many studies, to influence the survival and/or proliferation of certain cells dissociated in culture. The changes include raising the glucose levels in the medium to increase the neuron survival. Increasing potassium levels also increases neuron survival in dissociated cultures of embryonic chick dorsal root ganglia (Scott and Fisher, 1971). Nerve growth factor has been shown to be essential for survival and maintenance of dissociated sympathetic and some embryonic sensory neurons (Levi-Montalcini and Angeletti, 1963). This has been reviewed by Bottenstein (1983).

In the current experiments a concentration of 10% FCS was used in the culture medium. This provided a final concentration of around 300mg/100ml which is higher than would normally be found within the CSF of the ventricles at around this time (E15, 134 ± 21 mg/100ml). However, protein measurements of the culture medium showed that soon after the culture process had started, the foaming action of the gas which was continuously bubbled through the medium caused a reduction of the protein concentration (from 300mg/100ml to 247 ± 22mg/100ml in 24 hours) to one which was closer to that found *in vivo*. The overall composition of the FCS remained unaltered and was supplied from the same batch for all the experiments described in this thesis (see Methods).

During this developmental stage *in vivo* the CSF is contained within the ventricles and the spinal canal as well as surrounding the CNS (subarachnoid space). At around this time *in vivo* the communication between the ventricles inside and the subarachnoid space on the outside begins to increase (see

Proliferation of Cells In Vitro

Coronal sections through the neocortical wall show that after 24 hours in culture in the presence of 10% FCS a large amount of cell proliferation has occurred. Mitotic figures are seen at the ventricular surface, as *in vivo*. To examine this cell proliferation whilst in culture the thymidine analogue BrdU was introduced into the medium for a short period of time, immediately after the preparation was removed from the fetus.

The cycling time for nuclei within the ventricular zone of the neocortex in the E15/E16 rat is between 8 and 10 hours, (Hicks and D'Amato, 1968) and this increases to 18 hours at E18 (Waechter and Jaensch, 1972). The S-phase during these ages is thought to be relatively constant but thickening of the ventricular zone means that the subsequent stages of the mitotic cycle: G_2, M, G_1 and T are increased as the nuclei move through a greater distance to the ventricular surface where they divide and then move away towards the more superficial layers. During this time one cell is able to undergo one complete mitotic division forming two daughter cells. Using the DNA marker, BrdU, it appeared that the intermitotic nucleic cycling was also occurring *in vitro* although at a much slower rate. BrdU positive nuclei appeared in profusion close to the ventricle after 24 hours in culture (33°C). The staining within some of these cells is extremely weak and would suggest that these cells were undergoing the final part of the S-phase (DNA replication) when exposed to the marker, therefore only incorporating small amounts of the thymidine analogue. There are also a number of densely stained nuclei within the ventricular zone close to the ventricular surface and in the more superficial region of the ventricular zone but only after 40 hours in culture at 29°C or 24 hours at 33°C. It would appear therefore that the rate of cell proliferation in the cultured preparation is increased at the higher temperature of 33°C. The total amount of proliferation does not however appear to be increased at the higher temperature, merely the speed with which it occurs.

The process of cell division, *in vitro*, involving the cycling of the nuclei within this germinal layer appears to be occurring in the same way as it would *in*

vivo . These experiments clearly verify that the proliferation of the cellular DNA is occurring in the outer portion of the germinal zone (Angevine and Sidman, 1961; Sauer and Walker, 1959) and that following this replication there is a migration of the nuclei towards the ventricle prior to division. This is more prolonged *in vitro* with the arrival of the first labelled nuclei at the ventricular surface occurring after 19 hours at 33°C.

Hicks and d'Amato (1968) had noticed that cells taking up a label in the S-phase of their mitotic cycle can be placed into three groups: those that move towards the ventricle and divide, those which remain in the outer portion of this germinal zone and a certain portion of others which replicate their DNA and divide whilst moving directly out into the marginal (migratory zone). In the present work it is difficult to determine whether labelled cells have "escaped" the ventricular zone as the boundary between this region and those more superficial is unclear.

The division occurring at the ventricular surface *in vivo* is then followed by the migration of these cells to the outer regions of the neocortex. *In vitro,* the E15 cortex which has been in culture for 24 hours, does not appear to have any cells forming a cortical plate or a significant number of cells which have moved out beyond the subventricular zone and into the intermediate zone. A quantitative analysis of the changes occurring to the different layers *in vitro* was difficult as the interface between these regions was unclear. It may therefore be that despite the slowing of nucleic movements within the cortex, normal migration of cells out into the more superficial areas would still have been possible given a longer period of time in culture. It is clear however that in culture there is a proliferation of cells in the neocortex, particularly in the regions of the ventricular and subventricular zone. Also illustrated in Figure 3.2 (d) are mitotic figures some distance from the ventricular surface in a similar region to that found outside the ventricular zone *in vivo*, known as the subventricular zone. Therefore after 24 hours *in vitro* a secondary mitotic zone formed as it would *in vivo*.

Migration of neuronal and glial cells has been thought to occur in a radial fashion (Gadisseuz *et al,* 1989; Rakic, 1971) assisted by glial fibres lying radially across the cortex which supply a matrix along which the cells can move. Movement of neurons along radial glia fibres has been observed in culture (Hatten *et al,* 1984, 1986; Edmondsen *et al,* 1988, Edmondsen and Hatten, 1987), although the neurons appeared to move along these elongated processes until they reached the end where they then moved back again. Therefore *in vivo* the migrating neuron does not rely entirely on the glial processes for its migratory path to the correct position in the cortex.

According to Bayer and Altman (1992) the cortical plate in the rat first forms around E16 in the more ventrolateral part of the neocortex (also Raedler and Raedler 1978) and is made up of subplate neurons which are generated between E13 and E14. Cells labelled at E15 and studied whilst in culture for a 24 or 40 hour period, depending on the temperature, may not therefore reach the cortical plate or any position close to it, as this would not be expected *in vivo.*

Previous experiments have shown that subplate cells participate in the early functional circuits before the time of synaptogenesis in the cortical plate (Molliver *et al,* 1973; Kostvic and Rakic, 1980; König and Marty, 1981; Blue and Parnavelas, 1983a,b; Chun and Shatz, 1988, Kageyama and Robertson, 1993. Reviewed by Allendoerfer and Shatz, 1994). Labelling axons with the fluorescent tracer DiI in the internal capsule at a time when only subplate and marginal zone cells were present McConnell *et al* (1989) were able to label only these cells. The circuits that were studied in the cat neocortex were only transient and had disappeared when the 6 layers of the cortex had appeared (Chun and Shatz, 1988).

Therefore, the subplate cells may play a vital role in the formation of thalamocortical connections as well as the positioning of neurons in the correct region of the cortical layers. Ghosh and Shatz (1992) showed that the introduction of kainic acid, which ablates subplate neurons, into an area of the lateral geniculate nucleus disrupted the correct arrangement of cells into the six

layers. The present *in vitro* studies were done at a time of extensive fibre growth between the neocortex and other regions and if this had become disrupted or slowed in some way then the migration of cells out to the cortical plate may also be impeded. It was clear during these studies that regions of the isolated CNS deep within the brain; eg. the thalamic region, contained pyknotic nuclei indicating cell death. Fibre growth to and from this region has not been studied in these *in vitro* preparations but it would seem unlikely that this would continue as normal. This could be a contributing factor to the lack of cortical plate formation.

CSF-Brain Barrier

Electron micrographs taken of the ventricular surface and the pial surface show that both surfaces still appear to have maintained a tight barrier between the extracellular space around cells of the neocortex and the medium within the ventricles. At the pial surface, strap-junction-like elements linking the foot processes of glial end feet form the barrier on the outer surface and on the inner surface the characteristic strap junctions (see Introduction) between cells form the barrier at the ventricular surface. Junctions on the outer surface have not yet been characterised by either HRP or freeze fracture studies. The strap junctions at the ventricular surface have the same tortuous configuration both in the control preparations and those which have been cultured for 24 hours giving the same appearance as was described by Fossan *et al* (1985) and has been shown also in sheep fetuses (Møllgård *et al,* 1987) and human fetuses (Møllgård and Saunders, 1986). Therefore, this evidence suggests that in culture, as *in vivo*, the simple diffusion of substances within the fluid from either the ventricles or from the outside of the brain is restricted due to, what appears to be, a maintained CSF-brain barrier.

Staining for Fetuin and Albumin

Measurements of the total protein concentration of the fluid contained within the brain of preparations in culture show that the concentration in ventricles is similar to that found *in vivo*. Immunoelectrophoretic analysis of the individual proteins within the ventricles showed that the CSF had been replaced by the medium after the culture period (see Figure 3.9). Micrographs of coronal sections through the brain show an opening at the base of the brain allowing free access of culture medium into the ventricles (see Figure, 3.2 d). There was however a small amount of rat protein remaining in the ventricle and the total protein concentrations were not the same, suggesting that the medium and the CSF had not mixed thoroughly within the time period.

Immunocytochemical staining of coronal sections of the neocortex for the proteins, bovine albumin and bovine fetuin, both present in the culture medium, showed that the barrier was able to restrict movement of these proteins into the extracellular space of the neocortex, as *in vivo* .

Previous studies have demonstrated the presence of plasma proteins within cells of the CNS in rats (Benno and Williams, 1978), rat fetuses (Møllgård *et al*, 1987; Cavanagh and Warren, 1985), mice (Toran-Allerand, 1980), human (Møllgård and Jacobsen, 1984), human fetuses (Møllgård *et al*, 1988), baboon (Uriel *et al*, 1982), pig (Cavanagh and Møllgård, 1985), pig fetuses (Cavanagh and Møllgård, 1985), cow fetuses (Reynolds *et al*, 1987), sheep fetuses (Reynolds and Møllgård, 1985), pouch young wallabies (Dziegielewska *et al*, 1988). Of these species studied a few have been shown to synthesise certain proteins within specific cells of the CNS (Dziegielewska *et al*, 1986; Møllgård *et al*, 1988; Dziegielewska *et al*, 1993).

The fetal, plasma protein fetuin has been shown to be present within cells in the developing brains of many different species: sheep (Saunders *et al*, 1992; Dziegielewska *et al*, 1993; Møllgård *et al*, 1984; Reynolds and Møllgård, 1985), pig (Cavanagh and Møllgård, 1985), human (Dziegielewska *et al*, 1987), cow (Reynolds *et al*, 1987), rat (Sarantis and Saunders, 1986) and tammar wallaby

(Jones *et al*, 1988 and 1991). This fetal glycoprotein appeared first in the population of cells that migrate away from the ventricular zone and differentiate into the first cells of the cortical plate. Saunders *et al* (1992) showed that fetuin in the sheep is a marker for the early cortical plate cells which begin to differentiate within the primordial plexiform layer. In fact, in all species studied, the same population of cells show similar fetuin staining. The authors showed that these cells later contribute to the formation of the subplate as the cortical plate begins to thicken. These are the cells which provide the early scaffolding of the developing layers of the neocortex (Luskin and Shatz, 1985; Shatz *et al*, 1988). In the sheep the cells were also found to contain the specific mRNA for fetuin (Dziegielewska *et al*, 1993). The authors concluded that the presence of the fetuin within these cells was likely to be due to *in situ* synthesis as the transfer of the protein from the CSF and blood to the brain is unlikely because of the CSF-brain and blood-brain barriers (see Introduction).

In the rat however, the presence of fetuin within the early neocortex *in vivo* has not been shown. Sarantis and Saunders (1986) showed that fetuin appeared in cells of the upper intermediate zone and subplate by E21. These cells were described as being mature and staining was seen in the cell bodies as well as their processes. Until the present studies fetuin has not been seen in the early cortical plate cells of the rat (E15-E16). At this point it is important to mention that the antibody used in the study of Sarantis and Saunders (1986) was raised against the human glycoprotein α_2HS. Only recently were the human form of this glycoprotein and bovine fetuin found to be homologues belonging to the cystatin family and are now considered to be the same (see Brown *et al*, 1992 for review). The antibody used in the present study was raised against rat fetuin (see Methods) and does not appear to detect any of the glycoprotein in the neocortex at either E15 or E16. Using probes against the fetuin mRNA (Dziegielewska *et al*, 1995) did however indicate that between E15 and E16, when the cortical plate first appears, there was an increase in the level of mRNA signal in the cells in this region.

In vivo then, the present studies show that rat fetuin is synthesised in cells of the early cortical plate but it was not possible to localise the protein using the available techniques and antibodies. The concentration of the rat fetuin is likely to be low in these early cells if synthesis has only just started.

Evidence from the electron microscope shows that in the E15 neocortex and also the neocortex which has been cultured for 24 hours the CSF-brain or medium-brain barrier on the inside and outside appears to be continuous and undisturbed. Using immunocytochemical methods there does not appear to be any extracellular staining for either bovine fetuin or bovine albumin within the neocortex, which also verifies a maintained barrier. Therefore diffusion of fetuin or albumin to the extracellular space is restricted and cells containing fetuin in the neocortex were able to specifically take it up directly from either the ventricular surface or the pial surface (see Figure 3.5).

After 24 hours in culture at 33°C there appeared to be a specific population of cells in a region of the cortical plate which had stained positively for fetuin. This staining was not seen in any cells of this region at any time prior to this. At previous time intervals fetuin positive cells as well as cell processes were present in the outer part of the ventricular zone. After 24 hours *in vitro* the cells present within a region of the cortical plate showed a similar fetuin staining to that found in early cortical plate neurons of other species, but not previously demonstrated in the rat (see above). These cells have either arrived in this region during the period of culture (migrated) or were present throughout and after 24 hours *in vitro* have differentiated in some way as to be able to specifically take up the fetuin from the medium.

Albumin, a 69 kD protein abundant within the plasma of most species, is present within the plasma and CSF during development. The entry of this molecule into the CSF during development has been studied extensively in the rat (Habgood *et al,* 1992) and sheep (Dziegielewska *et al,* 1991) but only in the *Monodelphis* has this entry mechanism been closely examined at the time of cortical plate formation (see Chapter Two). The movement of this molecule from

the CSF into the brain was studied in prenatal and neonatal rats (Cavanagh and Warren, 1985) by injected sheep serum in the ventricles of animals of different ages. At E13 (designated as E14 by Cavanagh and Warren (1985) as fetuses were aged differently: the day following mating was designated as E1. *cf.* Methods) only one fetus was injected and albumin was not found within any cells of the neocortex. At E15 the authors describe the results as "variable" with only some fetuses showing the presence of sheep albumin in cells of the neocortex. The present studies have looked for the presence of bovine albumin in the E15 neocortex up to 24 hours after being maintained in culture. At no time during culture did the protein appear either intracellularly or extracellularly. This confirms a maintained barrier between the medium and the brain as well as the lack of specific uptake of the protein by cells of the neocortex, in contrast to bovine fetuin (see Figure 3.5).

The present studies therefore have shown that *in vitro,* cells of the neocortex were able to take up a specific protein, fetuin, from the medium. It has also been shown to be present within cells of the early cortical plate and these cells are known to synthesise fetuin *in vivo* (Dziegielewska *et al*, 1993).

Bovine fetuin which was carefully injected into the subarachnoid space as well as the ventricle of the same rat fetus, *in utero,* at E15 and E16 was also taken up by certain cells within the neocortex. This uptake was compared with the presence of the endogenous fetuin and also with the levels of signal for rat fetuin mRNA (see Figure, 3.6 and 3.7). At E15 and E16 certain cells within the ventricular zone appeared to have taken up the bovine fetuin and yet the endogenous proteins fetuin and albumin were absent from this and all other regions of the neocortex. If during the course of this experiment damage had been caused to either the pial or ventricular surface when the bovine fetuin was injected, endogenous albumin or fetuin would have appeared within the neocortex (Cavanagh and Warren, 1985). The two proteins are present on both sides of the neocortical wall but not within it, either intracellularly or

extracellularly. At E16 early cortical plate cells have only taken up bovine fetuin from the CSF spaces.

As mentioned before, fetuin in the past was shown to be present as well as synthesised in cells of layer VI in the fetal sheep (Dziegielewska *et al*, 1993) and a proportion of these cells are the known early generated subplate cells (Saunders *et al*, 1992). The correlation between these cells and the function of fetuin is at present unclear. It would appear that *in vitro* the thalamic regions were not well preserved, therefore cells and their associated fibres were likely to be absent, yet cells in the region of the cortical plate still took up fetuin from the medium as they would *in vivo* from the CSF. A possible function for fetuin in subplate cells, which remains to be investigated, is that it acts as a signal or chemoattractant for the incoming thalamocortical fibres.

General Discussion

A large part of the work presented in this thesis studied certain aspects of two barriers within the brain: the blood-CSF barrier and the CSF-brain barrier. This work has been presented in two parts. The first part using *Monodelphis domestica* as a model, has studied the barrier (to albumin) between the blood and the CSF. The second part using both an *in vitro* and *in vivo* model has studied the barrier (to protein) between the CSF and the brain. Using these *in vivo* and *in vitro* models, the ability of these barriers to exclude protein during the very early stages of brain development, when the cortical plate first starts to differentiate, was found to be both effective and well developed. During this time period the concentrations of protein within the CSF are known to be high and it would seem likely that the effectiveness of these barriers is crucial during normal brain development.

The measurement of total protein in the CSF from the lateral ventricles and *cisterna magna* of fetal rats at different ages has clarified the relation between the peak concentration of protein in lateral ventricular CSF and the initial stages of formation of the cortical plate. In species other than the rat it has been shown that there is a clear correlation between the two events (see Introduction). The present result shows that for CSF from the lateral ventricles (which is closest to the developing neocortex) the peak in protein concentration in the rat also corresponds to the initial stages of cortical plate formation.

Albumin Transfer into the CSF

Using the marsupial species *Monodelphis domestica*, during the initial phase of cortical plate formation, and increasingly so as development progresses, the movement of albumin from the blood into the CSF is restricted. The albumin molecule has limited access by simple diffusion but is also able to enter by a species specific mechanism. This mechanism seems only to operate during the early stages of brain development. Transfer of albumin was only investigated in one region of the CSF, the *cisterna magna*, and previous studies have shown it in

later stages of development, the level of transfer may differ between regions (Dziegielewska *et al*, 1991). This problem still remains unresolved at the very early stages of brain development. The present work has shown that in the rat there is a marked difference in the concentrations of protein between different regions of the CSF over the period of cortical plate initiation and formation. There is little doubt that the route of entry of albumin is through the epithelial cells of the choroid plexus (Dziegielewska *et al*, 1991) although the present work did not examine the route morphologically. Further work needs to be carried out to determine whether some aspects of the route change during development and account not only for the different levels of transfer but also for regional concentration differences.

The functional significance of a mechanism which operates to control the concentration of proteins within the CSF and its effect on neocortical development may be manifold and difficult to determine *in vivo*. Other proteins have been shown to be transferred specifically from the blood to the brain and CSF including: insulin (Pardridge *et al*, 1985; King and Johnson, 1985; Duffy and Pardridge, 1987), α-fetoprotein (Villacampa *et al*, 1984), IgG (Vollerthun and Möller, 1980). The functions of albumin, IgG and insulin within the body are well established in the adult. But it is not known what their functions are in CSF or within cells of the brain at this extremely early stage of development. A high concentration of protein within the CSF, which appears not to cross into the extracellular space through the CSF-brain barrier during the period of neurogenesis (see Chapter Two, Results), may exert a significant colloid osmotic effect. The high concentration of protein in the CSF may therefore have a very important role in terms of providing a physical force on fluids across the CNS. These forces could therefore determine the movement and direction of cellular migration and contribute to the shaping of the brain. Since the ventricular system of the CNS preparation did not expand, unlike the situation *in vivo*, it seems likely that continued CSF secretion by the choroid plexus into the ventricular system together with the osmotic transfer of water and solutes from brain tissue to CSF may be useful for shaping the developing brain.

In Vitro Studies

In vitro it appeared that there was a large proliferation of cells into the ventricles and that during this time in culture the barrier separating the brain from the medium in the ventricles had been maintained. This may be evidence that a change in the colloid osmotic pressure due to the culture medium can affect the growth and shaping of the brain. A relatively higher concentration on the outside of the brain and in the ventricles compared to *in vivo* has produced a large difference in the way cells of the ventricular zone proliferate.

Isolating the entire central nervous system and maintaining it in a viable state for a period of time in which normal cell proliferation appears to occur provides a convenient model in which to study the relationship between cells of the neocortex and certain CSF proteins in the surrounding medium. During the period in culture the barrier between the CSF and the brain, which has been studied extensively at the ventricular surface under the electron microscope, is functionally significant, limiting the movement of proteins such as albumin and fetuin from the medium into the brain. This barrier appears to be well maintained throughout the time period *in vitro* while there is a continuing proliferation of cells in ventricular zone and an apparent increase in the number of cells in the more superficial regions.

During the growth and development of the neocortex, either *in vitro* or *in vivo,* it would appear that the uptake of fetuin is limited to specific cells and their stage of development. Cells within the ventricular zone and cells in a region of the cortical plate appear to specifically take up the protein. *In vivo* this uptake in the more superficial region of the neocortex appears to be only present in cells in the lower cortical plate, the subplate. There also appears to be uptake by a population of cells migrating horizontally to the ventricular surface in the subventricular zone. The cells appear to be those migrating laterally to the more ventrolateral regions of the neocortex.

Manipulating the environment of the neocortex in order to study fetuin uptake and its role in neocortical development would be technically difficult using

an *in vivo* model. An *in vitro* model, in which the cells of the neocortex appear to behave in a similar fashion to those *in vivo*, provides an excellent opportunity to determine the importance and function that fetuin might have in cortical development.

The *in vitro* experiments have shown that the movement of a specific protein, fetuin, into the brain is a developmentally regulated phenomenon. Similarly the *in vivo* experiments have shown specific transfer of albumin from plasma to CSF. It would seem likely therefore that these proteins and other proteins in CSF are essential early in brain development. The brain is surrounded in CSF of high protein concentration during a very dynamic period in its development and yet the passage of protein (albumin and probably other proteins, *cf.* Dziegielewska *et al*, 1980b) from the blood to the CSF is strictly controlled. Free movement of these proteins into the brain is further restricted due to the presence of specialised cell junctions between apposed cells at the pial and ventricular surface. The blood-CSF barrier may be providing the CSF with a "rich soup" of specific nutrients from which cells of the neocortex can select what they require in order to sustain a rapid and organised growth. These proteins may be acting as simple carrier molecules for precursors required by the CNS or for their buffering capacity and ability to "soak up" unwanted metabolites. The proteins that are taken up may have more specific functions and this may be an important preliminary stage of development before these cells are able to synthesise an adequate amount of the protein for themselves.

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