THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN THE SURVIVAL OF ROSTRAL AND CAUDAL CELLS IN THE DEVELOPING CEREBRAL CORTEX OF THE RAT: AN IN VITRO STUDY

By

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ii. PUBLICATIONS

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iii. CONTENTS

iv. ABSTRACT

The cerebral cortex is generated through the proliferation of the epithelium lining the lateral ventricles of the telencephalon. Although the ventricular epithelium (VE) has a uniform appearance, evidence is mounting to suggest that the VE is heterogeneous population of cells. In support of this, we have demonstrated that dissociated cell cultures prepared from rostral regions of the embryonic cortex undergo cell death whilst their caudal counterparts survive for longer periods. Furthermore, our experiments have shown that in co-cultures, caudally derived cells are able to rescue rostral sister cells. This suggests that a factor is present in caudal cortical cells that is able to promote the survival of rostral cells. Evidence from basic fibroblast growth factor (bFGF) knockout animals has shown that there is a significant reduction in the thickness of the cerebral cortex indicating that bFGF may be one such signalling molecule. In an effort to identify neurotrophic factors involved in the survival of these cortical cells, we applied a range of growth factors and neurotrophins including bFGF to rostral cultures derived from embryonic day 17 cortices. Only bFGF significantly rescued the rostral cortical cells indicating that bFGF is a candidate factor produced by caudal cells. Caudal cells treated with genistein, to block the effects of bFGF, survived for shorter period than the untreated control cultures. We have described the distribution of FGFR 1 receptor and bFGF in rostral and caudal cortical cultures using immunocytochemistry, Western blotting and *in situ* hybridisation. We propose that caudal cells produce bFGF which acts in an autocrine manner to maintain their survival and is also the factor likely to be affecting rostral cells *in vivo*.

1.1. INTRODUCTION

The cerebral cortex is phylogenetically a new addition to the vertebrate central nervous system (CNS). Although the cortex of the reptilian brain is considered to represent a primordial neocortex, it is generally agreed that the neocortex is a structure unique to the mammalian brain. The size of the neocortex varies greatly among the various mammalian groups. The process of neocorticalisation, that is an increase is the size of neocortex relative to other brain structures, has been extensively investigated by Stephan and colleagues (Stephan *et al.,* 1981). It was concluded that a greater degree of neocorticalisation has occurred in primate brains as compared to insectivores. An index that relates the relative expansion of the cortex indicates that the human neocortex is 156 times larger than that of insectivores (Stephan and Andy, 1964). This remarkable fate of phylogenetic development reflects the extended degree of specialisation that has occurred in higher mammalian species, that of cognition and sophisticated perceptual abilities. The mechanism proposed to explain this relative expansion is discussed in the radial unit hypothesis described by Rakic (1988).

Histological examination of the neocortex reveals a distinct stratification. Meynert (1867-68) provided the first systematic description of cortical layering. Whereas, in the piriform and hippocampal parts of the mammalian cortex three layers can be distinguished, in the neocortex six layers are usually recognised. This fundamental cortical pattern was confirmed somewhat later by the observations of Brodmann on Nissl-stained sections (Brodmann, 1905). The six layers of the cerebral cortex are as follows: I) lamina molecularis, II) lamina granularis externa, III) lamina pyramidalis externa, IV) lamina granularis interna, V) lamina pyramidalis interna and VI) lamina multiformis (Fig. 1). Layer I is a cell sparse layer which contains the horizontally oriented Cajal-Retzius (CR) neurons, afferent fibres from a number of

subcortical areas, axon terminals of the Martinotti neurons and the apical dendritic bouquets of pyramidal neurons of lower cortical strata. The CR cells are a unique feature of layer I. Layer II is composed of smaller, densely packed cell bodies. The constituent somata belong to small pyramidal neurons with the apical dendrites directed towards the pial surface. The boundary between layers II and III is poorly delineated, the latter comprising again of pyramidal somata. Layer IV consists of small, densely packed, pyramidal and nonpyramidal cell bodies. Layer V is characterised by loosely packed neurons with triangular or polygonal shaped cell bodies. Two zones can be distinguished in layer VI; layer VIa with neurons resembling those of layer IV, and layer VIb bordering the subcortical white matter, consisting of horizontally oriented neurons.

Although the six-layered pattern is recognisable throughout the neocortex this structure is not homogenous. Differences in relative thickness and cell density of the various layers, in the size, shape and arrangement of the neuronal cell bodies are evident and have been used to divide the neocortex into cytoarchitectonic areas. Although clear differences can be seen in the cytoarchitecture of some regions of the cortex, the total number of anatomically distinct areas has been a matter of some debate. Estimates in the human cerebral cortex vary between 50 and 220 distinct areas. The scheme most commonly used for the cytoarchitectonic division in primates is that devised by Brodmann (1905). In many cases, the anatomically defined areas correspond to functionally distinct regions, as in the case of area 17, the primary 'visual' cortex, or area 4, the 'motor' area. Since there is a direct correlation between cytoarchitecture and functional specialisation, the question then arises as to how cytoarchitectonically similar areas are functionally distinct? The introduction of axon tracers made it possible to study the connections of the cortex. A further degree of

cytoarchitectonic parcellation came about when differences in the pattern of myelinated fibres were investigated. Other methods employed to examine the histology of the cerebral cortex include, pigmentoarchitectonics, chemoarchitectonics and pathoarchitectonics.

1.1.1. Cytology of the cerebral cortex

The cytology of the cerebral cortex was primarily investigated using the Golgi method. This method typically stains the entire cell body and processes of only a small proportion of neurons, thus greatly facilitating the examination of neuronal morphology. It was first applied to the CNS by Golgi in the 1870s and subsequently by Ramón y Cajal and his student, Lorente de Nó. Ramón y Cajal paid particular attention to the cerbral cortex and was able to describe accurately and in detail its cellular composition before the turn of the century (Ramón y Cajal, 1891).

Over the years, a number of attempts have been made to classify neurons of the cerebral cortex, but none of the classification schemes gained wide acceptance. It is now generally agreed that there are two broad morphological classes of cortical neurons: the pyramidal and nonpyramidal cells.

Pyramidal cells

Pyramidal cells, are the principal projection neurons of the cortex, make up approximately two thirds of all the neurons of the neocortex. They contain the excitatory neurotransmitter glutamate. Pyramidal neurons maybe found in all cortical layers except layer I. They demonstrate a variety of shapes, the most common being a conical cell body. These neurons typically possess a prominent apical dendrite that passes from the cell body vertically towards the pial surface giving rise to numerous

oblique and horizontal branches on its way and forming a terminal tuft in layers I/ II. Two or more basal dendrites emanate from the base of the soma and together with their branches form the basal dendritic field. An axon typically arises from the base of the cell body or occasionally from the proximal portion of the basal dendrite and extends towards the white matter giving rise to numerous collaterals forming part of the intrinsic cortical circuitry. A characteristic feature of pyramidal cells is that all their dendrites are covered with spines. The features described above are typical for a pyramidal cell residing in layer III and in layer V. In addition to these typical pyramidal cells, there are other cells which are modified in form but recognisably pyramidal. In layer II, the apical dendrites of the pyramidal cells are very short, so that the terminal tufts arise directly from the apex of the soma. Other modified pyramidal neurons that are frequently encountered in layer IV which differ from the typical ones in having very thin apical dendrites and cell bodies appearing more oval in shape (Lorente de Nó, 1949).

Pyramidal cells project to other regions of the brain or spinal cord and represent the output of the cortex.

Nonpyramidal cells

Approximately 15-30 % of the total neuronal population in the neocortex comprises the nonpyramidal cells. These cells, the local circuit neurons of the cortex, serve an inhibitory function utilising GABA as primary neurotransmitter whilst some also produce one or several neuropeptides (Parnavelas *et al.*, 1989). Golgi impregnation studies have shown that nonpyramidal cells are heteromorphic in nature. They vary in size and have a variety of dendritic field shapes. The various dendritic morphologies have been referred to as multipolar, bipolar and bitufted (Feldman and

Peters, 1978). Detailed analysis of dendritic morphology reveals that nonpyramidal neurons are largely free of spines, and so are often referred to as smooth neurons. The few spinous dendrites that are seen appear to be associated only with multipolar neurons.

Bipolar neurons occurring in layer I of the cortex of many different mammals are known as the horizontal cells of Cajal. These cells put out a few smooth dendrites which pursue a course parallel to the cortical surface, as too do their axons. Curiously, horizontal cells in layer I have been seen to have more than one axon. Immunocytochemistry reveals that these neurons are GABAergic and also contain the neuropeptide cholecystokinin (Alan and Peters, 1991).

Glial cells

The other major cell type that makes up the cerebral cortex is the neuroglia. These cells were first discovered by Virchow (1846), when he observed that non-neuronal elements were present in the walls of the ventricles and in the spinal cord, that were disimilar to connective tissue in the brain and that were present between neuronal cells. These cells were distinguished from smaller nerve cells because of their special stellate or spindle-shaped appearance. Virchow observed that these interstitial elements separated the nervous tissue from blood vessels. The neruoglia were perceived to be cells suspended in interneuronal matrix just as in the areolar connective tissue in other organs. This view was upheld until the advent of the electron microscope, when perfusion fixed material allowed investigators to actually see the interneuronal matrix to comprise of glial perikarya and their fine processes.

In addition to the ependyma, which lines the ventricular cavities and spinal canal, two further categories of supporting cells in the CNS are recognised, the

macroglia and the microglia. The macroglia comprise the astrocytes and the oligodendrocytes. Characteristically these cells differ from neurons, despite being ectodermally derived, in that they maintain the capacity to divide throughout life. The microglia, however, are considered by del Rio Hortega (1932) and others to have a mesodermal orign and enter the brain in the neonatal period. This has raised some controversy over their status as neuroglia. The microglia are smaller than either astrocytes or oligodendrocytes. They are capable of multiplying by mitosis under proper stimulation, such as in injury to the nervous system, and they have the ability to become phagocytes. Further classification of the macroglia based on light microscopic investigation divide the astrocytes into protoplasmic and fibrous, and the oligodendrocytes into interfascicular and perineuronal satellites.

Astrocytes

Ramón y Cajal (1913), using gold sublimate described astrocytes as star-shaped cells whose processes extend into the surrounding neuropil, some of which expand to abut against blood vessels with characteristic endfeet. Astrocytes present in the white matter have numerous fibrils and are classified as fibrous, whilst grey matter astrocytes have fewer fibrils, and thus termed protoplasmic. Astrocytes contain an abundance of glial fibrillary acidic protein (GFAP), one of a number of intermediate filaments.

Oligodendrocytes

Oligodendrocytes most commonly occur in the white matter where they may be aligned in rows between nerve fibres (interfascicular oligodendrocytes). They are also present in the gray matter, especially in the lower cortical layers, where they may

be associated with nerve fibres forming myelin or sometimes, with neurons (satellite cells).

Afferent and efferent connections

Afferent axons to the cortex aise in the thalamus and in other subcortical structures. Thalamic afferents can be distinguished as two types: fibres conveying specific information, originating in sensory relay nuclei of the thalamus forming dense terminal arbors in layer IV and deep layer III, and non-specific afferents orginating in the intralaminar nuclei of the thalamus. These latter fibres are thinner and give rise to diffuse innervation of the entire cortical thickness (Herkenham, 1980; Parnavelas and Chatzissavidou, 1981). Other subcortical afferents include basal forebrain projections providing cholinergic input to the entire cerebral cortex (Divac, 1975), the noradrenergic input arising from the locus ceruleus in the pons (Jones and Moore, 1977), serotonergic innnervation originating in the midbrain dorsal and median raphe nuclei (Parent *et al.,* 1981; Waterhouse *et al.,* 1986), and dopaminergic fibres arising in the ventral tegmental area (Lindvall and Bjorklund, 1974; Tork and Turner, 1981).

The organisation of cortical callosal connectivity has been studied using degeneration, autoradiography and HRP tracing techniques (Jacobson, 1970; Olavarria and Sluyter, 1985). The neurons that give rise to these projections as well as those that receive connections from contralateral hemisphere are found predominantly in layers II, III and V. In addition to callosal connections there are corticocortical projections connecting different corical areas ipsilaterally (Ribak, 1977).

Cortical efferents

Pyramidal neurons in the cerebral cortex project to subcortical structures including the spinal cord, thalamus, superior colliculus, pons and the striatum (Parnavelas *et al.*, 1989).

1.1.2. Development of the cerebral cortex

Overview of development

A key event in the development of the mammalian cerebral cortex is the generation of neuronal populations during embryonic life. In short, this occurs as a result of rapid proliferation of a group of germinal neuroepithelial cells lining the lateral ventricles of the developing forebrain during the last week of gestation in the rat. Cohorts of these cells exit the cell cycle. Postmitotic cells migrate out of the proliferating zone toward the pial surface, where the initial population forms the marginal zone, the future layer I. Subsequent generations of cells, guided radially by radial glia, come to settle in the CP in an inside out manner. Cells subsequently differentiate to acquire their adult layer specific phenotype, thereby forming layers II-VI. These neurons undergo further differentiation forming functionally and cytoarchitechtonically distinct cell populations, thus giving rise to the different cortical 'areas'.

Laminar organisation of the developing telencephalic vesicle

Ventricular zone

The entire cerebral cortex is derived from the psuedostratified columnar epithelium lining the lateral ventricles (LVs) of the forebrain, forming the VZ. These cells are derived from embryonic ectoderm and are distinguished as cortical primordium around embryonic day (E) 13 in the rat in the wall of the telencephalic vesicles. The ventricular epithelium spans the depth of the dorsal telencephalon, the apical ends anchored to each other by terminal bars at the lumen of the LV and the basal processes extending to beneath the pial surface (Sauer, 1935). The ventricular

neuroepithelium is able to undergo extensive proliferation. Light microscopic and autoradiographic studies have provided evidence for the positional changes that occur when the ventricular epithelium undergoes division. The latter studies confirmed Sauer's hypothesis, that during the cell generation cycle, neuroepithelial cells undergo a to-and-fro movement within the cytoplasm. This phenomenon became known as interkinetic nuclear migration. The use of radioactively labelled thymidine (^{3}[H] thymidine) incorporated into the nucleus showed that cells generating DNA descend from the lumenal surface of the VZ to aggregate at some distance in the synthetic zone. Cells at a later stage in the cycle, G2/M phase, ascend back to the lumenal surface, at the mitotic zone, and complete that round of the cycle. Investigators have also observed variation in the plane of cell cytokinesis, with evidence indicating that the plane of orientation somehow effects the restriction of potential of progenitor cells.

As development proceeds, the depth of the VZ increases in preparation for neurogenesis. Some cells leave the VZ and form a few of the first differentiated neurons of the cortex, the CR cells at the marginal zone (MZ) beneath the pial surface. Around E16, a new proliferative zone is evident dorsal to the VZ, this is the subventricular zone (SVZ). The thickness of the VZ continues to increase until E18. Thereafter the neuroepithelium progressively declines until no cells remain, at E21.

Subventricular zone

The SVZ becomes apparent on E15 in the early maturing ventrolateral cortex of the cerebral wall between the VZ and the IZ. This zone is easily observable, containing cells that have a relatively random orientation, both horizontal and vertical. Tritiated thymidine labelling reveals cells to be heavily labelled demonstrating that

this layer, too, is proliferative in nature. As development proceeds the thickness of the SVZ increases in contrast to its sister proliferative zone, the VZ. The SVZ proliferative properties differ from those of the VZ in that cells in the former do not undergo interkinetic movements during mitosis. The SVZ begins to diminish during the early postnatal period (Bayer and Altman, 1991). An important difference between the VZ and SVZ is the contribution each makes to the neuronal and glial populations of the forebrain. Tritiated thymidine birthdating studies have demonstrated that prenatally the VZ is the main source of neurons for the mammalian cerebral cortex, and after the diminution of the VZ, the SVZ continues to generate the glial cells postnatally (Paterson *et al.,* 1973). The SVZ has also been implicated in replenishing glial cells in adult life (Morshead *et al.,* 1994). Recently, using retroviral mediated gene transfer Luskin and colleagues (1993) have shown that distinct parts of the SVZ generate an immense number of neurons destined for the olfactory bulb.

Marginal zone

The MZ is the layer of cells that appears beneath the pial surface early on in cortical development. Layer I cells are the first neurons to differentiate. Recent evidence suggests that some of the neurons that come to settle in the MZ are not of cortical origin (Meyer *et al.,* 1998, Lavdas *et al.,* 1999). The neurons that appear in this subpial zone are large horizontally oriented well differentiated cells, the CR cells. Another group of differentiated neurons is seen in the SP (SP). Together with the MZ, they form the preplate (PP; Marin-Padilla, 1971, 1978). The SP cells give rise to the deep layers of the cortex, layer VI b or VII. As cortical development proceeds, the PP is split by the newly arriving neurons as they form the CP.

Intermediate zone

At the onset of neurogenesis, groups of neuroepithelial cells lose the capacity to divide and exit the VZ. The newly generated neurons, guided by radial glia cells, migrate vertically towards the pial surface. The IZ is the region in which these newly postmitotic cells sojourn. The IZ forms at E16 and continues to increase in size during neurogenesis. No mitotic profiles are seen in the IZ. Stensaas (1967) distinguished between upper and lower portions of the IZ. The upper portion contains horizontally oriented cells, and the lower part, vertically oriented cells. Most neurons that transit the IZ are assumed to be making their way toward the CP (Bayer and Altman 1991).

Cortical plate

Migrating neurons of the IZ are largely destined for the region just beneath the MZ, the CP. The CP is composed of densely packed, radially oriented cells, the majority of which are neurons. As neurogenesis proceeds, the thickness and packing density of the CP increases. The most striking feature regarding the formation of the CP is the order in which the neurons settle, giving rise to a neurogenetic gradient of development. Autoradiographic studies provide convincing evidence that the cerebral cortex neurons form in an inside-out manner. Neurons that are born first settle in the deepest layers, whilst the younger neurons settle more superficially. The exceptions being the neurons of the MZ and SP. Golgi stained preparations demonstrate that neurons arriving at the coritcal plate make connections via an apical dendrite in the MZ and await the arrival of their cousins. As the later cohorts arrive by-passing the already resident neurons, the existing pyramidal neurons are thought to increase the length of their dendrites and thus descend further beneath the newly arriving cells (Marin-Padilla, 1998). Experimental evidence supporting this hypothesis has come

from the findings of Pinto-Lord and Caviness (1979) in the reeler mutant mouse cortex where the fibre plexus in layer I is placed deep within the CP rather that superficially. In these mice, there is no 'inside-out' gradient as pyramidal cells will often grow 'apical' dendrites to contact the misplaced layer I. Finally, as the neurons differentiate the horizontal cellular layers and fibrous layers of the cortical grey develop.

Migration

Radial migration

An essential feature of cortical development is the movement of newly generated neurons out of the zones of proliferation. The idea that newborn neurons moved out of the VZ came after observing numerous mitotic figures in the VZ, their absence in the developing CP itself and the presence of bipolar cells oriented in the IZ. Thymidine autoradiographic evidence supports this hypothesis (Angevine and Sidman, 1961; Berry and Rogers, 1965; Hicks and D'Amto 1968; Shimada and Langman, 1970). Cells do indeed migrate, and not merely by passive displacement to the periphery by cells generated later.

Neurons in the process of migrating to the CP are radially oriented. Under the light microscope the nuclei appear dark and spindle-shaped and after arrival in the CP the nuclei are rounded, become pale and are seen to have multiple nucleoli (Ref. 160 McConnell, 1988). How these neurons arrive at their final destination has been a topic of intense debate. Morest (1970) suggested that the cell bodies of young neurons are translocated from the VZ to the CP within long, external radial processes that extend to the pia. A similar mechanism was suggested Berry and Rogers, where they proposed that it was the cell's nucleus that was translocated (Berry *et al.,* 1964; Berry

and Rogers, 1965). However, electron microscope studies have failed to support this hypothesis of cell migration. Rakic (1972, 1978, 1982, 1988) proposed a more plausible mechanism for neuronal migration. Using light and electron microscopial analyses, Rakic described the intimate apposition of neurons in the IZ with radial glial processes. He proposed that migrating neurons used the radial glia as guides and supports for migration. This theory has been used to explain the ontogentetic and phylogenetic growth of the cerebral cortex in the radial unit hypothesis. That is, the cortex develops from a series of 'proliferative units (precursor cells that form a pseudostratified column seperated by glial cell). Cohorts of cells produced in succession from these same proliferative unit migrate along radial glial fascicles. The neurons that arrive at the CP pass by each other and become arranged radially in the form of ontogenetic columns (Rakic, 1988). The migrating pathway and the ontogenetic column form the radial unit that extend from the ventricular to pial surface. Therefore, the developing neocortex can be considered as a mosaic composed of a large number of such radial units. The prediction of the radial unit hypothesis is that each proliferative unit can produce multiple neural phenotypes which terminate in a single ontogenetic column. Through this can the neurons of the cerebral cortex 'know' where to go. Rakic (1988) proposed that the cortical primordium is not a blank slate or *tabula rasa*, but in fact despite appearing quite homogenous, the primordium is in some way pre-specified, in what is termed a 'proto-map'.

Molecular mechanisms involved in migration of newborn neurons must enable the neuron to recognise a migratory pathway, allow displacement of the neuronal cell body along the pathway and signal the cessation of migration and detachment from the radial glial process. The initial recognition of glia by young neurons may come about as a result of neuron-glia cell adhesion molecules (see Review McConnell,

1988). Recently some potentially key molecules regulating the movement of neurons over glia have been identified (Rakic and Donoughue, 1999). Antibody purturbation studies on granule neurons of the cerebellum *in vitro* have demonstrated that the neural glycoprotein astrotactin provides a neural receptor system for migration along glial fibres (Edmondson *et al.,* 1988; Fishell and Hatten, 1991). Motility and navigation may be goverened by differential adhesion, since studies have shown neuronal preference for particular surfaces above others in cell culture (see Review McConnell, 1988). It is hypothesised that *in vivo* gradients exist that guide the movement of some of these neurons towards their final destination. The transient, CR cells located in the MZ have recently been shown to synthesise and secrete the glycoprotien reelin (Frotscher, 1997). This extracellular matrix protein has sequence similarities with cell adhesion moleclues such as tenascin and laminins, suggesting a role in cell migration and process outgrowth. Reelin has been implicated in acting as a stop signal preventing earlier generated neurons from growing further toward the pial surface (Frostscher, 1997). Anton *et al.,* (1996) have reported immunostaining of superfcial strata of CP, but not of the MZ using an antibody recognising an antigen of the embryonic CNS. Blocking experiments suggest that this molecule is important for the cessation of neuronal migration and the detachment of neurons. Further, release of proteolytic enzymes, such as plasminogen and plasminogen activator (Moonen *et al.,* 1982) abundantly expressed during the period of neuronal migration, at the growing tip of the leading process could aid in tissue penetration and thus faciliate the neuronal navigation through other cells and processes (Rakic, 1985). Instead of an outside signal, there maybe an intrinsic mechanism for the detachment of the neurons at their final destination.

Tangential Migration

Recent studies with recombinant retroviruses (Walsh and Cepko, 1990), chimeric and transgenic mice (Tan and Breen, 1993) and fluorescent labelling (O'Rourke *et al.,* 1992) has revealed that tangentially migrating neurons are not negligible in the embryonic neocortex (Tamamaki *et al.,* 1997). There are two hypotheses concerning the destination and fate of tangential cell migration in the IZ, one of which considers that the tangentially migrating cells will be incorportated into the CP and the other suggests that some of them will not join the CP. So far, tangentially migrating cells in the neocortex have been thought to originate in the VZ of the neocortex (O'Rourke *et al.,* 1992) or the VZ of the corticostriatal sulcus (Menezes and Luskin, 1994). However, recent investigators have demonstrated the origin of tangentially migrating cells destined for the IZ as the lateral and, more recently the medial ganglionic eminences (Tamamaki *et al.,* 1997; Lavdas *et al.,* 1999). Further, lineage and phenotypic differences are apparent amongst tangentially and radially migrating neurons, with the former predominantly GABAergic and the latter glutamatergic. These differences suggest lineage based mechanisms for early specification of certain progenitors to distinct dispersion pathways and neuronal phenotypes (Tan *et al.,* 1998).

Historical persepective of cortical development

A fundemental question in developmental neurobiology is how specialsied structures are derived from less differentiated cell types. His (1889) first described the existence of two major cell types in the VZ which he termed the "germinal matrix". He described the formation of cellular syncitia of germinal cells (neuronal precursor) and the spongioblasts (glial precursors). Shaper (1897) disputed His's interperatation

of light microscopial findings, instead he argued that the germinal cells His described were, in fact, epithelial cells in the process of division. Shaper also postulated that the cells of the neuroepithelium were "indifferent" elements that would give rise to both neurons and glia. Support for this latter notion came from observations reported by Sauer (1935). Sauer noted that germinal cells of the neural tube were anchored by thin cytoplasmic processes to the inner and outer surfaces on the neuroepithelium. He later proposed that the nuclei of the neuroepithelial cells underwent to and fro movements within the VZ during the mitotic cycle. Sauer referred to this phenomenon as interkinetic nuclear migration and described the neuroepithelium as a pseudostratified epithelium rather than a truly stratified one. Sauer's investigation concluded that the spongioblast and germinal cells were in fact the same cell in different stages of the cell cycle (Sauer 1935).

Autoradiography (Sidman *et al.,* 1965; Sauer and Walker, 1961), transmission electron mircoscopy (Hinds and Ruffet, 1971), and scanning electron microscopy (Seymour and Berry, 1975) confirmed the notion that cells were undergoing interkinetic nuclear migratory movements. Recent research, however, provides support for the notion that the neuroepithelium in the cerebral cortex is not a pseudostratified germinal matirix at all stages of development, rather one in which cells with different kinetic properties occupy different compartments.

1.1.3. Heterogeneity of the VZ

Despite complex cytoarchitectoninc diversity of the adult neocortex the developing cerebral cortex arises from a seemingly homogenous layer of neuropithelial cells. How is such a diverse and complex structure derived from simple sheets of undifferentiated progenitors? Currently under investigation are mechanisms

that control how and when a neuron is born, what are the fate determinants and how neurons attain their final destination. Specifically, this section reviews progress made in understanding the orgins of cellular diversity in the cerebral cortex and tackles the issue as to whether the developing neocortex is pre-specified to form specific functionally distinct areas or remains pluripotential throughout development. The use of transplantation, retrovirus and cell culture techniques are amongst the few approaches used to attempt to expose some of the underlying mechanisms of control of cortical development. Two contrasting views dominate the scene. The proto-map theory (Rakic, 1988) and the proto-cortex hypothesis (O'Leary, 1989).

Determination of laminar fate

One approach to examining progenitor cell commitment to producing progeny with specific cortical phenotypes involves transplanting VZ cells to novel environments and assaying the fates of their progeny. The fate of a neuron can be discussed in terms of final laminar destination, areal position, neurotransmitter phenotype, connectivity and expression of particular markers. Further, a precursor is said to be committed to a particular phenotype if, even under different environmental conditions, it continues to develop autonomously (McConnell, 1988). The correlation between a neuron's birthdate and its ultimate laminar fate raises the possibility that young cortical neurons may become committed to their fates early in development. An argument against this is that neurons born at similar times adopt similar identities because they are exposed to similar environmental cues in the VZ and during their migration to the CP.

Donor progenitor cells, labelled by injecting pregnant ferrets with ^{3}[H] thymidine were harvested and dissociated before transplantation to the germinal zone

of developing host animals. Weeks later the labelled progeny were visualised by autoradiography to determine their laminar position (McConnell, 1988). When cortical progenitors originally isolated from an immature brain at the time when progenitors would normally produce layer VI cells were transplanted into an older brain that is producing layer II cells, progenitors were expected to either demonstrate an intrinsic committment to produce neurons destined for layer VI or demonstrate the capacity to respond to the novel host environment by instead producing layer II neurons. About half of all ^{3}[H] thymidine labelled progenitors produced progeny which migrated preferentially to layers V and VI, layers appropriate to age of the donor cortex, illustrating early committment of some progenitor cells to populate specific layers in the developing cortex (McConnell, 1988).

Subsequent experiments demonstrated that the commitment of mitotic progenitors to produce neurons populating specific laminae is regulated by the evironment in a cell-cycle dependent manner (McConnell and Kaznowski, 1991). Progenitors that undergo S-phase in the new host brain adopt the same fate as endogenous precursors, while progenitor cells tha progress to G2/M phase before transplantation produce cells which occupy layers reflective of donor brain developmental stage (McConnell and Kaznowski, 1991). These experiment provide strong evidence for the notion that laminar fate is determined by environmental cues encountered just before the final mitosis. Laminar fate, therefore, seems to be specified by temporally regulated cues between the S- and M- phase of the cell cycle. However, when the experiment is conducted in the reverse, that is progenitors from older donors (fated to producing upper layer neurons) are transplanted into younger hosts (fated to be producing deep layer neurons), neurons fail to appear in the deep layers of the cortex (Frantz and McConnell, 1996). This suggests that differences arise

in the mechanisms specifying laminar fate in the maturing VZ. That is progenitors from older brains may lack the ability to respond to cues promoting deep layer neuronal differentiation.

Further, ablation of upper layer neurons by administration of a cytotoxic drug, which destroys dividing cells, to feotuses following the period of deep layer neurogenesis, shows that deep layer neurons do not compensate for the absence of upper layer neurons by altering their fate. This suggests that laminar fate is well established by the time the neuron reaches the CP (Sheppard *et al.*, 1995).

Cortical area specification

Branching from the theme of cell fate determination and progressing from laminar specification, investigators have become interested in mechanisms that specify areal fate. Because of the prevailing notion that the migration of cortical neurons from the ventricular surface to the CP is largely or strictly radial, it seemed possible that the areal identity of cortical neurons might be specified in the VZ. That is the VZ has inscribed in it a fate map that Rakic has termed the proto-map of cortical areas that will emerge later during development (Rakic, 1988). Two current points of discussion regarding areal specification exist. The first are the transplantation experiments performed by O'Leary and Stanfield (1989) in which a small piece of visual cortex from an E17 donor was placed in the rostral cortex of neonatal host. After maturation of the host, retrograde tracer labelling was used to assess the distribution of axonal projections of transplanted cells. The transplanted visual cortical neurons maintained axonal projections typical of their new site not of their donor origin. These experiments argue strongly in support of malleability of projections of cortical neurons, therefore challenging the notion of early parcellation

of cortical areas (discussed below). That is, the developing cortex has a uniform capacity to produce area-specific features (Schlaggar and O'Leary, 1991). This lead to the suggestion of a naïve 'proto-cortex' within the germinal zones, with more or less indistinct populations of neuroblasts generated across the tangential domain (O'Leary, 1989). According to this view, neurogenesis in the VZ serves only to generate the adult complement of neurons; this notion is reinforced by lineage tracing experiments. The earliest studies employing retroviral markers in the cerebral cortex (Luskin *et al.*, 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988) demonstrated that cells of potentially single clones could come to lie in multiple cortical laminae and wide dispersion of cells presumed to belong to one colony. Specifically, the observation that clonally related cells did not strictly adhere to radial migratory routes, but were able to migrate long distances tangentially, lead investigators to believe that neurons were able to cross functionally distinct areal boundaries. By this reasoning, the complex patterning of the adult cortex, with its various area specifc features of neuronal differentiation, is a developmental consequence of extrinsic cues from the local environment. Further support is provided by recent studies showing that certain neocortical neurons actually originate from outside the neocortical VZ by tangential migation into the cortex from the underlying ganglionic eminences (Anderson et al., 1999; Tamamaki et al., 1997; Lavdas et al., 1999). However, there is some ambiguity in the interpretation of lineage data, since this depends on knowing that cells widely separated belong to a single clone. For this reason the development of a library of proviral sequences that can be distinguished by the polymerase chain reaction represents a conceptually simple solution to the problem of differentiating cells of multiple clones. Experiments employing retroviral libraries have shown that progeny of a single progenitor may reside not only in different cortical layers but also in

functionally distinct domains (Reid *et al.,* 1995). The migration of ventricular progenitor cells observed by Fishell and colleagues (1993) or postmitotic cells in the VZ and SVZ coupled with the curved geometry of the rapidly expanding cortex suggests that progeny generated at different points along the ventricle might be expected to occupy multiple cortical regions.

The proto-map hypothesis, in contrast to the notion discussed above proposed by Rakic (1988), states that the VZ is a heterogeneous group of cells which possess a crude blueprint of future functional domains. This proto-map would initially direct association among neighbouring cortical cells, later attracting appropriate inputs, the net effect of extrinsic and intrinsic cues leading the final assembly of groups of cells in functionally specific domains. In support of this concept domain specific molecular markers as well as regional differences in rates of cell division suggest that distinct populations of cells exist within the cerebral cortex (Levitt, 1984; Ferri and Levitt, 1993; Arimatsu *et al.,* 1992; Dehay *et al.,* 1993; Polleux *et al.,* 1997a, b; Na *et al.,*1998; Nothias *et al.,* 1998). Furthermore, tissue culture, cell lineage and transplanting studies testify that cortical cells exhibit certain phenotypic, lamina and specifically, regional biases (Luskin *et al.,* 1988; Mcconnell, 1988; Parnavelas *et al.,* 1991; Ferri and Levitt, 1993; Tan *et al.,* 1998). For example, cell death between cortical areas is highly non-uniform (Finlay, 1992), and the length of G1 phase is regionally variant (Miyama *et al.,* 1997). Donoghue and Rakic (1999) have recently identified a molecule whose expression corresponds to particular presumptive cortical areas during their formation. Using the monkey cortex, the role of ephrins and their receptors was investigated. Members of Ephrin A family were seen to be expressed in distinct patterns within the embryonic cerebral wall. The fact that well defined patterns of gene expression are present early in cortical development before

innervation of CP by thalamic afferents suggests that the expression of this group of molecules is regulated by programs intrinsic to the cells. Cohen-Tannoudji and colleagues generated an *H-2z1* transgenic line using regulatory elements from a major histocompatibility complex class I gene linked to Escherichia coli *lacZ* gene (Cohen-Tannoudji *et al.,* 1992). The pattern of beta-galactosidase expression in *H-2z1* had a striking expression pattern correlating to the somatosensory area of the neocortex. Later work on this transgenic line revealed that commitment to the expression of the somatosensory area specific marker expression occurs well before birth and coincides with the onset of neurogenesis even before the birth of non-GABAergic neurons. That is regionalisation had already begun by E13.5 mouse cortex.

Within the neocortical analage differences in the density of the proliferative population and differences also in cell cycle kinetics in adjacent regions appear to anticipate the final differences in neuronal density which will be characteristic of the adjacent areas 17 and 18 (Dehay *et al.,* 1993). Horizontal compartments within the proliferating VZ produced by faster and slower cycling of different populations of progenitor cells have been reported (Altman and Bayer, 1990), suggesting that the spatial organisation of the germinal zone may foreshadow the production of different cell phenotypes (Acklin and Van der Kooy 1993). In support of this notion, measurements of the cytokinetic parameters and the fractional neuronal output of laterally located early maturing regions has been examined over the course of neurogenesis in the mouse. Compared to medial later maturing portion of the VZ, Miyama *et al* (1997) described the changing length of G1 over the latero-medial gradient of development. They suggested that this gradient in T_{G1} is the cellular mechanism for positionally encoding a proto-map of the neocortex in the proliferating ventricular epithelium.

Recent support for early regionalisation of the cortex comes from descriptive studies of molecular expression. Several molecules have been described that are expressed by neurons located in discrete cortical areas. The limbic system associated membrane protein (LAMP) is restricted to regions of cortex including the perirhinal, cingulate and prefrontal areas that classically are considered limbic (Levitt 1984); few or no neurons in non-limbic cortical regions such as the somatosensory and visual cortex express this protein. Latexin expression crosses cytoarchitectonic boundaries but is localised only in neurons of the infragranular layers of the cerebral cortex (Arimatsu *et al.,* 1992, 1994; Hatanaka *et al.,* 1994). TBr-1 exhibits a complex expression pattern that is not confined to specific areas. During the middle and late period of neurogenesis, however, TBr-1 is expressed in deep layers of the cortex but is present in a rostral-dense to caudal-sparse gradient in superficial layers (Bulfone *et al.,* 1995). FGF7 expression in the forebrain is observed in the VZ during a restricted period of embryonic development (Mason *et al.,* 1994). More strikingly FGF 7 expression within the cortex is limited to presumptive parietal and frontal regions. The family of growth factors, the heregulins are also expressed in unique patterns in the cerebral wall (Marchioni *et al.,* 1993). Beta adrenergic binding sites in the SP distinguish the primary and secondary visual areas of the overlying cortex well before these areas are identifiable cytoarchitectonically (Lidow and Rakic, 1994). Delimitation of the visual cortices, auditory and somatosensory cortices are observed in a restricted period of developments by α bungratoxin binding (Fuchs, 1989), and α7 nicotinic receptor messenger RNA (Broide *et al.,* 1995). Such investigation lead to the proposal that the combinatory expression of early genes may be the mechanism by which regional organisation of the cortex is achieved (Levitt *et al.,* 1997).

Early genes that broadly define the entire cerebral cortex have also been described, for example, *Emx1* and *Emx 2* (Simeone *et al.,* 1992). *Emx 2* gene for the transcription factor EMX 2 is expressed throughout the entire developing cortex at early corticogenesis. As neurogenesis proceeds, EMX 2 expression becomes confined to the VZ. A distinct anteroposterior gradient of intensity of labelling is observed (Gulisano *et al.,* 1996). It has also been reported that *Pax 6,* a paired box gene shows a rostral-to-caudal gradient like expression pattern in the VZ (see Gulisano *et al.,* 1996). Protooncogene expression also reveals heterogeneity between radial glia and surrounding non-stained proliferating cells (Johnston and Van der Kooy 1989). Together these reports provide sound evidence for the heterogenous nature of the developing cerebral cortex, and argue favourably for the proto-map theory.

Heterogeneity in the rostral-caudal axis

Of particular interest to the current study is the formation of the cerebral cortex in the rostro-caudal axis (Fig. 2) . Components of the nervous system which demonstrate an existing axis, for example, spinal cord axons which display a rostrocaudal, positional bias in their innervation of sympathetic ganglia and intercostal skeletal muscles. In an effort to identify molecular mechanisms that play a role in the rostrocaudal mapping of the sympathetic system, Suzue *et al.* (1990) produced a number of monoclonal antibodies that display a rostrocaudal bias in their binding to sympathetic and/or dorsal root ganglia and to elements in intercostal muscles. The staining distribution of antibody ROCA1 represented a graded decline in binding along the adult sympathetic chain (Suzue *et al.,* 1990). The finding of such antigens supports the notion of a rostrocaudal positional marking system in the CNS.

Further, the cytogenesis in the cortex is not uniform throughout. A number of gradients are observable. Besides growth and differentiation there are also neurogenetic gradients in which a spatial distribution of neurons results according to the timing of their origin. These gradients include the radial gradient, transverse gradient and the longitudinal gradient. Bayer and Altman (1991) have at length described the various neurogenetic gradients. Specifically, neurons in the anterior region are older than neurons in posterior region in every layer. Exposure of embryonic rats to ionising radiation has devastating effects on the developing nervous system, revealing regional differences in susceptibility to death in the CNS, with the cortex being the most vulnerable component. Specifically, patchy collapse of the cortical neuroepithelium was more pronounced in the anterior than in the posterior cortex (Bayer and Altman, 1991). These regional differences in patchy neuroepithelial collapse are thought to reflect regional gradients in cortical maturation. Alternatively, the differences which are pronounced under ionising maybe due to intrinsic differences in susceptability of anteriorly positioned cells. The propensity of data demonstrating regional variation with respect to genes and molecular expression, supports the concept that such differences give rise to susceptability. Further, Finlay (1992) has demonstrated inhomogeneities in cell death in the cerbral cortex, whilst others (Miyama et al., 1997) have shown differences in components of the cell cycle time in different regions of the developing cortex (Fig. 3).

Further, several studies have demonstrated the presence of immunoreactive epidermal growth factor receptor (EGFR) in selected regions of the embryonic and adult brain, such as the frontal cortex (Gomez-Pinella *et al.,* 1988; Werner *et al.,* 1988; Kornblum *et al.,* 1997). Several mice carrying the null mutation in the EGFR gene (Miettinen *et al.,* 1995; Sibilia and Wagner 1995; Threadgill *et al.,* 1995) have

been generated. The analysis of these mutant mice reveals a complex role for the receptor during embryonic and postnatal development. Brain defects in EGFR mutant mice and atrophy specifically in anterior cerebral cortex have been described (Threadgill *et al.,* 1995; Sibilia *et al.,* 1998). During embryogenesis, no gross structural abnormalities have been described. By postnatal day (P) 13, the forebrain size of the mutants is dramatically reduced with the loss of a major part of the frontal cortex (Sibilia *et al.,* 1998). Increased in situ end labelling was first detected in frontal cortex between P4 and P5. In addition, c-fos expression was increased in the degenerating region. The degeneration is not attributed to persistent perinatal hypoxia. Further, the distribution of FGFR1 mRNA appears to show a rostro-caudal regional differences in the cerebral cortex, with caudal cells expressing higher detectable levels than more rostral brain regions (Wanaka *et al.,* 1991). A recent report describes the distribution of a transcription factor, ARNT 2 (Drutel *et al.,* 1996; Hirose *et al.,* 1996; Drutel *et al.,* 1999) and its role in preventing cell death (Drutel *et al.,* 1998). Of particular interest is the distribution in the adult in which higher levels of expression were found in more posterior than anterior regions of the cortex.

1.1.4. Factors effecting the development of the cerebral cortex

A number of factors appear to have influential roles in the development and decision making of the progenitor cells in the cerebral cortex. Such factors fall into several categories, although functionally there is a considerable degree of overlap; these include extracellular matrix molecules, neurotrophins, neurotransmitters and growth factors. The following is a brief synopsis of the current knowledge regarding control and development of the nervous system.

Extracellular matrix

Amongst the vast array of extracellular matrix proteins that have been implicated in the regulation of proliferation, differentiation and survival (see review Pearlman and Sheppard, 1996; Hunter *et al.,* 1992) of cortical neural progenitors are the collagen family, laminins, fibronectin, proteoglycans, and tenascin, (for review see Reichardt and Tomaselli, 1991, Letourneau *et al.,* 1994). There is increasing evidence that an interaction between extracellular matrix molecules and a variety of growth factors is critical in regulating proliferation and differentiation of neural precursor cells. For example, the ability of cilliary neurotrophic factor (CNTF) to induce the stable differentiation of type 2 astrocytes from the O-2A progenitor cells *in vitro* requires the presence of the extracellular matrix (Lilien and Raff, 1990). Perhaps the best characterised matrix/growth factor interaction is that between fibroblast growth factor (FGF) and the heparan sulphate proteoglycans (HSPG). The interaction modulates the subsequent binding of FGF to its signal transducing receptor (Rapraeger *et al.,* 1991; Yayon *et al.,* 1991), and thus regulates the activity of the growth factor. The interaction of epidermal growth factor (EGF), transforming growth factor α (TGFα) with collagen type IV in regulating the differentiation of limbic cortical phenotype *in vitro* was described to be a key determinant in the initial decision making of neocortical progenitor pool (Ferri and Levitt 1995; Eagleson *et al.,* 1996). Indeed, collagen alone has been documented to influence the differentiation of neuronal precursor cells as well as affect the astroglial progenitors (Ali *et al.,* 1998).

Neurotrophins

During development of the central and peripheral nervous systems in vertebrates, many more neurons are generated than ultimately survive and make

functional connection with their targets. Neuronal survival is assumed to be regulated by target-derived factors, a concept referred to as the neurotrophic hypothesis. This theory predicts that as neuronal populations undergo developmental cell death, the neurons compete for a limited quantity of a neurotrophic factor or factors produced exclusively by their target tissues. Nerve growth factor (NGF) was the first neurotrophic factor demonstrated to be required for normal development. NGF was discovered over 45 years ago as a diffusible substance capable of inducing neurite outgrowth in explants from sympathetic and sensory ganglia (Levi-Montalchini, 1987). NGF can be considered as the prototypic neurotrophin that defines the properties and functions of this class of growth factors. There are two unique features of the actions of NGF on neurons. NGF can regulate the differentiation of neurons. Secondly NGF is synthesised at a considerable distance from the cell body by peripheral tissues or other neurons (target tissues) that are contacted by axons of the NGF-sensitive neurons. In the periphery, the tissue sources of NGF are typically non-neuronal cells while, in the CNS, they are synthesised predominantly by neurons under physiological conditions (Theonen, 1995). During development, a retrograde flow of NGF is established, transporting NGF from the target into the nerve terminal and up the axon to the cell body (Theonen and Barde, 1980). Thus, the neurons that establish this flow avoid the cell death pathway whilst those that do not, undergo apoptosis (Barde, 1989). Recent studies on the expression and actions of the NGF family indicate that in addition to target derived factor acquisition, autocrine and non-target derived paracrine modes of factor presentation are likely to be important (Bothwell, 1995). Antibodies raised to NGF, when injected into new-born rodents, specifically destroy the peripheral sympathetic nervous system (Cohen, 1960), while prenatal exposure to anti-NGF also causes the death of sensory neurons of spinal

ganglia. Subsequent isolation of brain derived neurotrophic factor (BDNF; Barde *et al.,* 1982), and the development of molecular clones (Leibrock *et al.,* 1989) revealed that the amino acid sequence of BDNF was about 50% identical to that of NGF. BDNF was found capable of supporting the survival of sensory but not sympathetic neurons. The structural homology to NGF lead to the concept of the neurotrophin family. Subsequently molecular cloning techniques lead to the protein purification of neurotrophin-3 (NT-3; Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et la.,1990) and neurotrophin-4/5 (Snider, 1994; Bothwell, 1995). More recently, two novel neurotrophins from the platyfish and carp have been cloned, neurotorphin-6 (Gotz *et al.,* 1994) and neurotrophin-7 (Lai *et al.,* 1998).

Essentially the differences in these neurotrophins arise in their functional abilities to promote survival of distinct neuronal populations *in vivo* and *in vitro*. Explants of chick peripheral ganglia have been used to distinguish the biological activities of NGF, BDNF (Davies *et al.,* 1986) and NT-3 (Maisonpierre *et al.,* 1990a, b; Hohn *et al.,* 1990). Both NGF and BDNF promote survival and differentiation in cultures of dissociated ganglionic neurons (Lindsay and Rhore 1985) and prevent naturally occurring cell death in specific peripheral ganglia when administered to developing avian embryos (Hofer and Barde 1988).

Neurotrophins exert their physiological effects by binding one of two classes of transmembrane glycoproteins on responsive cells: protein tyrosine kinase-type receptors (members of the Trk family) and a smaller binding protein containing a short cytoplasmic tail of unknown function- the p75 low affinity NGF or neurotrophin receptor, $p75^{LNTR}$ (Meakin and Shooter 1992).

Although well established mediators of growth and survival in the PNS, a growing body of evidence demonstrates neurotrophins as survival factors of the adult

CNS (Buck *et al.,* 1988; Maisonpierre *et al.,* 1990; Freidman *et al.,* 1991; Kaisho *et al.,* 1992 Miranda *et al.,* 1993; Temple and Qian, 1995). However their role in the development CNS is not clear. BDNF and NT-3 and their receptors Trk B and TrkC are abundant in the developing cerebral cortex at an early age (reviewed Klien, 1994). Immunocytochemistry reveals the presence of these proteins in migrating neurons of the IZ in the embryonic neocortex. BDNF was shown to be involved in activity dependent survival of embryonic cortical neurons suggesting a role in the pruning of cortical neurons during development (Ghosh *et al.,* 1994). NT-3 has been shown to stimulate the differentiation of calbindin positive neurons in the embryonic hippocampas (Collazo *et al.,* 1992). Antibodies raised to NT-3 have been shown, for neocortex and hippocampus, to significantly reduce the numbers of neurons in bFGF expanded neuroectodermal cell cultures, whilst recombinant NT-3 can significantly increase neuronal differentiation.

The idea that neuronal survival and differentiation might be regulated by many endogenous and/or target derived factors during the neurogenesis of the cerebral cortex lead Fukumitsu *et al.* (1998) to investigate the pattern of expression of NGF, BDNF and NT-3 and their respective high affinity full length receptor. Specifically, BDNF and NT-3 are expressed simultaneously in CR cells, SP, and in ventricular progenitor cells during early development, and these observations indicate the involvement of autocrine and/or local paracrine actions of BDNF and/or NT-3 in the formation of the cerebral cortex (Fukumitsu *et al.,* 1998).

In addition to the neurotrophins, a number of other polypeptide factors have been shown to possess neurotrophic activities. These include CNTF (Ip and Yancopoulos 1996), glial cell line derived neurotrophic factor (GDNF, Lindsay and

Yancopoulos 1996), insulin like growth factor (Dore *et al.,* 1997), and basic FGF (bFGF, Walicke, 1989).

Basic fibroblast growth factor

The isolation of biologically active factors from the CNS which promoted fibroblast proliferation has been documented since 1950s. The FGF family also known as heparin binding growth factors consist of at least 17 structurally related proteins binding to the sulphated glycosaminoglycan heparin, and by their overlapping mitogenic and neurotrophic actions on a variety of mesodermal, ectodermal and neuroectodermal cell types (Burgess and Macaig, 1989). Although FGF have been implicated in a variety of physiological and pathological processes the specific function of each FGF *in vivo* is not yet clear (Basilico and Moscatelli 1992).

An increasing amount of evidence suggests a pivotal role for FGFs in various proliferative and differentiative aspects of embryonic development ranging from mesoderm induction and formation of muscle, cartilage and bone to vascular development (Slack 1990; Baird and Walicke 1989; Gospodarowicz *et al.,* 1990). Increasingly, FGFs signalling is being reported to play an important role in embryonic CNS development (reviewed Eckenstein 1994). Studies using *Xenopus laevis* embryos revealed roles for FGF signalling during the establishment of initial posterior neural tube identities (reviewed by Sasai and DeRobertis 1997). A significant degree of interest in the function of FGFs in the nervous system was promoted by observations that acidic FGF (aFGF; FGF-1) and bFGF (FGF-2) could support the survival of hippocampal and cerebral cortical neurons *in vitro* (Walicke *et al.,* 1986; Morrison *et al.,* 1986).

The identification of FGF came after extracts of pituitary and brain stimulated the growth of 3T3 cells. The activity was demonstrated to be a result of two closely related proteins. One of them, aFGF which has an acidic pI(5.6) and elutes from heparin Sepharose with 1 M NaCl (Thomas *et al.,* 1984; Bohlen *et al.,*1984). The second, bFGF which has a basic pI (>9.0), elutes from heparin Sepharose at 1.5 m NaCl and has 55% sequence homology to a FGF (Esch *et al.,* 1985). Using molecular genetic approaches, other members, of the FGF family have also been identified; for example the FGF-3 gene was identified as the preferred integration site of mouse mammary tumour virus (Smith *et al.,* 1988), FGF-4 as the *hst* oncogene (Taira *et al.,* 1987) and the karposi sarcoma oncogene (delli-Bovi *et al.,* 1987). Transfecting the FGF gene into 3T3 fibroblasts resulted in the biochemical purification of FGF-7 as a mitogen for keratinocytes (Finch *et al.,* 1989) and FGF-8 as an androgen induced mitogen for mammary carcinoma cells (Tanaka *et al.,* 1992), and FGF-9 as a mitogen for primary astrocytes (Miyatomoto *et al.,* 1993). The majority of FGFs contain 30-40% amino acid homology to aFGF and bFGF (reviewed Eckenstein, 1994).

Basic FGF is a pleiotropic factor that induces proliferation of most mesoderm and neuroectodermal cells. It is also a potent chemotactic factor for fibroblasts and endothelial cells, can promote or induce cell differentiation, and is a potent angiogenic and neurotrophic factor (BikFalvi *et al.,* 1997).

The introduction of heparin-affinity chromatography facilitated the isolation of sufficient quantities of bFGF for structural studies. Bovine pituitary bFGF was found to be 146 amino acid protein with a molecular weight of 16, 400 (Esch *et al.,* 1985), while human placental bFGF had 157 amino acids with a molecular weight of 17, 500. However, smaller forms of both bovine and human FGF have been identified (Moscatelli *et al.,* 1988). The shortest molecule that retained biological activity lacked

the first 15 amino acids present in bovine pituitary bFGF (Esch *et al.,* 1985). In some cases, these truncated forms of bFGF seen to be generated by proteases released during the isolation procedure (Klagsburn *et al.,* 1987). Basic FGF has been purified from various tissues including hypothalamus, brain, retina, kidney, adrenal glands, thymus, corpus luteum, placenta, macrophages, prostrate and pituitary (see review Gospodarowicz *et al.,*1986).

Several forms of bFGF are known to exist (Baird *et al.,* 1986, Gospodarowicz *et al.,* 1987). Structural studies have shown that basic FGF is a single chain polypeptide composed of 146 amino acids which can also exist in truncated form. The truncated form as mentioned above is as potent as native bFGF. Thus, the NH_2 terminal FGF region is neither involved in biological activity nor in binding to cell surface receptors (Gospodarowicz *et al.,* 1985). Basic FGF possesses two binding domains for heparin (Esch *et al.,* 1985), one being located near the NH_2 terminal region, while the other is near the carboxy terminal (residues 18-22 and 107-110).

Different molecular weight forms of bFGF corresponding to approximately 18, 22.5, 23 and 24 kDa can be translated from a single human bFGF mRNA transcript have been identified (Florkiewicz and Sommer, 1989). The higher molecular weight forms represent amino terminal extensions of the 18 kDa bFGF and appear to be initiated at leucine codons upstream of the first methionine. Amino acid sequencing of the placental form of human bFGF yielded a protein with an amino terminal sequence predicted from the cDNA to be of high molecular mass. Indeed, Moscatelli *et al.* (1987) isolated a 25 kDa form of bFGF from the guinea pig brain. This protein was as active as 18 kDa human bFGF and was cross reactive with antibodies to 18 kDa human bFGF (Moscatelli *et al.,* 1987). Western blots and immunoprecipitations from numerous cell lines reveal bFGF forms with molecular weights 24, 22.5 and 22 kDa in

addition to 18 kDa form. Giordano *et al* (1992) proposed that by existing in multiple molecular weight forms, bFGF can achieve its diverse biological effects.

Distribution of bFGF in the nervous system

Basic FGF has been found in all organs, solid tissues, tumours and cultured cells examined. Although bFGF was reported to be found in serum (Mormede *et al.,* 1985), the validity of the assay used has been questioned. It is unclear whether ubiquitous distribution of bFGF in cultured cells represents *in vivo* distribution.

A variety of *in vitro* and *in vivo* studies have illustrated that bFGF can influence all major cell types in the brain, including astrocytes, oligodendrocytes and multiple neuronal cell types. Basic FGF has been shown, using immunocytochemistry to have a widespread distribution in the adult rat brain, with different regions expressing distinct patterns. Astrocytes have been shown by in-situ hybridisation, to contain the highest levels of bFGF mRNA while oligodendrocytes, do not appear to contain significant levels of bFGF immunoreactivity (Gonzalez *et al.,* 1995). In neurons, low levels of FGF mRNA and immunoreactivity to bFGF has been localised to the perikaryon, whilst selected populations of neurons, such as CA2 field in the hippocampus show considerably high levels of bFGF mRNA.

Basic FGF in the Adult CNS

Basic FGF is present in relatively high levels in the CNS. It was initially thought that bFGF is expressed solely by astrocytes and that it was absent in neuronal cell populations (Eckenstein *et al.,* 1991a, b). Initially, a biological assay based on measuring the stimulation of mitogenesis in cultured AKR-2B cells was used to determine the levels of FGF-like bioactivity in extracts prepared from different areas

of adult rat CNS (Eckenstein *et al.,* 1991A and B). To differentiate bioactivity related to aFGF and bFGF, heparin dependence was used. The former protein requiring the presence of heparin to exhibit activity. In the normal adult CNS, levels of heparin independent activity (bFGF) were distributed relatively evenly whilst no heparin independent activity was found in the PNS. Basic FGF was found in similar levels throughout the CNS and in the PNS (Eckenstein *et al.,* 1991), reflecting the results of the bioactivity.

Controversy exists over the exact distribution of the bFGF protein in adult and embryonic CNS (discussed below). These discrepancies are likely to be due to the vast array of antibodies used to this protein, the potential for cross reactivity of other members of the FGF family and the diverse tissue preparation techniques. In addition, there is lack of experiments that apply alternative methods to characterise each antibody type.

Grothe *et al.,* (1995) have used ribonuclease protection assay to confirm the presence of bFGF in CNS.There is general agreement that astrocytes of the adult CNS contain abundant bFGF immunoreactivity (Kuzis *et al.,* 1995; Eckenstein *et al.,* 1991; Gonzalez *et al.,* 1995; Emoto *et al.,* 1989; Gomez-Pinella *et al.,* 1992; Woodward *et al.,* 1992). Immunocytochemistry and insitu hybridisation for bFGF confirmed the presence of bFGF in the cerebral cortex (Matsuyama *et al.,* 1992; Gonzalez *et al.,* 1995). Basic FGF mRNA was predominantly found in cells of layers II, III and V with high levels of immunoreactivity localised in cell perikarya (Gonzalez *et al.,* 1995; Matsuyama *et al.,* 1992). This is in contrast to the findings of Eckenstein *et al* (1991) who described that only astrocytes of the cortex to contain bFGF. Highest levels of mRNA for bFGF are found in cortex, hippocampus and spinal cord (Riva *et al.,* 1991).

Basic FGF in the cortex

Despite controversy as to the exact distribution in the adult CNS, there is a general consensus from studies that bFGF is present in the adult CNS. The bFGF story is further complicated by attempts to characterise its developmental distribution in the CNS. Here, current literature on the distribution of bFGF in the developing cortex is reviewed.

Although bFGF can be purified from different organs, its exact distribution in these tissues is unknown. While expression of bFGF is high in the adult brain, in peripheral tissues bFGF mRNA is low to undetectable (Mergia *et al.,* 1986). Several studies have shown that the gene for bFGF is expressed in early embryonic development (Heine *et al.,* 1987; Kimelman *et al.,* 1987; Moscatelli *et al.,* 1987).

Logan *et al.* (1985), using mitogenic activity of crude brain homogenates to estimate FGF levels, found a marked increase in FGF content in the developing rat brain from embryonic to early postnatal stages, but these studies did not distinguish between aFGF and bFGF. Using the heparin binding properties of these factors, the selective examination of changes in aFGF and bFGF during rat brain development were subsequently examined (Caday *et al.,* 1990). Mitogenic activity tested on Balb/c3T3 cells showed a 13-fold increase in developing brain from embryonic to third postnatal week. High levels being sustained in the mature brain.

This activity, determined by heparin affinity columns, was ascribed to aFGF and bFGF. The former demonstrating a greater degree of activity in the embryonic period compared to postnatal periods, suggesting low levels of bFGF in embryonic rat brain. However, this study failed to ascertain regional differences in FGF activity coincident with neural outgrowth and synaptogenesis and with astrocytes and vascular

proliferation. Correlated with these findings significant amounts of 1.8kb bFGF mRNA are found during embryogenesis, but multiple mRNA species are expressed in postnatal brain; the 6.0kb mRNA being the most abundant (Powell *et al.,* 1991). In support of this, RNA protection assay with a cRNA complementary to the coding region of bFGF mRNA demonstrated low levels of bFGF mRNA in all brain regions in new born rats, increasing thereafter to reach a peak of expression around P21 (Riva *et al.,* 1991).

Multiple molecular weight forms of bFGF are regulated during development of the rat brain. The 22 kDa form is not present in the embryonic CNS, but is expressed in neonates and adults, whilst the 18 kDa form peaks during embryonic development and is minimally expressed in the adult. Additionally, bFGF mRNA was significantly greater in embryonic rat brain than adult (Ernfors *et al.,* 1990).

A more comprehensive investigation in bFGF localisation has demonstrated, in contrast to earlier publications, the presence of strong bFGF immunoreactivity between E16 and E17 in the cortex and striatum of rat and elsewhere (Weise, 1993). No bFGF immunoreactivity was detected in the cortex of the newborn animal. Between E13 and E15 bFGF immunoreactivity was found exclusively within the cortex. Specifically CP staining intensity and the number of immunoreactive cell bodies was higher than the VZ (70% in CP, 20% in IZ and SVZ, and 50% in VZ). Deep CP also showed intense labelling at E17 (Weise *et al.,* 1993). In support of these latter findings, Gomez-Pinella (1994), using immunohistochemistry, showed that bFGF was transiently expressed in different cellular phenotypes throughout development. By E18, bFGF immunoreactivity emerged in CP and SP regions of the cortex. The use of the RNA protection assay confirmed the presence of bFGF mRNA

in E16 and E17 rat CNS. However, the exact regional distribution within the brain was not described (Grothe and Meisinger, 1995)

More recently, the generation of mice with homozygous deletion of individual genes has highlighted new and specific roles of individual gene functions as well as reveal complex redundancies in the function of molecules within a given gene family. Homologous recombination in embryonic stem cells to produce bFGF knockout have elucidated a physiological role of bFGF *in vivo*. Specifically the brains of bFGF -/- show abnormalities in cytoarchitecture of the neocortex and a significant reduction in the number of neurons in the motor sensory area of the cortex. This indeed highlights that bFGF is necessary for the development of the cortex (Ortega *et al.*,1998; Dono *et al.*,1998; Vaccarino *et al.*,1999). These latter authors have demonstrated specifically the distribution of bFGF by in-situ hybridisation. They found that bFGF was expressed within VZ of the cortex during neurogenesis and that there was a marked diminution of protein over the proliferating ventricular epithelium as neurogenesis ceases. Indeed this was confirmed by immunostaining for bFGF in coronal sections of cerebral cortex at E14.5 and E16, with the CP and VZ being immunoreactive (Dono *et al.*,1998).

In summary, bFGF has been shown to be present within the neocortex during embryogenesis. Specifically, the CP and VZ are highly immunoreactive. Such a distribution correlates with significant events during corticogenesis providing evidence for a critical role for bFGF in cortical development.

Effects of bFGF

The presence of bFGF in a vast array of tissues during development and adult CNS tissues is highly suggestive of a multiplicity of roles in embryogenesis and tissue regulation. The multiple molecular weight forms are a proposed mechanism by which such roles could be mediated. To add to this is, the range of growth factor receptors to which bFGF can bind.

In early embryonic development, the basic body plan arises because cells in the different regions of the egg become programmed to follow different pathways (Nieuwkoop, 1969; Slack, 1983). Various inductive events follow that lead to the formation of differentiated cell types. It has been proposed that this process of regional specification arises from the action of inducing factors or morphogens (Smith *et al.*,1985). Slack and colleagues (1987) investigated the possibility that bFGF mimics the effects of some of the mesoderm inducing signals, and that such inductive events could be mediated specifically through bFGF. These results were confirmed by Kimelman and Kirschner (1987) who reported the presence of mRNA encoding a protein highly homologous to bFGF in Xenopus leavis embryo. Therefore, bFGF can act as a differentiating factor in early embryos and instruct tissue destined to form ectodermal structures to differentiate instead into mesoderm.

FGF has been show to be required for myogenic differentiation of myoblasts (Seed and Hauschka, 1988). In tissues such as cartilage, bFGF has been reported to promote chondrossification and its presence in bone matrix indicates that it plays a role in the development and growth of osseous tissue (Hauschka *et al.*,1986). Infusion of FGF promotes the resumption of mitotic activity in denervated newt forelimb blastema following amputation of an appendage (Gospodarowicz and Meschner, 1980), suggesting that bFGF plays a role in limb regeneration. Furthermore, bFGF has

been implicated in mitogenic regulation of granulosa cells and their terminal differentiation (Gospodarowicz and Bialecki, 1978), whilst other studies have demonstrated the requirement of bFGF in vascular endothelial proliferation (Gospodarowicz.. *et al.*,1978).

Basic FGF has also been attributed with a role in the development of the lens (McAvoy *et al.*,1991), in neural induction (Xu *et al.*,1997), maintenance of vascular tone (Zhou et al, 1998) and as a morphogen for the anteroposterior axis of nervous system in *Xenopus* (Kengaku and Okamoto, 1995). As well as conducting a physiological role in development, evidence is emerging for important roles for bFGF in the adult. This is not surprising considering the mounting body of evidence demonstrating the distribution and localisation of bFGF in a vast array of cells of the nervous system of the adult. Togari *et al.* (1985) first reported that bFGF acts as a differentiation factor for rat pheochromocytoma (PC-12) cell line by inducing both neurite outgrowth and ornithine decarboxylase activity. Basic FGF in the adult striatum induces the proliferation of EGF generated precursors (Gritti *et al.*,1995). The local synthesis, concentration, localisation and bioactivity of bFGF at the site of injury in brain also suggests that this growth factor may contribute to the cascade of cellular events that occur in CNS wound repair (Logan *et al.*,1992; Baird and Walicke 1989; Logan 1990). As well as mediating the proliferation of non CNS tissues, bFGF has been shown to stimulate the proliferation of O2A cells (Mckinnon *et al.*,1990), astrocytes (Morrison and deVillis 1981) and oligodendrocytes. Basic FGF stimulates the survival and transmitter metabolism of cultured central and peripheral neurons. *In vivo*, the protein prevents ontogenetic neuron death in the cilliary ganglion (Dreyer *et al.*,1989). Furthermore, in model systems of the PNS and CNS, exogeneously applied bFGF prevents lesion induced neuron death (Anderson *et al.*,1988; Otto *et al.*, 1989),

and promotes cell proliferation and synthesis of several glial specific proteins. Evidence (Ray *et al.*,1993) indicates that FGF has a concentration dependent action on immature hippocampal neurons in culture, resulting in either survival or proliferation. Similarly, the fate determination of precursors of the developing cerebral cortex appear to be regulated by differing levels of bFGF (Ghosh and Greenberg 1995). The transient appearance of bFGF in different neuronal phenotypes described by Gomez-Pinella (1994) appears to be consistent with the basic mechanism involved in the process of naturally occurring neuronal death and apoptosis. Induction of dependency and then withdrawal of trophic support appear to trigger apoptosis in selective neurons. Basic FGF may be is proposed as a candidate for the regulation of neuronal cell survival during development.

It has been found that systemic levels of bFGF regulate neurogenesis in the newborn rat, with factors apparently crossing the blood brain barrier to stimulate mitosis, acting via a unique endocrine-like pathway. Twenty five percent of proliferating hippocampal neurons were stimulated *in vivo* following a subcutaneous injection of bFGF (Wagner *et al.,* 1999). Extensive work on the effect of bFGF in neurons *in vitro* include stimulating development of dopaminergic neurons in culture (Kushima *et al.*,1992), protecting striatal neurons from NMDA receptor mediated cytotoxity, promoting both the survival of cholinergic neurons (Kushima *et al.*,1992), and the development of cholioreceptive neurons from foetal cerebellum in culture (Kushima *et al.*,1992), and promoting the survival of peptide containing neurons of rat hypothalamus (Ishikawa *et al.*,1992). Such neuroprotective activities *in vitro* appear also to be true in numerous *in vivo* models of injury where an FGF target cell is involved. Basic FGF protects hippocampal and cortical neurons against iron induced degeneration (Tanaka *et al.*,1992). In addition, it has protective effects

following hippocampal ischaemia (Nakata *et al.,* 1993), and promotes the survival of ventral mesencephalic neurons in nigral transplants (Mayer *et al.,*1993).

Role of bFGF in cortical development

As highlighted above, a considerable body of evidence now exists demonstrating that diffusible signals play an important role in the earliest stage of nervous system development (reviewed Jessell and Melton, 1992). The differentiation of neuroectoderm from the surrounding ectoderm can be influenced by mesoderm derived signals. The identification of molecules such as follistatin and noggin as likely candidates for such early neuronal differentiation have been proposed (Lamb *et al.,*1993; Hemmati-Brivanlon and Melton, 1994). In a similar vein, investigators are keen to understand the mechanisms underlying the cellular events in the neurogenesis of the cerebral cortex. The presence of bFGF mRNA and protein during important events during corticogenesis make it a likely candidate for mediating such events.

A marked increase in levels of mitogenic activity (bioassay) of 3T3 cells in the presence of extracts from developing rat brain have been observed with identification of 18 kDa and 22 kDa forms of bFGF in such extracts (Moscatelli *et al.,*1987). Significant levels of bioactive bFGF have been detected in both plasma and cerebrospinal fluid (Baird 1994; Wagner *et al.,* 1999); furthermore, a soluble extracellular domain of the FGF receptor has also been described in both fluid compartments (Hanneken *et al.,*1995) suggesting molecular mechanism to stabilise bFGF levels in these compartments. These finding suggest that extracellular bFGF is synthesised in the brain (Emoto *et al.,*1989; Caday *et al.,*1990; Woodward *et al.,*1992).

As well as promoting the survival of peripheral sympathetic, parasympathetic and sensory neurons (Eckenstein *et al.*, 1990), bFGF can promote the survival of large numbers of central neurons, particularly within the cerebral cortex (Morrison *et al.*, 1986; Walicke *et al.*, 1986; Sendtner *et al.*, 1991). The strong immunoreactivity observed in the CP compared to the VZ (Rickmann and Wolff, 1985) suggests that bFGF exerts trophic and/or differentiating effects on developing neurons rather than mitogenic effects (Weise *et al.*, 1995) which showed that bFGF stimulates the *in vitro* survival of rat embryonic cortical striatal neurons (Walicke 1988).

Further support for the notion that bFGF acts as a survival signal for cerebral cortical neurons in primary cultures experiments of Morrison *et al.* (1986), in which continuous exposure to bFGF was observed, lead to a marked degree of survival and neurite outgrowth in a dose dependent manner. Basic FGF's effect on apoptosis of primary cultured cortical neurons from embryonic rats induced by ionomycin (a potent Ca^{++} ionophore) were examined. Ionomycin causes sever toxicity on cultured central neurons which involves an active process requiring novel RNA and protein synthesis followed by shrinkage of cells, condensation of chromatin and DNA fragmentation. Takei *et al.* (1995) found that bFGF inhibited apoptotic cell death of ionomycin induced neurotoxicity in a dose dependent manner.

Isolated neuroepithelial cells from E10 mouse embryos were found to proliferate extensively in the presence of bFGF, whilst in the absence of bFGF all cell in the culture die within 6 days (Murphy *et al.*, 1990). Interestingly, a dose response effect was observed, with high concentrations of the growth factor promoting differentiation of cells into mature neurons and glia. These findings support earlier *in vitro* assays demonstrating mitogenic activity of bFGF on cerebral neuronal precursors (Gensburger *et al.*, 1987). Ghosh and Greenberg (1995) conducted a series

of experiments to determine whether proliferation of cortical cells was regulated by growth factors. Using BrdU labelling, dissociated cells formed clusters which increased in size following exposure to bFGF at concentrations postulated to be at physiological levels. Significantly, however, bFGF led to proliferation only if cells which formed clusters after plating and not single cells, suggesting that the ability of bFGF to promote cell proliferation appears to require cell-cell contact among precursor cells (Ghosh and Greenburg, 1995). In support of this, Nadarajah *et al.* (1998) applied bFGF to progenitor cells derived from embryonic cortex *in vitro* and observed an increase in the expression of the gap junction protein, connexin 43. These results infer that gap junctional channels provide a direct conduit for mitogen release in response to bFGF to effectively regulate proliferation during corticogenesis (Nadarajah *et al.*, 1998). Further support for the role of bFGF in proliferation of embryonic cortical cells is provided by the report by Cavanagh *et al.* (1997). A marked increase in BrdU labelling in the presence of bFGF without alteration of the length of cell cycle phases was noted. Marked clones grew significantly larger under treated conditions. Basic FGF apparently stimulated the division of quiescent progenitor cells which otherwise would have differentiated or undergone cell death. Thus, bFGF promotes the proliferative capacity and survival of cells and delays differentiation (Cavanagh *et al.*, 1997).

Besides its effects on a large population of neuroepithelial cell, bFGF specifically increases the numbers of glutamate positive neurons in cultures derived from E12.5 rat embryos (Vaccarino *et al.*, 1995), and the number of precursor cells expressing OTX2 (Robel *et al.*, 1995). GABAergic neuronal precursors were stimulated under the influence of bFGF (Deloulme *et al.*, 1991; Antonopoulos *et al.*, 1997).

Marked neuronal deficiency in the cerebral cortex (frontal motor-sensory) was observed in FGF-/- mice and is due to a consequence of an early developmental defect (Ortega *et al.,* 1998; Dono *et al.,* 1998). It is proposed that this defect is as a result of increased or prolonged wave of PCD that occurs in newborn animals, specifically due to the lack of bFGF, an antiapoptotic factor (Ortega *et al.,*1998). In contrast to previous observations, Dono *et al.* (1998) found that BrdU pulse labelling was normal, however a fraction of the labelled neurons failed to colonise target layers in the cortex. This suggests a role for bFGF in migration during the patterning of the vertebrate neocortex. In *Drosophilla*, FGF signalling also controls morphogenetic movements such as those occurring during gastrulation, neurogenesis and tracheal development (Klambt *et al.,*1992; Gisselbrecht *et al.,*1996). Basic FGF could also act as a chemoattractant since it is found abundantly in CP (Weise *et al.,*1993). It may act indirectly on neuronal migration along radial glia by modulating adhesive properties (Kinoshita *et al.,*1993). Indeed cell motility has been directly correlated with bFGF release from migrating cells (Mignatti *et al.,*1991). Bartheld (1996) investigated the possible transport of bFGF through axons.

Microinjection of bFGF into cerebral ventricles or rat embryos at the beginning of cortical neurogenesis resulted in an increase in both volume and total number of neurons within cerebral cortex (Vaccarino *et al.,*1992). This increase resulted in an increase in total number of CP neurons, whilst evidence of increase in glia is not found, consistent with the fact that gliogenesis occurs postnatally (Vaccarino *et al.,* 1999). A concomitant increase in density of GABA and glutamate containing cells was also observed, the latter greater in number than the former following the injection of bFGF. Importantly, examination of the adult cortex in para-sagittal sections revealed an enlargement particularly in the anterior-posterior axis. It

is suggested that, in the absence of any change in dying cells, bFGF does not affect significantly progenitor cell apoptosis. Northern blot analysis in bFGF mutants reveals that other FGF proteins investigated do not alter their expression (Zhou *et al.,* 1999). Basic FGF can influence the lineage of precursor cells from neuronal to that of glial (Qian *et al.,* 1997) as well as neurotransmitter subtype (Vaccarino *et al.,* 1995).

Mechanism of secretion of bFGF

Although bFGF lacks a conventional secretory signal peptide sequence, reports suggest that naturally occurring cell death in the early neural tube at Hamburger-Hamilton stages 15-18 in the chick suggests that bFGF might be released by cell lysis (Homma *et al.,* 1994). Alternatively, an intracrine mode of action might yield functional responses in the absence of bFGF release (Yayon and Klagsbrun 1990). There is evidence, however, that bFGF may be secreted by some cells employing a mechanisms that remains to be characterised (Mignatti *et al., 1991*). Recent speculation has it that the induction of bFGF release is activity dependent. (Greenough et al 1999). Basic FGF may be released when the plasma membrane is compromised (Mason 1994). Recent evidence suggest that FGF2 can be secreted through an alternative energy dependent non-endoplasmic reticulum/Golgi pathway (Mignatti *et al.,* 1992; Florkiewicz *et al.,* 1995).

Since this factor is localised essentially in neuronal cells, these cells may both secrete and respond to bFGF. Thus, bFGF may function in an autocrine fashion (Gensburger *et al.,*1987). The notion that bFGF participates in autocrine regulation is supported by the observation that it is localised in compartments within cells in culture, predominantly in the cytoplasm of astrocytes and the nuclei of neuronal cells (Vijayan *et al.,*1993; Eckenstein *et al.,*1991). This is not to say that it cannot be

exported from cells to act in a paracrine fashion or by axonal transport (Bartheld, 1996). For example, baby hamster kidney cells will not initiate neuronal differentiation of neural crest progenitors unless they have been transfected with bFGF (Dai and Peng, 1992).

FGF Receptors

Functions of FGFs are mediated by different types of receptors including high affinity receptors designated FGFR1, FGFR2, FGFR3 and FGFR4. These are four transmembrane proteins with intrinsic tyrosine kinase activity (Basilico and Moscatelli, 1992; Goldfarb 1996; Szebenyi and Fallon 1999). FGFR1 was first isolated from a chicken embryo ligand receptor complex yielding useful amino acid sequences for the receptor (Lee *et al.*, 1989) which revealed striking homology to two different partial cDNA clones (termed flg and bek) coding for tyrosine kinases. Standard molecular cloning techniques were then used in rapid succession to first isolate full length cDNA clones coding for FGFR1 (Lee *et al.*, 1989; Ruta *et al.*, 1989). This was followed by the identification of 3 additional different sequence homologous FGFR genes, FGFR2 (Dionne *et al.*, 1990' Hattori *et al.*, 1990; Houssain *et al.*, 1990), FGFR3 (Keegan *et al.*, 1991) and FGFR4 (Partanen *et al.*, 1991).

Analysis of the amino acid sequence of the receptors revealsed that all four receptors were highly similar, approximately 820 amino acids long (Eckenstein *et al.*, 1994). The extracellular aspect is formed by an amino terminal hydrophobic signal peptide followed by 3 immunoglobulin like domains followed by a singly hydrophobic transmembrane region about 380 amino acid distant from the amino terminal followed by the intracellular part of the receptor, characterised by a tyrosine

kinase domain (Dionne *et al.*,1990). A large number of different splice variants have been isolated and characterised to date (Johnson and Williams 1993).

Northern blot, PCR and RNA protection experiments have demonstrated that the main receptor genes expressed in the CNS are the IIIc splice variants of FGFR1, FGFR2 and FGFR3 (Werner *et al.*,1992). In situ hybridisation for FGFR in adult rat CNS revealed a widespread distribution amongst specific subsets of neurons. Intense signals were detected in hippocampus and pontine cholinergic neurons (Wanaka *et al.*,1990), adding support to the notion that bFGF or FGF like molecules effect neurons of the CNS. In particular, these findings are consistent with survival effects of FGF in cultures of hippocampal neurons (Walicke 1988; Mattson *et al.*,1989).

The presence and significant involvement of bFGF and other FGF proteins within the developing CNS is highly predictive of the presence of a receptor mediating such functions. Indeed an increasing amount of evidence is mounting demonstrating the distribution of FGFR in the developing CNS. The presence of FGF mRNA in developing organs have been examined employing immunocytochemistry (Gonzalez *et al.*,1990), immunoprecipitation (Seed *et al.*,1988), and Northern blot analysis (Hebert *et al.*,1990). A temporal profile of receptor expression was conducted (Wanaka *et al.*,1991). From the earliest stage (E9), a widespread distribution of FGFR mRNA was observed in various embryonic tissues with varying degrees of intensity. FGFR1 was found to be abundant in the CNS.

FGFR1 transcripts were predominantly located in the VZ between E12 and E17, that is within the proliferating epithelium of the cerebral cortex. At later stages, FGFR1 was absent in the mantle/marginal zones. These findings are suggestive of a mitotic role for FGFR in early CNS development. At E14, the primordial hippocampal cortex begins to express mRNA for FGFR1 (Wanaka *et al.*,1991). These

findings support a role for bFGF in the survival of hippocampal neurons in culture (Walicke 1988).

It is interesting to note that FGFR1 mRNA was located to the cortex in E17 embryonic para-sagittal sections. More intense labelling was observed in a regional dependent manner, with the posterior cortex displaying more intense labelling than anterior cortex (Wanaka *et al.*, 1991; Vaccarino *et al.*, 1999; Weise *et al.*, 1993; Grothe *et al.*, 1990).

The distinct immunoreactivity of central neurons between E15 and E17 was also reported by Weise *et al.* (1991). However, these authors failed to reproduce the exact pattern observed previously. Instead, it was reported that distinct cells within the VZ demonstrated intense immunolabelling. FGFR1 is present in the early proliferating ventricular epithelium (Vaccarino *et al.*, 1999). At later stages of neurogenesis in the rat (E17), FGFR1 mRNA and protein are down regulated and a corresponding increase in receptor distribution is found in the neurons of the CP. A distinct gradient of receptor distribution is notable (Vaccarino *et al.*, 1999).

1.1.5. Cell death in the nervous system

During normal development of the vertebrate nervous system, large numbers of neurons die. This naturally occurring cell death has been documented for many years in both the central and peripheral nervous systems of various species (Clarke 1985; Cowan *et al.*, 1984; Oppenheim 1991). In mammals, cell death has been studied extensively in spinal cord (Nurcombe *et al.*, 1991; Banker 1982; Comans *et al.*, 1987), and retina, lateral geniculate nucleus; and superior colliculus (Arees and Astrom 1977; Sengelaub and Finlay 1982; Finlay and Pallas, 1989). Naturally occurring cell

death has also been found in the neocortex of the hamster (Finlay and Slattery 1983) and rat (Ferrer *et al.,* 1990; Ferrer *et al.,* 1992).

Cell death has been classified according to two types, termed necrosis and apoptosis. Necrosis is a passive process which is induced by various environmental stresses such as certain toxins, changes in osmolarity or pH, hypoxia or viral infection. In contrast, apoptosis is an active process of cell death that functions also in the normal control of development and cell homeostasis. Apoptosis differs from necrosis in its morphological and biochemical aspects. Ultrastructural changes of apoptotic cell death are well characterised. Cells undergoing apoptosis exhibit shrinkage of the cell body, compaction of the nucleus and condensation of the chromatin at early stags. Membrane blebbing and fragmentation of cells have also been observed thereafter (Kerr *et al.,* 1972a, b; Wyllie 1980). Apoptosis is known to require *de novo* synthesis of RNA and proteins.

The development of an organ or an animal requires a numbers of cellular events to occur. Proliferation, differentiation, migration and cell death. These factors determine the cell number and organisation of an animal or organ. All three mechanisms depend on intracellular mechanism that are regulated by extracellular signalling molecules produced by other cells. The signalling molecules can either activate or inhibit the intracellular mechanisms; they can operate locally or systemically, bound to cell surfaces or associated with the extracellular matrix (Raff 1996). The cerebral cortex is no different. In much of the literature published on the development of the cerebral cortex, little attention has been given to the role of cell death in 'shaping' the cortex. However, PCD might have a greater role than previously implicated (Voyvodic 1996). A difficulty in investigating the prevalence and distribution of dying cells in situ has been the absence of specific cell death

markers. The technique based on the transfer of biotinylated UDP element to the fragmented ends of DNA using the terminal transferase enzyme (Gavrielli *et al.,* 1992; Wijsman *et al.,* 1993; Wood *et al.,* 1993) has allowed the accurate detection of dying cells in the cerebral cortex. Furthermore, other conceptually similar techniques have been developed and have been collectively termed in-situ end labelling (TUNEL). With these techniques, the double stranded DNA breaks produced during PCD are identified.

The issue of how much cell death actually occurs is of current debate. Blaschke *et al.,* (1996a, b) report massive normal cell death in the developing neocortex of the mouse using a new highly sensitive assay. Their results show the presence of a large number of dying cells in the postmitotic upper layers of the cortex as well as dying cells within the cortical VZ, a region not previously associate with cell death (Blaschke *et al.,* 1996). According to their report, over 50% of cells in both the VZ and the CP appear to be undergoing death during the period of neurogenesis. More recent studies provide further support for the results of Blaschke *et al.* (1996) in which the prevalence of PCD in neuroproliferative regions has been described (Thomaidou *et al.,* 1997; Chun and Blashcke 1997; Blaschke *et al.,* 1998). The widespread distribution of dying cells detected within the cerebral wall was shown to be fairly uniform across the rostro-caudal dimension. The peak of cell death occurred around E14.

The cellular and molecular mechanism underlying neuroproliferative apoptosis are not known, but are thought to be distinct from those regulating PCD in more mature neuronal regions. Within the cerebral cortex, Blaschke *et al.,* (1998) speculated a candidate molecule regulating cell death was the growth factor like lipid, lysophosphatidic acid (LPA). An LPA receptor gene, *vsg-1* has been found to be

expressed in the VZ of the cerebral cortex (Hecht *et al.,* 1996) which places it at the correct time and location to mediate at least some signalling aspects of cortical apoptosis.

Interestingly, a new approach to the examination of cell death in the proliferative neuroepithelium comes from the examination of a novel marker *png-1,* which identifies newly postmitotic neurons (Weiner and Chun, 1997). Combined ISEL+ with in situ hybridisation for the *png-1* gene, resulted in a positive correlation between the start of differentiation and the onset of PCD. This correlation suggests a link between the onset of PCD in a given VZ and the initial generation of postmitotic neurons form the VZ (Blaschke *et al.,* 1998). Further support from studies into dying proliferative cells comes from Thomaidou *et al.,* (1997) in which cumulative labelling with BrdU showed that 71% of TUNEL labelled cells had taken up BrdU before undergoing cell death. The proposal that the cells use the same machinery in the opposing processes suggests that the cells utilise this pathway to eliminate cells carrying mutation which would be produced in large numbers by proliferating tissue. Alternatively, this could be an indication that one of the two processes is dependent on the other (release of FGF2 by dying cells promotes the proliferation).

The cellular mechanisms that initiate the death of individual neurons are under investigation and will be discussed briefly below. However, the death of individual cells affects the organisation and survival of whole neuronal populations surrounding the individual neurons in question. In an influential early examination of cell death and its role in embryogenesis, Glucksmann (1951) distinguished three classes of cell loss; morphogenetic, phylogenetic and histogenetic. The first is described as sculpting some aspect of the gross morphology, for example the closure and opening of lumen between tissues. The third for the removal of vestigial or larval organs and the second

for the loss of cells that have ceased proliferation and begun differentiation (Glucksmann, 1951). Obviously these three categories do not cover neuroproliferative death, thus a re-examination of the definitions might be required.

Regional variation in cell death in the nervous system

The developing retina is relatively uniform in terms of cell density. By the end of development, most species possess a highly non-uniform pattern of density in the form of an area centralis, visual streak or fovea (reviewed in Finlay, Wikler and Sengelaub 1987). Senglaub and colleagues noted that the incidence of pyknosis in the retina as expressed with respect to local cell density was higher in the periphery (Senglaub and Finlay, 1982) than the centre. Subsequent investigations examined whether the disproportionate cell loss was a general feature of retinal development. The quokkas, grey kangaroos, cats, and humans appeared to demonstrate this differential cell loss (Beazley *et al.,* 1989; Dunlop and Beazley, 1987; Wong and Hughes 1987; Provis 1987). Even in the ferret and the gerbil, pronounced spatiotemporal non-uniformities in cell death were observed (Hender *et al.,* 1988; Winkler *et al.,* 1989). Why there is a disparity in cell loss in the retinal periphery compared to the central regions remains to be determined? One possibility proposed is that it is a result of the order of cell generation. Alternatively, activity dependent sorting may give rise to this phenomenon (Finlay, 1992). Further examination of the visual pathway revealed that there is regional cell loss in the dorsal lateral geniculate body (Sengelaub *et al.,* 1985). The superficial grey layer of the superior colliculus had elevated cell loss in the periphery (Sengelaub and Finlay 1982). Detailed examination of this phenomenon revealed that differential cell death did not result because of interactions between the components of the visual pathway (retina or tectum), but

rather a cell intrinsic mechanism. Differential cell death has previously been described in the cerebral cortex (Finlay and Slattery 1983; Finlay 1992). Layer specific cell death was observed particularly amongst the first generated neurons of the cortex, the SP (Luskin and Shatz 1985; Kostovic and Rakic, 1990). Differences across cortical areas have also been observed in terms of dying cells. Late in neurogenesis, following the completion of neuronal migration and the formation of the CP, marked regional differences in the incidence of pyknotic cells can be seen. The differences correlate highly and negatively with the eventual numbers of cells per unit column: the visual cortex has few degenerating cells (Finlay and Slattery, 1983).

Trophic dependence

Death of cells by apoptosis has often been associated with target derived trophic factor deprivation (Levi Montalacini and Angeletti, 1968). Neurons are generated in excess in the developing vertebrate nervous system. Superfluous and inappropriately connected neurons are eliminated in a phase of cell death that begins shortly after neurons innervate their target fields (the neurotrophic hypothesis). Thus, growing axons compete for limiting amounts of neurotrophins that the target tissue produces.

A growing body of evidence suggests however that non-target derived influences can regulate PCD. These include incoming afferents, glia derived and extracellular matrix molecules, as well as other local cell-cell interactions between neurons and non-neuronal cell may play a role in promoting the survival of developing neurons (Walicke 1989; Barde *et al.,* 1989).

Functions of neuronal cell death

Some of the functions attributed to neuronal death that have been identified so far include removal of cells with transient functions; removal of redundant cell in a lineage; established neuronal circuitry; creation of specific structures; formation of morphogenetic patterns; elimination of cellular phenotypes; the creation of region specific differences in neuron numbers, and the removal of potentially harmful cells (Kallen 1965; Glucksmann 1951; McConnell *et al.,* 1989; Luskin and Shatz 1985 Oppenheim, 1991, for review).

1.2. AIMS OF STUDY

It is now widely accepted that the developing VZ of the cerebral cortex is a heterogenous population of cells. A strong body of evidence is accumulating to suggest that there are differences amongst cells residing in different regions of the cortex; regional expression of genes, transcription factors, receptors and various proteins involved in the regulation of neurogenesis. We propose that these differences portray future areal distinctions of the adult cerebral cortex. Such regional identities are thought to be under the control of intrinsic information regulated by cell lineage and environmental determinants in the form of cell-cell interaction, spatiotemporal presence of growth factors and neurotrophins.

In this investigation, we sought to determine differences, primarily, in the survival of cells over the period of neurogenesis from two different regions of the developing cortex, rostral (presumptive frontal) and caudal (presumptive occipital) cortex. Employing cell culture, we isolated separately the rostral and caudal regions from rat cortices. Counts of dead cells determined the proportion of cells undergoing programmed cell death. Cell survival assays determined the extent of cell survival and proliferation. Thereafter, we investigated the interaction of rostral and caudal cells using a co-culture set up. We also investigated the degree of proliferation and differentiation amongst cultures from different ages using standard BrdU incorporation assay and immunocytochemistry.

In addition, we sought to test the role of bFGF in the survival of rostral cortical cultures. Using immunocytochemistry, in situ hybridisation, SDS-PAGE and Western blotting we describe the distribution of bFGF and FGFR 1 in the developing cerebral cortex. Further, we tested the survival of cortical cells in cultures obtained from loss of function bFGF -/- mutant mice.

The final aspect of the present study was to elucidate the type of cell death occurring. Employing in situ end labelling (TUNEL) and transmission and scanning electron microscopy we categorically define the mode of cell death occurring to be apoptotic.

2.1. METHODS

2.1.1 Preparation of dissociated cortical cell cultures

Separate cultures

To determine differences in cell death and cell survival of rostral and caudal cells of the cerebral cortex, dissociated cortical cultures were prepared from E14-19 Sprague Dawley rat embryos. Embryos from pregnant females were culled by cervical dislocation and placed on ice cold Dulbecco's Modified Eagles Medium (DMEM, pH7.4). The procedure was then performed under sterile conditions. Embryos were decapitated, the skin and meninges removed, and the cerebral cortices removed by dissecting away the striatum and presumptive hippocampus. The rostral, intermediate or caudal regions, as shown in figure 3 were then dissected and collected separately in labelled 35 mm petri dishes. The excised tissue pieces were incubated in DMEM containing 0.25% trypsin (ICN) and 0.001% DNAse I (Boehringer) for 30 min at 37°C. The tissue was incubated in Hank's Balanced Salt Solution (HBSS, Ca^{++} and Mg^{++} free, ICN) containing 0.05% trypsin and 0.002% DNAse I and 0.5 mM ethylenediaminetetraacetic acid (EDTA, Sigma) for 10 min at 37°C. Trypsin was inactivated by incubating tissue pieces in 10% heat inactivated foetal calf serum (HIFCS, Gibco) in DMEM:F12 (Sigma) at room temperature for 10 min. The tissue pieces were delicately tritruated using a 1000 microlitre pipette until no intact pieces were visible. The cell suspension was centrifuged at 1000 rpm for 3 min. The supernatant was discarded and the cell pellet re-suspended in 1-2 ml DMEM:F12 (depending on the size of the pellet). The solution was trituated again to obtain a cluster free suspension. The cell density was determined using a haemocytometer. Cells were plated on poly-L-lysin (PLL, 10 μg/ml, Sigma) and laminin (10 μg/ml,

Sigma) coated 13 mm diameter glass coverslips at a density of 2 X 10^5 cells per coverslip. Cells were allowed to attach for 30-50 min at 37°C.

The cells were subsequently grown for 24 hr in 5 % HIFCS in DMEME:F12 supplemented with 1 % L-glutamine (L-glut, GIBCO) and 1 % penicillin/streptomycin (pen/strep; GIBCO) at 37°C in a humidified incubator 95 % air/5 % CO_2. Cell growth was monitored using an inverted microscope. Culture media was replenished at 3-4 day intervals.

At the end of the culture period, most cultures were fixed in 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) and stored at 4°C until processing. Cultures harvested for scanning and transmission electron microscopy (SEM and TEM) were fixed separately. See below for details of procedures.

Co-cultures

Mixing cortical regions

To determine whether rostral cells could be rescued by caudal cells, cells isolated from rostral and caudal regions were co-cultured in a number of experiments from E16, E17 and E19 rat embryos. In these experiments, rostral and caudal cells were prepared as described above. In addition to plating rostral and caudal cells on different coverslips, cells were mixed in equal proportions (1 X 10^5 rostral cells + 1 X 10^5 cells caudal cells per coverslip), plated on coverslips and cultured under the same conditions as control cultures. Cells were subsequently fixed in 4 % PFA at 1, 2, 3, or 4 days in vitro (DIV).

Separate co-cultures

To determine whether a soluble diffusible factor produced by caudal cells was able to rescue rostral cells, cultures from rostral and caudal regions of E16, E17 and E19 cortices were isolated and plated on coverslips at 2 X 10^5 cells per coverslip. Using a 6 well plate, 2 coverslips from each group were cultured together in 1 ml of 5 % HIFCS with L-glut and pen/strep for 24 hr. The medium was subsequently changed to 1% HIFCS with L-glut and pen/strep.

Heterochronic co-cultures

As caudal cells rescued rostral cells in co-cultures, the following experiment was designed to ascertain whether this was an age related phenomenon. Dissociated cells taken from E16 and E19 rostral regions were co-cultured in 6 well plates with cells from caudal regions of E17 rat cortices. Cultures were maintained in standard culture medium as above.

Cultures from mice homozygous for a loss-of-function bFGF allele

To determine the role of bFGF in the survival of cells of the cerebral cortex, and its role in mediating the rostral cell rescue, mice lacking bFGF were used. A pilot study was carried out in embryonic cultures derived from bFGF knockout mice. The mice obtained were kindly donated by Dr. Rosanna Dono (Dono *et al.,* 1998). Briefly, the first coding exon of the murine bFGF gene encodes three protein isoforms which are translated by alternative initiation. Therefore, the complete first bFGF coding exon and parts of the com 5'- 3'-flanking intronic sequences were replaced by the neomycin resistance gene in the opposite transcriptional orientation using homologous recombination in embryonic stem (ES) cells. Chimeric mice were

generated from correctly targeted ES cells and crossed to C57BL/6J mice for a germline transmission. Subsequently, homozygous bFGF-/- mice were obtained in a mixed C57BL/6JX129/Sv genetic background. Genotyping was performed at birth.

Matings

Timed pregnancies were set up by crossing bFGF -/- females with bFGF -/- males and the dates recorded. Several females were placed together in the evening and checked for plugs the following morning. Plugged females were removed and the unplugged mice remained in the cage with the male until a plug was seen. Wildtype mice were also set up in a similar fashion. Mice in these experiments were culled by cervical dislocation, uteruses removed and the procedure for culturing was carried out as for rat embryonic cultures. On average one pregnancy gave rise to 8-12 embryos, sufficient for 1 X 24 well plate. Age matched +/+ and -/- were used. Cultures were grown strictly under the same conditions as those established for rat cultures and 3 coverslips were fixed from each region each day for 3-4 DIV. Cells were stained with PI and counts were made at the fluorescence microscope.

Different cell densities

Cells were plated at different densities to evaluate whether plating density would determine the survival outcome. E17 rostral and caudal cultures were prepared as described above. Cells were plated at a range of densities, 1 X 10^5, 2 X 10^5, 3 X 10^5 and 4 X 10^5 cells per coverslip. Cells were incubated under standard culture conditions. Cultures were fixed at 1, 2, 3 and 4 DIV in 4 % PFA.

Different culture conditions

To exclude the possibility that the differential cell (rostral) death phenomena observed was independent of culture conditions, E17 rostral and caudal cell cultures were prepared as described above and grown under different conditions. The following is a list of the culture conditions used;

1. 10 % HIFCS in DMEM:F12 and glut, pen/strep
2. 5 % HIFCS in DMEMF:12, glut and pen/strep
3. 1% HIFCS in DMEM:F12, glut and pen/strep
4. 5% HIFCS in DMEM:F12 and glut
5. 1% N2 Neural supplement, glut and pen/strep
6. DMEM:F12 alone
7. Basal Medium

The development of the cultures was observed on the inverted microscope. The approximate ratio of dead and dying cells to living cells was made and recorded. Cultures were also fixed at 1 and 3 DIV.

Effect of neurotransmitters

Various neurotransmitters were used to observe the survival response of E17 rostral cells. The neurotransmitters used were; Noradrenaline (NA; 100 μM), Dopamine (DA; 50 μM), acetylcholine (Ach, 100 μM), 5-hydroxytrptamine (5-HT, 200 μM) and monosodium glutamate (MSG, 50 μM). Cells were fixed at 3 DIV. Control cultures used were rostral untreated cells.

Effect of growth factors and neurotrophins

The survival response to growth factors and neurotrophins of cells in rostral cultures was tested. The following growth factors and neurotrophins were added to standard culture medium on the day of plating and removed the following day; basic fibroblast growth factor (bFGF; 10 ng/ml), nerve growth factor (NGF, 50 ng/ml), epidermal growth factor (EGF, 10 ng /ml); brain derived neurotrophic factor (BDNF, 50 ng/ml) and neurotrophin-3 (NT-3, 50 ng/ml).

Observations of the cultures were made each day and approximate proportions of surviving and dying cells were made and recorded at the inverted microscope. Cells were fixed at 1 and 3 DIV for all factors. Cultures treated with bFGF were also fixed at 2 DIV.

Cell tracker labelling

To determine which group (rostral or caudal) underwent cell death in mixed cultures, cells were labelled with cell tracker before mixing. Following dissociation and before plating, the cell suspension was divided into 2 aliquots. One aliquot from each group was treated with bisbenzamide (5 mM) or cell tracker blue (5 μg /ml) and then incubated at 37°C for 30 min. Following gentle trituation, cells were centrifuged at 1000 rpm for 3 min and re-suspended in fresh media. For mixed cultures, equal numbers of unlabelled rostral and labelled caudal cells were mixed together and plated at the control density. The reverse experiment was also set up in which unlabelled caudal cells were mixed with labelled rostral cells were cultured together. Control cultures set up were labelled and unlabelled cells from each group plated on separate coverslips.

Proliferation assay

The rate at which cells divided was determined by assessing the rate of uptake of a thymidine analogue, 5'-bromo-3'-deoxyuridine (BrdU, 10^{-5}M). Cultures derived from E15, E16, E17 and E19 were pulse labelled with BrdU at 1 DIV for 2 hr prior to fixation. BrdU was prepared at a stock concentration of 10^{-3} M in 0.07 N NaOH in water, pH 7.4, and filtered through a 2 μm sterile filter.

MTT survival assay

The survival of cortical cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma). A 5 mg/ml stock solution of MTT was prepared in GBSS and stored in aliquots at -20°C. Rostral and caudal cultures were prepared from all ages. 0.5 mg/ml of MTT was added to the cultures and incubated for 2 hr. The culture medium was removed and 1 ml of dimethylsulphoxide (DMSO) was added. The resultant blue coloured solution was collected in labelled centrifuge tubes and assayed using a spectrophotometer set at wavelength 540 nm. A minimum of 3 samples were collected from each culture at each time point. The experiments were repeated on 2-3 separate culture preparations. Cultures exposed to growth factors and pharmacological agents were also assayed in this way.

Basic FGF treatment

In some experiments, bFGF was added to E17 rostral cultures at increasing concentrations to assess the dose dependent survival effect using the MTT assay described above. The concentrations used were 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 20 ng/ml, 30 ng/ml and 100 ng/ml. Cultures were treated on the day of plating. The

medium was changed to 1% HIFCS containing the growth factor on the following day. Control cultures were prepared alongside treated cultures to compare the survival of the rostral cells under treatment conditions.

Rostral cells obtained from E17 embryos were treated with 30 ng/ml of bFGF and sustained in 5% HIFCS for 24 hr and subsequently grown in media containing 30 ng/ml bFGF and 1% HIFCS. Cultures were fixed in 4% PFA at 1, 2, 3 and 4 DIV. Some cultures were fixed in 2% glutaraldehyde for electron microscopy.

Aminoadipic acid treatment

To inhibit glial cell proliferation and differentiation in caudal cultures, 2 mM of α-aminoadipic acid (AAA) was added to culture media. A stock solution (20 mM) of AAA was prepared in concentrated HCl. The optimal concentration for use in tissue culture was determined by culturing cells in varying dilutions of the stock and observing them at the inverted microscope.

Cytosine aribinoside treatment

In order to assess the relative importance of cell division on cell survival, a mitotic inhibitor was used. Rostral and caudal cells were cultured in 0.01 mg/ml cytosine arabinoside (AraC) on the day of plating. Stock solution of 10 mg/ml AraC was prepared in DMSO and stored in aliquots at –20°C. The optimal concentration used was determined by exposing the cortical cells to increasing concentrations of AraC and observing the degree of cell death occurring 24 hr after treatment. Rostral and caudal cells treated with optimal concentration of AraC were fixed in 4% PFA at 1 and 3 DIV respectively.

Combined AraC and bFGF treatment

To observe the effect of bFGF on the survival rather than the proliferation of cortical cells, rostral and caudal cells were cultured with bFGF in the presence of AraC, an inhibitor of proliferation. The optimal concentration for use was determined using the MTT assay and counts to assess cell ratios. Control cultures were treated with either bFGF 30 ng/ml alone or AraC 0.01 mg/ml alone.

Genistein treatment

To block the effect of bFGF action via the tyrosine kinase receptor FGFR1, 5 mM genistein were applied. Increasing concentrations of genistein were added to the caudal cells on the day of plating. Stock samples of genistein were made up as 50 mM stock and stored at –20°C. Cultures were observed each day under the different conditions. Toxic concentrations of genistein caused cells to die by 24 hr *in vitro*. Control and treated cultures were fixed in 4% PFA. MTT assays were also performed at 3 DIV on treated and untreated culture groups.

Conditioned medium

Conditioned medium was obtained to assess whether a substance produced by caudal cells continued to exert its survival effects on rostral cells. E17 caudal cultures were prepared and maintained in 5% HIFCS and then changed to 1% HIFCS for a 3 day period. At day 3, fresh rostral cultures were prepared and plated. Conditioned medium was recovered from the caudal cells and added to the new rostral cultures. An estimate of cell death was made using the inverted microscope.

Preparation of coverslips

Coverslips (13 mm diameter) were washed in chromic acid overnight and washed in water until all traces of acid had been removed. After a brief rinse in distilled water, coverslips were sterilised by heating in 70% ethanol. Before coating the coverslips were rinsed in sterile distilled water and laid out in large petri dishes to dry. Solutions of 10 μg/ml of Lam and10 μg/ ml of PLL was prepared in sterile distilled water. A solution of 50-60 μl of Lam/PLL solution were placed on the coverslips. The coverslips remained at 4°C overnight, were washed briefly and stored for 24 hr before use.

Immunocytochemistry

Immunocytochemistry was performed on cultures and on brain sections.

All cultures were fixed in 4% PFA for 20-30 min and stored in PBS at 4°C. Briefly, cultures were blocked with 5% normal goat serum (NGS) in 0.3% Triton-X/PBS for 30 min. The primary antibody was diluted in 0.3% Triton-X/PBS and 50 μl were placed on each coverslip. Coverslips were kept in a humidified chamber and incubated at 4°C overnight and then washed 2 x 10 min in PBS. Goat anti-mouse or goat anti-rabbit antibodies were diluted 1:100 –1:200 in PBS. Coverslips were then incubated in secondary antibody at room temperature for 1-1.5 hr, washed in 2 X 10 min PBS and incubated in the third layer (standard avidin-bitoin conjugated to horse radish peroxidase (ABC, Vector; 1:100) or elite ABC (1:50) diluted in 0.1 % triton-X/PBS for 1 hr. The ABC solution was pre-incubated for 30 min prior to use. A final 2 X 10 min wash in PBS was carried out and cells visualised by reacting in 0.05% diaminobenzidine (DAB) in 0.1 M tris buffer and 0.3% hydrogen peroxide. The intensity of the reaction was enhanced on occasions using nickel sulphate crystals.

The length of the reaction varied from 10-30 seconds. The reaction was stopped by immersion in PBS. Coverslips were mounted in 50% glycerol/PBS and sealed with nail varnish.

Peptide competition controls

Immunocytochemistry for bFGF and FGFR1 performed on cultures, wax embedded sections and fixed frozen sections. Samples from each group prepared in parallel with peptide competition controls. The antibodies to bFGF and FGFR1 were diluted in the presence of excess of their respective peptides and placed on the cultures or slides as described above. Tissue thereafter was processed as described above.

Antibody omission controls

Primary antibody to bFGF and FGFR1 were omitted in sample preparations as controls for the immunocytochemical procedure and subsequently processed as described above. Immunocytochemistry was also carried out on sections of adult and embryonic cortical tissue. Adult brains were perfused fixed in 4% PFA in 0.1 M phosphate buffer (PB), pH 7.4, dissected out and postfixed for 2-3 hr, and subsequently washed in PBS for 30 min. Some adult brains were dissected prior to fixation and coronal slices cut and immersed in 4% PFA/PB overnight. The fixative was then replaced by PBS. Tissue slices were then either embedded in wax or frozen, as described below.

Embryonic brains were dissected from the heads of E14, E17 and E19 embryos and fixed by immersion in 4% PFA for 2-24 hr. They were then washed in PBS for 30 min and were embedded in wax or frozen.

Wax embedding

Fixed, adult brains were cut in the sagittal plane along the midline and further sliced into coronal pieces. Tissue pieces were dehydrated in an ascending series of ethanol (2 X 2 hr in each concentration), and kept in chloroform overnight. They were then put through several 30 min changes of molten wax, and infiltration aided by a vacuum pump for 30 min. The brains were orientated and the wax allowed to set at room temperature overnight. Sections, 6-12 μm in thickness, were cut, collected in a water bath warmed to 37°C, and mounted on PLL coated slides.

Embryonic brains were processed in a similar fashion except that tissue was immersed in the ascending series of ethanol for 2 X 30 min each alcohol. After immersion in chloroform they were infiltrated in wax. Two 30 min changes were made and infiltration in the vacuum pump was performed for 20-30 min. Brains were set in wax in a sagittal orientation and mounted on blocks for cutting. Sections 6-10 μm thick, sections were mounted on PLL coated slides.

Both adult and embryonic wax sections were processed further for immunocytochemistry or histological staining. Briefly, slides were placed in histoclear and re-hydrated in a descending series of ethanols and then placed in PBS. For histological analysis, embryonic sections were immersed in Meyer's haemalum (Boehringer) for 6-8 seconds or stained with toludine blue. After all staining procedures were completed sections were dehydrated and mounted in DPX and allowed to dry.

Embedding for Cryosectioning

Fixed brains or blocks of tissue were immersed overnight in 14 % sucrose/PBS at room temperature. Brains were immersed in OCT and mounted in a sagittal orientation on a pre-chilled mounting block and frozen by cooling over liquid nitrogen. Tissue not used immediately was frozen in OCT and stored at –80°C. 10-15 μm thick sections were cut and collected on either PLL or silane coated slides (Sigma). Slides were kept at –20°C before use.

Fresh brains were quickly dissected in cold GBSS and mounted in OCT on pre-cooled blocks and rapidly frozen by immersion in isopentane cooled on dry ice. Brains were orientated in the sagittal plane and 10-25 μm sections were cut placed on slides, and stored at –20°C before use.

For immunocytochemistry, slides were allowed to reach room temperature for 30 min. Sections were then encircled with a grease pen (DAKO) and processed as described above. Fresh frozen tissue sections used for immunocytochemistry were air-dried for 2-3 hr and fixed by immersion in 4% PFA/PB for 30 min or by immersion in pre-chilled 95% ethanol:5% acetic acid for 5 min, or pre-chilled methanol or acetone for 10 min. Sections were then washed briefly in PBS and further processed for immunocytochemistry. Fresh frozen sections used for radioactive oligonucleotide in-situ hybridisation were air dried for several hours, fixed for 5 min in fresh 4% PFA, washed for 5 min in PBS and dehydrated in 70% ethanol and 100% ethanol. Slides were stored in 100% ethanol until required. Fresh frozen tissue used for DIG (non-radiocative) in-situ hybridisation were air dried for 30 min to 1 hr, immersed in 4% PFA for several hours, washed in PBS and dehydrated as described above.

Preparation of coated slides

Glass microscopic slides were immersed in concentrated chromic acid overnight, followed by thorough washing in running tap water (3-4 hr) and finally rinsed in distilled water. Cleaned slides were then immersed in PLL, diluted 1:10 for at least 10 min at room temperature, and allowed to dry overnight.

BrdU labelling

BrdU was used as a marker of cell proliferation. 10^{-5}M BrdU was added to the cultures for 2 hr prior to fixation in 4% PFA. Cells were thereafter washed in PBS and incubated at room temperature in 2.5 N HCl for 30–60 min and subsequently washed in 0.1 M borate buffer for 15-30 min. Cells were then washed in PBS for 5 min and processed further. Cells were blocked for 30 min in 5% NGS in 0.5% triton-X/PBS and then incubated in monoclonal anti-BrdU diluted 1:500 in 0.5% triton-X/PBS overnight at 4°C.

TUNEL

Cultures were fixed in 4% PFA for 0.5-1 hr, rinsed in PBS and processed for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL). Cells were incubated in TDT buffer (Boehringer) for 15min, and subsequently incubated in the following for 1 hr at 37°C in 20 µl/ml 5 X TDT buffer, terminal transferase enzyme (0.2 µl), biotin-6-dUTP (1 µl); cobalt chloride 5 µl (25mM), made up in double distilled water. Following incubation, coverslips were rinsed in distilled water and washed 1 X 5 min in PBS. The cultures were then incubated in streptavidin-flurothicyanate (FITC; 1:100) for 1 hr, and subsequently washed in PBS for 2 X 10 min. Coverslips were also counterstained with propidium

iodide (PI, 5 μg/ml) and mounted in citifluour, an aqueous mounting medium. Labelling was analysed using a laser-scanning confocal microscope.

2.1.2. In-situ hybridisation

Plasmid mini prep

Basic FGF and FGFR 1 plasmids were transformed into DH5α *Eschericia coli* (*E. coli*) cells to propagate plasmids. Twenty ng of each plasmid (Dr. A. Baird and Dr. A. Logan) were used in the transformations. Cells were incubated at 42°C for 30 seconds and 1 ml of Luria-Bertani (LB) broth was added. A further 1 hr incubation at 37°C was followed by plating on LB containing 60 μg/ml of ampicillin. Cultures were incubated overnight at 37°C.

Plasmid maxi prep

QIAgen plasmid kit was used to make preparations of plasmid. Briefly 10-15 ml of the overnight culture of *E.coli* were harvested and 0.4 ml of cell suspension buffer containing RNase A was added to the pellet and cells were re-suspended until homogenous. After the addition of 0.4 ml of cell lysis buffer, the suspension was mixed gently by inversion 5 times. The suspension was incubated at room temperature for 5 min. To neutralise the solution, 0.4 ml of neutralisation buffer was added and the solution mixed by inversion. The mixture was then centrifuged at approximately 12000 g at room temperature for 10 min. The supernatant was pipetted and loaded into the equilibration column. The solution was allowed to drain by gravity flow. The flow-through was discarded. The column was washed x2 with 2.5 ml wash buffer and allowed to drain by gravity flow. The flow through was discarded. Plasmids were eluted by the addition of 0.9 ml elution buffer to the equilibration

column and allowed to drain through by gravity flow. To precipitate the DNA 0.63 ml of isopropanol was added to the eluate, mixed and centrifugated at 12000 g for 30 min at 4°C. The supernatant was carefully discarded and the plasmid DNA pellet washed with 1 ml of 70% ethanol and further centrifugated at 12000 g for 5 min at 4°C. The ethanol was removed and the pellet air dried for 10 min. The purified DNA was dissolved in 50 µl TE buffer.

Preparation of DIG labelled riboprobe

The following steps were involved in the preparation of the DIG labelled riboprobe; (1) preparation of DNA template, (2) precipitation and purification of DNA, and (3) transcription of riboprobe. All conditions for probe preparation were DNAse RNAse free.

The DNA templates were prepared from vector pBlueskript KS plasmids with a 350 bp fragment of the transmembrane region of rat FGFR 1 cloned into the EcoR1/KpnI sites (gift from Dr. A Baird) and vector pBlueskript SKMIB+ with an insert of 477 bp of the coding fragment of RobFGF 503 clone at the XbaI restriction site.

FGFR 1 antisense and sense DNA templates were obtained by incubating 10 µg of plasmid in EcoR1 (5 U), and Kpn1 (5 U) respectively with the appropriate enzyme buffer made up to 50 µl in water and 10 µg/ml BSA and incubated for 2 hr at 37°C. The basic FGF antisense and sense cDNA template strands were obtained by incubating the plamid with the cDNA insert in the above conditions with Xba1 (5 U) and Xho1 (5 U) respectively.

Following linearisation, 1 µl of each template was removed and added to 1 µl of gel loading buffer (containing bromophenol blue), made up to a volume of 5 µl in ultra pure water and run in a 1% agarose/TAE/ ethidium bromide gel.

Phenol chloroform extraction

An equal volume of Tris EDTA (TE) saturated phenol was added to the DNA sample in a 1.5 ml microcentrifuge tube and vortexed for 15-30 seconds (until the solution appeared milky). The sample was then centrifuged at 4°C for 13 000 rpm for 30 min to separate the phases. Approximately 90% of the upper (aqueous) layer was carefully removed to avoid proteins at the aqueous:phenol interface. At this stage, the aqueous phase was extracted a second time using equal volumes of 1:1 TE-saturated phenol:chloroform, centrifuged and placed in a clean tube. No phenol:chlorofrom was transferred.

Concentration of DNA

To concentrate the DNA, 2.5 times the volume of 95%ethanol/0.3 M sodium acetate was added to the DNA sample in a microcentrifuge tube, mixed by inversion and placed at –20°C overnight. The sample was then centrifuged at 13000 rpm in a microcentrifuge for 15 min at 4°C and the supernatant decanted. The precipitate was allowed to air dry briefly. The sample was washed in 70% ethanol and allowed to air dry. The pellet was then re-suspended in 10 µl of TE buffer and stored at –70°C. The final concentration of the DNA was 1µg/µl.

***In vitro* transcription**

The DIG labelled riboprobe was prepared from 1 μg of purified DNA. The following were added in this order on ice to set up the transcription;

	FGFR1 antisense	FGFR1 sense	bFGF antisense	bFGF sense
Ultra pure water	5	5	7	5
5X transcription buffer	4	4	4	4
0.1 M DTT	2	2	2	2
Linearised plasmid (1 μg/μl)	1	1	1	1
DIG 10X NTP RNA labelling mix*	2	2	2	2
Rnasin ribonuclease inhibitor	1	1	1	1
T3 or T7 RNA Polymerase	5(T3)	5(T7)	3(T7)	5(T3)
Total	20 μl			

* DIG labelled UTP, unlabelled ATP, CTP and GTP.

The mixture was incubated for 2 hr at 37°C, and 1 μl removed and run in a 1% agarose gel to test for transcription. A 10 fold more intense band indicated synthesis of 10 μg of riboprobe. The mixture was then incubated in RNAse free DNASe I for 15 min at 37°C. The DIG labelled riboprobes were precipitated in 25 μl 10 M ammonium sulphate, 200 μl 100% ethanol and 50 μl ultra pure water, mixed by inversion and placed on dry ice for 30 min (or –70°C overnight). The mixture was centrifuged 1000 rpm for 20 min at 4°C. The resultant pellet was washed in pre-chilled 70% ethanol and allowed to air dry. The RNA pellet was then re-suspended in 50μl of diethylpyrocarbonate (DEPC) treated water and stored at –70°C.For in situ hybridisation, 2- 3 μl of the DIG labelled ribroprobe was used.

In-situ hybridisation RNA:RNA labelling

Great care was taken to ensure that no RNAase contamination occurred. Instruments were washed thoroughly in ethanol and gloves worn throughout the procedure. Tissue sections were cut and fixed as described above. Slides were then immersed into 0.1 M HCl (fresh) for 5-10min, followed by 2 X 10 min washes in PBS.

Pre-treatment and hybridisaiton

Tissue was permeabilised with 0.07M triethanolamine pH 8 to which was added 0.25% acetic anhydride just before immersion of slides, and stirred rapidly for 10 min and then washed in PBS 2 X 10 min. Sections were dehydrated through an ascending series of ethanols (5 min in each). Slides were then removed and tissue sections delineated with a grease pen. The prehybridsation mix was diluted 1:1 ratio with deionised formamide, and approximately 400 μl was placed onto each slide and incubated in a humidified chamber at 37°C for 3 hr. The slides were blotted, the hybridisation mix pipetted onto the slides and incubated at 60°C overnight for bFGF and FGFR1 riboprobes (AS and S). Slides were removed and washed in preheated (55°C) 2 X SSC 2 X 30 min and then 0.2 X SSC for 2 X 30 min.

Immunological detection

Slides were washed for 5 min in buffer 1 (described below). Sections were then blocked in 1% blocking reagent diluted in buffer 1, followed by 5 min in buffer 1. Anti-DIG-alkaline phosphatase was diluted 1:5000 in buffer 1 and incubated at 4°C for 2 days. Two further 15 min washes of the slides in buffer 1 followed by a 5 min wash in buffer 2 were carried out at room temperature. The colour reaction was

performed by utilising the dye generating red-ox reaction in which colour soluble products nitroblue- tetrazolium chloride/ 5-bromo-4-chloro-indolyl-phosphate-toludine salt (NBT/BCIP) are reduced and oxidised respectively to produce a blue precipitate. One tablet of NBT/BCIP (Roche international) was dissolved in 10 ml of distilled water, and then placed on the slides at 4°C overnight. The composition of the starting solution was 0.4 mg/ml NBT, 0.19 mg/ml BCIP, 100 mM Tris buffer pH 9.5 and 50 mM MgSO4. The tissue sections were set through an ascending series of ethanols for dehydration and through histoclear before mounting in DPX.

Radioactive *in-situ* hybridisation

Probe synthesis

Oligonucleotides (2 µl) were end-labelled with ^{35}S-dATP (1.5 µl), n a 5X (2 µl), DEPC treated water (2 µl) using terminal transferase enzyme (1.5 µl). Addition was achieved at 37°C in a water bath for 1 hr. Following incubation, 40 µl TE were added to each labelled probe and the probes spun down in sephadex G25 columns (prepared at the time of use) to remove any unlabelled probe. Radiolabel was measured by placing 2 µl of probe into 4.5 µl of scintillation fluid in a vial and counts recorded. Probes with counts less than 100 000 per µl were discarded. Probes with adequate counts were stored at –20°C with 2 µl of DTT for up to 1 month.

Tissue preparation for radioactive in-situ hybridisation

Embryonic day 17 rat brains were isolated and rapidly frozen. Briefly, isopentane was pre-chilled and tissue dry mounted in a sagittal position by immersion into the isopentane. Sections 25 µm thick were cut at –13°C in the cryostat. About 16 sections were mounted onto silane coated slides. Slides dried at room temperature for

2 hr and fixed in 4% PFA for 5 min, transferred into DEPC treated PBS for a further 5 min and then briefly dehydrated in 70% ethanol for 5 min. Stored slides were kept in 100% ethanol at 4°C.

Hybridisation

Slides were removed from ethanol and allowed to air dry for 1-2 hr. The hybridisation buffer containing formamide, 20X SSC, 0.5 M, 0.1 M sodium pyrophosphate, 100 X Denhardt's, HSDNA, 5 mg/ml polyadenylic acid, dextran sulphate in DEPC water, was warmed to 42°C. Probes and unlabelled oligoneucleotides were thawed. The slides were placed in a humidified hybridisation chamber. Probe and DTT were added to the hybridisation mix and 100µl pipetted onto each slide. Parafilm was laid over the slide to ensure all sections had been covered with hybridisation solution. Control sections were covered with hybridisation buffer containing unlabelled probe. The hybridisaiton chamber was sealed and placed overnight at 42°C.

Washes

Slides were washed twice in 1 X SSC for 30 min at 55°C, and once in 0.1 SSC for 1 min at room temperature. Sections were dehydrated in 70% and 100% ethanol, allowed to air dry for 2 hr and placed in a cassette with Biomax film (Kodak).

Visualisation

One week following exposure in X-rays, cassette autoradiographs were developed and examined. Scans of autoradiographs were taken. Slides were subsequently dipped in photographic emulsion and placed in the dark for 2 months.

Using Nucloetide Query database, desalted oligonucleotides for bFGF (ACACTTAGAAGCCAGCAGCCGTCCATCTTCCTTCATAGCCAGGTA) and FGFR1 (CCCGCATCCTCAAAGGAGACATTCCGTAGATGAAGCACCTCCATT) were generated.

2.1.3. SDS-PAGE Western blotting

Preparation of samples for Western blots

Embryonic (E17) cortices were removed and dissected to obtain the rostral and caudal regions as described above. The tissue pieces were placed in freshly prepared lysis buffer containing 10 mM HEPES pH 7.8, 1.5 mM MgCl2, 10 mM KCl and a cocktail of protease inhibitors (leupeptin, 100 μM; pepstatin, 2 μg/ml; PMSF, 1 μM; DTT, 5μ M and aprotonin, 9 μg/ml). Cells were vortexed vigorously in lysis buffer for 1 min and incubated on ice for a further 10 min, vortexed again and centrifuged at 10000 rpm in a microcentrifuge for 2 min. The supernatant (crude cytosolic fraction) was removed and placed in a clean microcentrifuge tube to which was added an equal volume of resolving sample buffer (RSB) containing 10% beta-(2)-mercaptoethanol, 2% SDS, 60 mM Tris pH 6.8, and 0.1% saturated bromophenol blue made up in distilled water, mixed by vortexing briefly and stored at –20°C. The pellet was repeatedly homogenised and centrifuged to obtain a membrane protein fraction. Briefly, the pellet was re-suspended in 1 mM $NaHCO_3$ buffer, filled up to half the initial volume and centrifuged at 3000 rpm for 15 min at 4°C. This step was repeated twice more and the supernatants pooled and spun at 10 000 rpm for 15 min at 4°C. The resultant pellet (membrane fraction) was re-suspended in 250 μl of 1 mM $NaHCO_3$ buffer.

The membrane fractions were then subjected to alkali extraction to free integral proteins of plasma membrane fraction according to Hertzberg (1984). Thus, equal volumes of 40 mM NaOH solution (final concentration 20 mM) was added to the membrane fraction followed by mixing and centrifugating at 12 00 rpm for 15 min at 4°C. The resultant pellet was re-suspended in 1 mM $NaHCO_3$ buffer and washed by spinning at 12 000 rpm for a further 15 min. The resultant solution was diluted 1:1 in RSB, mixed and stored at –20°C.

Separation of proteins using SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A solution of 12% resolving gel was prepared by mixing 4.9 ml of distilled water, 6 ml of 30% arylamide mixture, 3.8 ml of 1.5 M tris (pH 8.8), 150 µl of 10% SDS, 150 µl of 10% ammonium persulphate and 6 µl TEMED. A solution of 5% stacking gel was prepared by mixing 3.4 ml of water, 830 µl of 30% acrylamide mixture, 630 µl of 1 M tris (pH 6.8), 50 µl of 10% SDS, 50 µl of 10% ammonium persulphate and 5 µl of TEMED.

The glass plates of the Western blot apparatus were assembled according to the manufacturer's instruction and the resolving mixture was poured into the gap between the glass plates, followed by an overlay of 1 ml of water. After polymerisation (30 min), the overlaid water was discarded and the stacking gel was poured onto the polymerised resolving gel. This was immediately followed by the insertion of a teflon comb into the stacking gel solution. Samples were thawed and boiled at 100°C for 3-10 minutes to denature proteins. Samples were loaded onto the gel and run as standard.

Western blotting

The resolved proteins in the gels were electorophoretically transferred onto nitro-cellulose membrane, sandwiched between 3 mm Whatman filter paper and soaked in the transfer solution containing tris, glycine, SDS and methanol. The transfer of proteins from the gel to the membrane was carried out at room temperature for 2 hr at a standard current.

Immunolabelling of blots

Membranes were blocked with 1% dry skimmed milk in PBS containing 0.1% Tween-20 (BST) for 30 min and subsequently incubated with either bFGF (1:500) or FGFR1 (1:1000) antibody overnight at 4°C. Detection was by electrochemiluminescence (ECL). The film was developed in the dark for 20 min.

2.1.4. Electron microscopy

Transmission electron microscopy

Cells were grown on Melinex sheets (ICI) cut to the approximate size of a 13 mm diameter glass coverslip for use in electron microscopy. The coverslips were sterilised as described above and coated with Lam/PLL overnight, rinsed and dried. Rostral and caudal cells were grown and fixed at 1 or 2 DIV using a 2% glutaraldehyde in1 M sodium cocodylate buffer (SCB). Tissue was fixed for 1-2 hr at room temperature in freshly prepared fixative. The specimens were washed in 0.1 M SCB for 2 X 10 min, osmicated for 15 min in 1% osmium tetroxide (OsO_4) made up in 0.1 M SCB at 4°C for optimum penetration of osmium into the cells. Excess OsO_4 was washed off in SCB for 5 min and the samples washed for a further 2 X 10 min in 0.1 M sodium acetate. Cells were stained with 2% uranyl acetate made in 0.1 M

sodium acetate at 4°C. A further 10 min washes in 0.1 M sodium acetate and 5 min wash in water followed. Specimens were dehydrated in an ascending series of ethanols for 2 min each and finally 4 changes in absolute ethanol for 5 min each. Cultures were placed in ascending mixtures of Araldite and absolute ethanol; 25:75, 50:50 and 75:25 and incubated at room temperature for 45 min. The mixture was removed and the specimens soaked in fresh Araldite resin for 24 hr at room temperature on a rotator. Polymerisation was allowed to take place at 60°C. The composition of the resin mixture used was 10g dodecenyl succinic anhydrite, 10 araldite (CY212), 0.8 g dibutyl pathalate (plasticiser) and 0.4 ml of benzyldimethylamine.

Thin sectioning

The Melinex was carefully removed and a block of tissue cut from the resin embedded culture. After the initial trimming of blocks, ribbons of ultra-thin sections of 80 nm were cut with wet glass knives and collected on carbon coated copper grids of 200-mesh. Sections were then counter-stained with 3% aqueous uranyl acetate in 50% ethanol and Reyonld's lead citrate each for 30 min to enhance the contrast. During staining the uranyl acetate was covered to prevent the breakdown that is caused by exposure to light. Grids were then floated on drops of lead citrate solution on parafilm in a petri dish for approximately 2 min. Pellets of sodium hydroxide were added to absorb moisture. Grids were washed for 10 seconds in distilled water and allowed to dry. Tissue was analysed with the JEOL 1010 electron microscope. Ultrathin sections were cut from cells in both the caudal and rostral regions obtained from E17 cultures. In 2 separate experiments, 6coverslips were analysed. A variety of morphologies were examined and photographed.

Scanning electron microscopy

Cultures of E17 rostral and caudal cells were prepared as described above on glass coverslips coated with Lam/PLL and fixed at 1 and 2 DIV. The primary fixative used was the same as that used in the transmission electron microscopic methods, above. Cultures were subsequently post-fixed and osmicated in 1% OsO_4 in 0.1 M SCB for 15 min, washed in 0.1 M SCB for a further 10 min and dehydrated through a series of ascending alcohols, 2 min in each and 3 X 5 min in absolute ethanol. Cells were immersed in hexamethyldisilaziane for 2 min and allowed to air dry. Coverslips were then attached with double sided sticky tape to round aluminium stubs and sputter coated with gold. Specimens were viewed at the JEOL 5410 LV scanning electron microscope.

Photography

Colour plates

Bright field colour images were obtained using Kodak 64 T Ektachrome colour reversal film at the Leica microscope. Exposures of less than 1 second proved to give the optimal results in terms of colour balance and detail. Slides were subsequently scanned in and processed in photoshop to prepare plates. Images were printed at Epson stylus inkjet colour printer

Double immunofluorescent images were obtained after scanning tissue at the confocal microscope. Images were stored on optical discs and CD and subsequently processed in Adobe photoshop 4. Images were printed at Epson stylus inkjet colour printer.

Immunofluorescent images were obtained by photographing slides with the Leica epifluorescence microscope using 1600 ASA Provia push processing colour

reversal film. Slides were scanned into the computer and stored on optical discs. Images were printed at Epson stylus inkjet colour printer.

Black and white light micrographs were photographed using Ilford PanF 50 ASA film. DAB stained material was photographed in most cases with the assistance of a green filter to enhance contrast.

Counting

All counts were made at the Leica epiflourescence microscope using a X 40 lens. A 1 cm^2 graticule was placed in the eye piece to assist counting. Five non-overlapping fields of view were selected per coverslip and counts were made of dead or immuno-positive cells against all cells or living cells respectively present in that field of view. DAB immuno-stained material was examine under the bright field filter. Phase contrast images were examined under the phase contrast filter. Nomarski images were examined using the polarising filter. Fluorescence staining was examined using a fluorescence lamp and various filters.

Data analysis

Most cultures were prepared from 3 different pregnancies. Three coverslips were obtained from each culture at each time point, counts were therefore obtained from 3-9 coverslips. A Student's t-test was used to analysed differences between rostral and caudal groups. Significance was considered when the probability or p-value was calculated to be less than <0.05 (5%).

3.1. RESULTS I

The developing cerebral cortex arises from a seemingly homogenous group of neuroepthelial cells lining the lateral ventricles of the forebrain. Evidence is mounting to suggest that the VZ is not a blank sheet or tabula rasa, but rather that inhomogeneities in it give rise to areal and regional differences found in the adult cerebral cortex. The present study investigates differences in the survival of different regions of the developing cerebral cortex using an in vitro model.

3.1.1. Cell survival assay

The survival of cortical cells was assessed by the MTT assay based on a protocol by Mossmann (1983). The colorimetric assay relied on the ability of viable, not dead or dying neurons to convert a soluble yellow tetrazolium bromide dye into an insoluble formazan product that was easily visible within living cells. The blue product was then solubilised in DMSO and the optical density (OD) measured using a spectrophotometer set at wavelength 540 nm. OD measurements were also obtained for DMSO.

In order to observe any differences in the survival of cells obtained from the different regions of the developing cerebral cortex, 3-6 samples were obtained from each region at each time point and assayed. Three pregnant rats were used in assessing the survival of cells in these experiments.

E19 rostral cells survive for shorter periods than caudal cells. Cerebral cortices were dissected from E19 rat brains, as described elsewhere, and tissue pooled from the litter for each experiment. The cortical explants were further dissected to isolate rostral and caudal cortex. Approximately, one fifth of the anterior end of the cortex was cut to obtain rostral tissue and posterior end to obtain caudal tissue

(Figs.4A, 5). Cells were plated at 2 X 10^5 cells per lam/PLL coated glass coverslip and cultured in 24 well plates. Previous work demonstrated that laminin was the most permissive substrate for the growth of embryonic cultures when tested against collagen type IV and fibronectin. Cultures were grown in the presence of 5% HIFCS to begin with and changed subsequently to lower serum condition (1% HIFCS).

Immediately after dissociation cells were observed with an inverted microscope. Most cells appeared to have lost all processes or neurites they might have had *in vivo* (Fig. 5). Most cells had rounded up whilst a few had one or two short processes protruding from the cell body. Dissociated cells from E19 cultures appeared to be larger than cells obtained from other ages, however no specific measurements were performed. Cultures prepared from E19 rostral or caudal regions grew for relatively short periods of time compared to cells obtained from other ages. Since both cell groups had relatively short survival times, several time points were examined. The assay was performed at 3 hr, 20 hr, 24 hr and 26 hr *in vitro* (after plating). OD measurements revealed that cells grew well for the first 20 (Fig. 4A). The survival of cells was approximately equal in both rostral and caudal cultures at 3 hr. By 20 hr, the survival of caudal cells was greater than survival of cells in rostral cultures, however both continued to show an increasing rate of survival reflected in the increase in the OD measurements. However, between 20 hr and 24 hr OD measurements from rostral cultures demonstrated a decline in the survival. Although the survival of caudal cells too, began to decline, the proportion of cells surviving remained greater than rostral cultures. By 24-26 hr there was a further reduction in the survival of rostral cells whilst, with OD measurements returning to basal levels (DMSO) by 48 hr. However, the caudal cells continued to survive for longer, although approximately at the same rate as when they were plated. A decline in the survival of caudal cells was seen after

48 hr, by 48 OD measurements returned to basal levels, that is all cells in caudal cultures have degenerated.

The OD measurements were converted into proportions of cells surviving in the two different regions compared to when they were first plated. At peak survival, around 20 hr, approximately the same proportion were metabolising in rostral cultures. There was an approximately 40% ($p<0.05$) increase in the proportion of cells surviving in caudal cells.

E17 rostral cells survive for shorter periods than caudal cell. Dissociated primary cell cultures were prepared from rostral and caudal cells obtained from E17 cortices as described elsewhere. Cells appeared rounded with a halo like appearance immediately after dissociation (Fig. 6). Nearly all the cells had lost their neurites. Cell survival estimates were obtained using the MTT colorimetric assay described above. Cells in rostral and caudal regions had similar OD readings 3 hr after plating, demonstrating that cells survived soon after dissociation; the survival was approximately the same in rostral and caudal cultures, reflecting that the cells had indeed been plated at equal densities in both sets of cultures (Fig. 4B). At 1 DIV there was an increase in the survival of cells in both rostral and caudal cultures. The increase in rostral cells was somewhat less than in caudal cells. By 2 DIV, a decline in the survival of rostral cells was observed as seen by a significant reduction ($p<0.05$) in the OD measurements. However, survival of caudal cells continued to increase significantly. Rostral cell survival diminished over time. By 3 DIV, in the majority of cultures examined and included in the results, there was a dramatic loss of rostral cells, with only a few cells remaining on the coverslip. When examined at 4 DIV, the OD readings have returned to basal levels, whilst survival of caudal cells remained on

the increase. Caudal cells were seen to survive for the whole period of examination of cultures up to 6 DIV.

An examination of the proportions of cells surviving in rostral and caudal cultures reveals that whilst the proportion of surviving cells peaked at 1 DIV by approximately 40% in rostral regions, there was a greater than 100% increase in the survival of cells in caudal regions. By 2 DIV, over a 3.5 fold increase in the percentage of surviving cells was observed, and by 6 days in culture nearly 5 times the number of cells were actively metabolising, whilst no cells were present in by 4 DIV in rostral cultures.

E16 rostral cells survive for shorter periods than caudal cells. Rostral and caudal cells were obtained from E16 cortices. Following dissociation, cells were immediately plated as described above. All cells had a rounded appearance and lacked neuritic processes.

OD measurements at 1 DIV revealed that cells had established growth. There was no difference in the survival of cells at 1 DIV (Fig. 7A). However, a significant difference in the OD measurements were observed at 2 DIV ($p<0.05$). A significant reduction in the survival of rostral cells was observed whilst a mild increase in the survival of caudal cells was seen. At 3 DIV, a further diminution in the survival of rostral cells was observed whilst a mild increase was observed in caudal cultures. At 4 DIV, no cells remain alive in cultures prepared from rostral regions of the developing cortex whilst an increase in the survival of caudal cells was observed. Although there was an apparent decrease in the survival of caudal cells at 6 DIV, the reduction was not statistically significant.

An examination of the proportions of cells surviving demonstrated that there was a small increase (10.3%) in the percentage of cells surviving in caudal cultures

over by 2 days, and almost 42% decrease in the proportion of cells in rostral cultures. Caudal cell survival increased by 37% by 4 DIV.

E15 rostral cell survive for shorter periods than caudal cells. Rostral and caudal cells were obtained from E15 cortices and plated as described elsewhere. Cells appeared rounded and very small in comparison to other ages examined immediately after dissociation.

The colorimetric assay described above revealed that cells had been plated at approximately the same density in both rostral and caudal cultures (Figs.7B, 8). There was no significant difference at 1.5 DIV between rostral and caudal cell groups in terms of survival. Observations were made over 6 days. Microscopic examination did not reveal any obvious difference in the survival. When assayed at 6 DIV, however, significant reduction in the OD measurement revealed a decrease in the survival of cells in rostral cultures below levels seen at 1.5 days. Caudal cultures continued to survive, although no significant increase in the survival was noted. A reduction of over 50% of surviving cells in rostral cultures was observed at 6 DIV.

E14 rostral and caudal cells have a similar survival rate. Rostral and caudal cells obtained from E14 cortices were plated as described above. Although no assays were performed on cultures obtained from this age, preliminary observations revealed that no obvious difference in the survival of cells in rostral and caudal cultures.

Summary 1

Cells derived from rostral regions of the cortex survive for significantly shorter periods than cells obtained from more caudal regions at all ages. However, the period for survival of rostral cells changes with age. Cultures of rostral cells obtained from older embryos (E19) survive for much shorter periods than cultures prepared

from younger (E15-17) embryos. Preliminary observations suggest that there was no difference in the survival of cells obtained from embryonic cortices obtained from very young cortices (E14). There was an increase in survival of caudal cells, the increase being more pronounced in cultures derived from younger animals (E16-17) than those prepared from older embryos (E19).

Cell death

Increased cell death in E19 rostral cultures compared to caudal cultures. Embryonic day 19 cultures were prepared from cells derived from rostral and caudal regions of the developing cerebral cortex as described elsewhere. Cultures were fixed at 3 hr, 20 hr, 24 hr, 28 hr, 32 hr and 48 hr in 4 % PFA and stained with PI. Counts of dead cells were made at each of these time points in rostral and caudal cultures. A few hours after plating, there was very little difference in the proportion of cells dying in rostral and caudal cultures (Fig. 9A). The proportions of cells dying remained low, below 20% in either region soon after plating (3 hr). There was an increase in the percentage of cells undergoing cell death in each region. By 20 hr, just over 20% of the cells in both rostral and caudal regions had died, there appeared to be no significant difference between these two proportions. However between 20 hr and 24 hr there was a cascade of cell death in rostral cultures. Approximately 71% ($p<0.01$) of the cells in rostral cultures degenerated whilst their caudal counterparts had a relatively low proportion of dying cells, approximately 23% ($p<0.05$). After 24 hr *in vitro* the proportion of cells dying continued to increase reaching on average, 93% by 32 hr in rostral cultures. Although there was an increase in the percentage of cells dying in caudal cultures, the rate of cell death was slower. Approximately 60% of caudal cells had died at 32 hr, significantly lower than the portion of dying cells in

rostral cultures. By 48 hr, all the rostral cells had died, with only a few caudal cells remaining alive.

Increased cell death in E17 rostral cultures compared to caudal cultures. Rostral, intermediate and caudal cultures were prepared from E17 rat cortices as described elsewhere. Cultures from each group were fixed at 4 hr, 1 DIV, 2 DIV, 3 DIV, 4 DIV and 6 DIV in 4% PFA. Cultures were stained with PI and estimates of the proportions of dead cells made (Figs. 9B, 10 and 6). Shortly after plating at 4 hr, less than 5% of cells in rostral and caudal cultures died. A greater proportion of cells derived from intermediate area of the developing neocortex underwent cell death. At 1 DIV, an increase in the proportion of dying cells in all three cultures was observed. No significant difference in the proportions of dying cells was seen in either rostral or caudal cultures. The proportion of dying intermediate cells remained higher than cells in rostral and caudal sister cultures. At 2 DIV, an almost 4 fold increase to 85% of dead cells was observed in rostral cultures. This proportion was significantly greater than the percentage of dying cells in either intermediate or caudal cell cultures (24% and 11% respectively, $p<0.01$). There was no statistically significant change in the proportion of dying caudal cells, neither was there any significant change in the fraction of dying intermediate cells. The percentage of dying rostral cells continued to increase from 94 % to 100% at 3 and 4 DIV respectively. At 3 DIV, over one third of the intermediate cell population had degenerated whilst the fraction of dead caudal cells remained steady at approximately 12-16%. By the fourth day in culture, 100% of the intermediate cultures had degenerated. The proportion of dying cells in caudal cultures remained constant (low) even when examined at 6 DIV.

Increased cell death in E16 rostral cultures compared to caudal cultures. Rostral and caudal cells derived from E16 embryonic cortices were cultured as described elsewhere. Cultures from both rostral and caudal regions were fixed at 1 DIV, 2 DIV, 3 DIV and 4 DIV. Estimates of dead and dying cells were made on cultures stained with PI (Fig. 11A). There was no significant difference between the proportions of dead cells in rostral and caudal cultures when examined 1 day after plating. The percentage of cell death seen in rostral and caudal cultures was approximately 11-13%. A slight increase in the fraction of cells dying was observed at 2 DIV. At 3 DIV, 60.6% of rostral cells had died whilst only 17.7 % of caudal cells had died ($p<0.05$). Counts made at 4 DIV revealed that a mild increase in the percentage of caudal cell death (22.9%) had occurred whilst rostral cells had continued to die with on average 95% cell loss ($P<0.01$).

Cell death in E15 rostral and caudal cultures. Cultures of rostral and caudal cells were prepared from E15 cortices. Cultures were subsequently fixed in 4% PFA at 1 DIV, 3 DIV and 6 DIV (Fig. 11B). Cell death estimates were made on cultures stained with PI at 1 and 3 DIV only since cells cultured for longer periods became extremely confluent making counts ambiguous. No difference in the percentage of dying cells in rostral and caudal regions was seen at these two time points. There was no obvious difference in the fraction of dying cells in cultures examined at 6 DIV.

Summary 2

Proportions of dying cells were estimated at different time points from rostral and caudal cultures obtained from several different ages. The results of dead cell estimates closely reflect the trend seen in cell survival assays. A greater fraction of dead and dying cells were seen in cultures obtained from rostral regions of the cortex

in comparison to cultures prepared from caudal regions in all ages examined with the exception of E15. The rate of cell death increased with increasing embryonic age, with all rostral cells diminished by 4 DIV in E16 cultures and 3 DIV in E17 cultures. The rate of increase in the proportion of dying cells was rapid in cultures derived from older embryos, with all rostral cells having completely degenerated by 32 hr. Estimates for fractions of dying cells in E15 cultures were unavailable due to the confluent nature of the cultures. Caudal cells in younger embryos have a low fraction of dying cells that remained fairly constant throughout the period of culture. E19 caudal cultures demonstrated greater vulnerability. The fraction of dying caudal cells increased rapidly after 28 hr in culture.

In order to examine whether the differences in survival of rostral and caudal cells was a result of differences in proliferative ability, rostral and caudal cultures were investigated to determine rates of cell cycling at different ages.

3.1.2. Rate of cell proliferation in rostral and caudal cultures

The rate of cell proliferation was monitored in two ways. The first, employing BrdU incorporation over a specific interval of time (1 hr). BrdU was taken up by dividing cells during the S-phase of the cell cycle and incorporated into the DNA instead of thymidine. By adding BrdU to the culture medium for 1 hr before fixation, the rate of cell division was assessed in all ages in rostral and caudal cortical cultures. BrdU was added to rostral and caudal cells at 1 DIV in all ages. The rate of proliferation was over 4 days in cultures prepared from E17 rat cortices and for 3 days in E15 derived cortical cultures. Estimates of the proportions of dividing cells were made. The second method employed to assess the proliferation was to utilise the MTT colorimetric assay data.

BrdU uptake in cortical cultures reflects the trend in proliferation seen in vivo

Rostral and caudal cultures were prepared from all ages and pulsed with BrdU at 1 DIV. The fraction of cells cycling in both rostral and caudal cell cultures decreased with age; cultures derived from younger embryos having the greatest proportion of proliferating cells whilst only a few cells remained in the cell cycle in older embryonic cultures. This trend reflects closely the *in vivo* situation, in which the proportions of dividing BrdU positive cells decreased with increasing age of the embryo (Figs. 12 and 13). Approximately 27.4% of E15 rostral cells were dividing by 1 DIV, significantly lower than the proportion of dividing caudal cells (36.4%, $p<0.05$). The difference appeared to reflect that expected in the rostro-caudal

neurogenetic gradient *in vivo*, with more anterior cells leaving the cell cycle, whilst the more immature caudal cells underwent more frequent divisions. Unexpectedly, however, there was no significant difference seen in the percentages of BrdU positive cells in rostral and caudal cultures prepared from E16 or E17 embryos, in contrast to what was observed *in vivo* with respect to the neurogenetic gradient. That is, that fewer cells were cycling in the mature anterior portion of the cortex than the immature posterior portion (Bayer and Altman, 1991). The fraction of dividing cells in cultures derived from E16 brains was approximately 23% in both rostral and caudal regions, significantly lower than the rate of proliferation in E15 cultures. The rate of BrdU uptake in rostral and caudal cells in E17 cultures was approximately equal in both regions (17.1% and 16% respectively) and lower than the rate observed in E16 cultures. The rate of proliferation in E19 cultures at 20 hr *in vitro* was significantly lower than all other ages examined. Rostral cells had approximately 5.5% cells dividing whilst in caudal cultures marginally fewer cells were cycling (2.8%).

Calculating the difference in the proportion of BrdU positive cells in each region between consecutive ages, it was possible to estimate the predicted fraction of cells leaving the cell cycle. Approximately 4.8% of cells were leaving the cell cycle in rostral cultures at each age, whilst a greater fraction were leaving in caudal cultures (13.6%). Approximately 5.8% E16 rostral cells leave the mitotic cycle and 6.9% leave in caudal cultures. It was estimated that half of the difference between E17 and E19 cells leaving the cell cycle would be the proportion expected at E18. Between E17 and E19 an average of 7.5% rostral cells have ceased to divide whilst a greater portion of caudal cells exit (11.2%). This suggested that more cells at any one time point were leaving the cell cycle in caudal cultures than rostral cells, suggesting that the rate of

differentiation was greater. This was in contrast to that predicted by the neurogenetic gradient.

Uptake of BrdU decrease over the time in E15 and E17 cultures

Pulses of BrdU were given to rostral and caudal cells in cultures prepared from E15 cortices at 1 DIV, 2 DIV and 3 DIV. Estimates of percentages of cells labelled with BrdU were made (Fig. 14B). Over time, there was an overall decrease in the proportion of cells entering the mitotic cycle from an initial 27.8% (rostral) and 36.4% (caudal) at 1 DIV to 10.8% (rostral) and 11.1% (caudal) at 3 DIV amongst E15 cultures. It is pertinent to note that the approximate 3 fold reduction in BrdU labelled cells was not solely due to the reduction in proliferation alone but also due in part to the increasing proportion of cell death. Since there was also a reduction in the ratio of cells immunopositive for BrdU amongst caudal cultures, it was clear that caudal cells have a greater capacity to survive.

E17 rostral and caudal cortical cultures were pulse labelled with BrdU for 1 hr before fixation. Pulses were given at 4 hr, 1 DIV, 2 DIV, 3 DIV and 4 DIV. BrdU immunoreactive cells were counted as described elsewhere (Fig. 14A). Almost immediately after plating (4 hr), 17.5% rostral cells and 13.4% caudal cells were proliferating. At around 1 DIV very little difference in the proportion of proliferating cells were seen between rostrally and caudally obtained cells. However, by 2 DIV there was a small increase in the proportion of dividing cells in rostral cultures (19.9%). [note at this stage an increase in death occurs], in contrast to the decrease in cycling cells in caudal cultures (12.6%). Proliferating caudal cells continued to decrease over the culture period examined. The complete loss of rostral cultures by 3 DIV results in no uptake of BrdU when pulsed at this time point.

Summary 3

The proliferating fraction of cells was assessed *in vitro* in both rostral and caudal cell groups using a thymidine analogue, BrdU. One hour pulses were administered at each age examined following one day in culture. Clearly, with increasing age there was a gradual reduction in the proliferative population. At E15, a notable difference in the rostral and caudal populations was apparent, reflecting the difference expected from the rostro-caudal neurogenetic gradient. There was no apparent difference in uptake of BrdU at E16 and E17 amongst rostral and caudal cell groups. A dramatic reduction in the fraction of cells entering mitosis was observed by E19 with a small but significant, difference between rostral cells and caudal cells, the former having a marginally greater population of dividing cells.

Cultures of E17 and E15 examined over time revealed that the population of cycling cells reduces with time in culture.

Rate of proliferation assessed by MTT assay

The proliferation of rostral and caudal cells prepared from different ages were assessed over several days in culture using the MTT assay. In all ages examined a greater degree of proliferation was observed in cultures derived from caudal cortex than rostral cortex. At E15, a marginal increase (5%) in the OD measurements of caudal cells after 6 days in culture was observed (Fig. 7B). However, since no intermediate stages were observed, it was difficult to ascertain the peak proliferate stage at this age. By 6 DIV, the OD readings had reduced significantly (by approximately 50%), to below readings taken just 1.5 days after plating, indicating that a reduction in proliferation in addition to the reduction in survival had occurred.

E16 cultures were examined over 6 days (Fig. 7A). A moderate increase in the rate of proliferation of caudal cells over the period examined was observed. By 4 days in culture, a 37% increase in the OD measurements was observed. That is, cells were not only surviving, but also proliferating at a steady rate. An average 12% turnover of caudal cells over the first 3 days examined. Thereafter, there was a significant reduction in the OD measurements, indicating that proliferation and survival of caudal cells had decreased. Since the effect was cumulative, one of three possibilities were assumed; 1. that the percentage of cells entering the cell cycle increased over time, 2. that the proportion remained constant, or 3. the proportion decreased. In the first instance, a steeper increase in the OD measurement would be expected, this would be shallower in the second instance and lower still in the latter case scenario (see Discussion).

A greater difference in the OD value was observed between cultures of rostral and caudal cerebral cortex prepared from E17 rat foetuses (Fig. 4b). That is, the gradient of increase in the proliferation/survival of cells in caudal cultures was not only greater than rostral sister cultures, but also greater than those of other ages examined. A 4.8 fold increase in the number of cells present in caudal cultures was observed. This increase was a reflection of survival and the cell's ability to continue to proliferate. The rate of proliferation far exceeds the rate of cell death since the graph continued to ascend over the 6 day period examine. The gradient of the graph suggests that the proportion of proliferating cells remained relatively high over the first 2 days in culture and then the numbers of cells cycling either levels off or decreases (in keeping with the data from the BrdU incorporation index). The rostral cells appeared to proliferate early in culture, and they may have continued to do so, however, at around 2 DIV the fraction of cells dying appear to exceed that of those

replenishing the current number, resulting in a reduction in the apparent population of cells present.

At E19, an increase in the OD was observed over the first 20 hr whereas there was no apparent change in rostral cultures. An approximate 40% increase in the proportion of cells surviving also indicated that cells were not only surviving but were proliferating also. The proportion of cells able to proliferate in caudal cultures appeared to exceed that of rostral cultures; or that the cell death occurring in rostral cultures was over and above the number of cells proliferating since significant change in the number of cells in these cultures around 20 hr *in vitro*. After 24 hr, the proliferation of caudal cells diminished or the occurrence of cell death increased or both. By 32-48 hr it was obvious that the occurrence of cell death far exceeded the proliferation capacity of cells in both rostral and caudal cultures since OD measurements drop to basal control (DMSO) levels.

Summary 4

The MTT assay allowed the detection of a change in the proliferation as well as the survival of cells in rostral and caudal cortical cultures. An increase in the OD and hence the percentage survival reflected a cumulative increase in the number of cells present in the culture. This was interpreted as indicating a dynamic population of cells, that is cells were proliferating as well as surviving. Caudal cells from each age underwent a considerable amount of proliferation over time in culture, whereas proliferation and cell death equalled each other in rostral cultures examined early in the culture period at each age examined. The ratio between cell death and cell proliferation changed in rostral cultures, more acutely in older embryonic cultures, with the occurrence of cell death overriding the rate of proliferation.

Assessment of cell survival and growth in rostral and caudal cultures plated at differing densities in order to determine whether cell plating density affected the survival of cells in the different regions of the developing cortex. That is, whether there was a differential need for cells to be in contact with other cells in order to maintain survival and or proliferation.

Cell density

E19 rostral cells were prepared from rat cortices dissociated as described above. Cells were subsequently plated at $1x10^5$, $2x10^5$ and $4x10^5$ cells per coverslip. Observations of cultures at these differing densities were made with the inverted microscope at 24 hr *in vitro*. All rostral cells had died by 24 hr in the lower density cultures, whilst higher density cultures demonstrated a moderately greater number of surviving cells. Caudal preparations were not made.

Different media conditions were tested to examine the possibility that rostral and caudal cells were responding differentially to the culture medium and its serum content.

Different media conditions

Cultures from rostral and caudal regions of the developing neocortex were obtained from E17 rat brains. After plating, cultures were subjected to different growth conditions. Cultures were grown in 1) serum free 1% N2 supplemented media, 2)10% HIFCS, 3) 5% HIFCS, 4) 1% HIFCS, 5) DMEM F12 alone and 6) L-glut free media. All media contained 1% L-glut and 1% pen/strep except where specified differently.

In all growth conditions, except cultures incubated in DMEM F12, the differential survival phenomenon of rostral and caudal cells was observed at around 3

control cultures had died, there was a low proportion of dying cells in the mixed cultures. The comparison between the proportion of dying cells in mixed cultures to those dying in caudal cells cultured alone was difficult to make using the inverted microscope since so few cells appeared to be undergoing cell death.

Embryonic day 17 rostral and caudal cells were mixed in equal proportions and cultured together on the same coverslip. Rostral and caudal cells were cultured alone to act as internal controls for the experiment. Cultures were examined each day with the inverted microscope and coverslips from each group were fixed in 4% PFA at 1 DIV, 2 DIV, 3 DIV, 4 DIV and 6 DIV. Counts of dead cells were made after staining with PI to ascertain the ratios of dead to living cells (Fig. 15 and 10). There was no obvious increase or decrease in the proportion of cells undergoing cell death, an average of 15.6% of cells over the 6 day culture period were seen to be dead. There was no significant difference in the proportions of cells dying between mixed cultures and internal rostral or caudal control cultures after 1 day of culturing. The difference becomes apparent when the fraction of rostral cells dying doubles from 15.8% to 32.9% over just one day, at 2 DIV, whilst little or no difference in the proportion of dying cells was apparent between caudal and mixed cultures. Over the course of development *in vitro* the rostral cells continued to die rapidly whilst death in caudal and rost/caud cells remained low. This suggests that there was some interaction between rostral and caudal cells that resulted in the rescue of rostral cells which otherwise, alone, would under go cell death.

The same experimental design was set up for rostral and caudal cells derived from older embryos (E19). Rostral cells were mixed in equal proportion with caudal cells and plated on the same coverslip. Rostral and caudal cells from each experiment were also grown separately to act as internal controls. Although cultures were not

fixed and counted, qualitative estimates were made of cells dying in mixed cultures using the inverted microscope. A short time (20 hr) into the culture period, no difference was observed in the relative fraction of dying cells. Over time, cells in both rostral and rost/caud cultures degenerated. There appeared to be some delay in the rate of cell death in mixed cultures compared to rostral cultures alone. At around 28 hr, more cells appeared to be dying in rostral cultures than in rost/caudal cultures, and even less were seen in caudal cells cultured alone at this time point. When cultures were examined at 32 hr an extensive amount of cell death had occurred in both rostral and rost/caud cultures, somewhat less than in caudal cultures alone. By 48 hr, all cells in all groups had almost completely degenerated.

Summary 5

Rostral and caudal cells were prepared from E16, E17 and E19 cortices. Equal proportion of cells from rostral and caudal regions were mixed and plated on the same coverslip in addition to rostral and caudal cells being cultured separately in each experiment. Qualitative comparisons of the proportions of dead cells were made at each age between cells cultured alone and clusters obtained from mixing the two cell groups. Quantitative analysis was also carried out on E17 at various days in culture. At E16 and E17, there was a significant reduction in the proportions of dead cells compared to rostral cells cultured alone. Results from the E17 study revealed that there was no significant difference between the percentage of dead cell in mixed cultures compared to caudal cell cultured alone, suggesting that an interaction between caudal and rostral cells had occurred resulting in a diminished population of dying cells. This reduction in cell death was not observed in co-cultures of E19 rostral and caudal cells.

Cell tracker labelling

In an effort to determine which cells were undergoing cell death in mixed cultures, rostral and caudal regions of the developing neocortex of E17 embryos were removed and dissociated. Half of the rostral cells and half the caudal cells were suspended in DMEMF12 containing cell tracker blue or bisbenzamide and incubated for half an hour in order to label cells from the two different regions. Rostral and caudal cultures that were not to be labelled underwent similar processing as those cells being labelled to maintain consistency within the experiment. After a number of centrifugations cells were plated on lam/PLL coverslips as described. In addition, rostral labelled cells were plated with equal proportions of unlabelled caudal cells and vice versa. Cells were cultured for 3 days in most circumstances. Cultures in which any one group of cells had died after just one day of culturing were discarded. Cells were fixed in 4 % PFA at 1 and 3 DIV. Cultures from each group were examined. It was anticipated that rostral labelled dead cells would be compared against unlabelled caudal cells in mixed cultures to determine whether there was a specific reduction in the death of rostral cells compared to caudal cells. However, these experiments proved inconclusive as a result of leaching of the dye into adjacent cells.

The reduction in the fraction of cells dying in mixed rostral and caudal culture lead to the development of another culture set up to test whether caudal cells rescued rostral cells from cell death by a cell to cell contact mechanism or via some diffusible survival signal.

3.1.4. Caudal cells rescue rostral cells in separate co-cultures at E17

Rostral and caudal cultures were prepared from E17 embryonic cortices. Rostral and caudal cells were grown on separate coverslips in the same well of a 6 well plate dish. Two coverslips from each region were placed together in the same well. Cultures were observed over several days with the inverted microscope and cultures were fixed at 3 DIV, stained with PI to quantify the fraction of dead cells in the cultures. Rostral and caudal cells were also grown on separate coverslips in separate wells in 24 well plates, enabling a comparison to made between cultures grown together and cultures grown separately. Observations with the inverted, microscope early in the culture period, revealed that there was little difference in the proportions of dying cells in rostral or caudal cultures grown either separately or together. However at 3 DIV a striking difference between rostral cells cultured in the presence of caudal cells and rostral cells cultured alone was observed (Fig. 16 and 17). When quantified, it was noted that at 3 DIV nearly all of the cells in rostral cultures had died (93%, $p<0.01$). However, in the presence of caudal cells the percentage of dead cells found in rostral cultures was over 4 fold lower (21.8%, $p<0.01$). The proportion of dead cells in caudal cultures was not examined quantitatively, but no obvious difference was detected between caudal cells cultured alone or in the presence of rostral cells. The percentage of dying cells in rostral cultures grown in the presence of caudal cells was approximately the same as the

percentage of dying cells in caudal cells cultured alone (21.8% and 19.5% respectively).

Rostral and caudal separate co-cultures at E16 and E19

Rostral and caudal cells were obtained from E16 embryonic cortices. Cells from each region were cultured at a density of $2x10^5$ cells per coverslips as described previously. Two coverslips from each region were placed together in 6 well plates. Rostral and caudal cells were also grown separately in different wells in 24 well plates.

Cells were examined with the inverted microscope throughout the culture period. Cells were not fixed in each of these experiments. Estimates of relative proportions of dying cells were made based on morphological appearance of degenerating cells. Over the first 2 days in culture, no differences were observed in the proportion of dead cells between rostral cells cultured alone or in the presence of their caudal counterparts. No apparent difference in the dead cell population was seen in caudal cells grown separately or in the presence of rostral cells. By 4 DIV, all rostral cells had degenerated whether cultured alone or in the presence of caudal cells.

Similar experiments were performed to examine the interactions of rostral and caudal cells prepared from E19 embryonic cortices. Cultures were plated at $2x10^5$ cells per coverslip. Two coverslips growing cells from each region were grown together in the same well of a 6 well plate culture dish. No differences were apparent between the proportions of dead cells in rostral cultures grown alone and rostral cultures grown in the presence of their caudal counterparts. No differences were apparent when caudal cultures were examined and compared to caudal cells grown in the presence of rostral cells.

Furthermore, in order to reproduce the results observed in separate co-cultures of E17 rostral and caudal dissociated cultures, ratios of caudal to rostral coverslips were altered in E19 experiments. In some experiments, 3 caudal coverslips were placed with 1 rostral coverslip and vice versa. No difference in the proportions of dead cells in each region cultured together compared to respective regions cultured separately were observed at the end point. The end point was the time at which rostral cells grown separately had neared a 100% degeneration.

In a number of experiments rostral and caudal cells derived from E19 embryonic cortices were plated at higher densities and subsequently cultured together in the same well. Cells were plated at $3x10^5$ and $4x10^5$ cells per coverslip. No difference in the population of dying cells was apparent when 2 rostral coverslips were grown in the presence of 2 coverslips growing caudal cells was observed over the culture period examined (28 hr).

As the above experiments revealed that E17 caudal cells could successfully rescue distant E17 rostral cells via a diffusible survival signal, the following experiments were designed to test whether rostral cells from other ages were able to respond in the same manner to signals arising from E17 caudal cells.

3.1.5. Co-cultures of rostral and caudal cell from different ages

E17 caudal cortical regions were dissociated and plated at $2x10^5$ cells per coverslip. Cultures were incubated whilst rostral cultures were prepared from E16 or E19 rat cortices. Subsequently, E19 or E16 rostral cells were grown in the presence of E17 caudal cells. In addition, rostral and caudal cultures from each age were also cultured for each experiment to act as internal controls for the experiment. The

relative proportions of dead cells in rostral and caudal cultures were monitored at each time point.

E16 rostral cells cultured in the presence of E17 caudal cells. E16 rostral cells were cultured in the presence of equal density of E17 caudal cells. Cultures were examined each day. E17 caudal cultures were prepared in the morning from one pregnancy and, on the same day in the afternoon, E16 embryos were obtained to prepare rostral cortical cultures. E16 rostral cells in co-cultures were compared to rostral cells cultured separately, but obtained from the same embryonic age group. There were no apparent differences between the proportion of dying cells between rostral cells cultured alone or together with E17 caudal cells. The difference in the survival of E16 rostral and E16 caudal cells as observed in earlier experiments still remained strikingly obvious. The apparent ratio of dead cells in E17 caudal cells seemed not to be affected by co-culturing with E16 rostral cells.

E19 rostral cells cultured in the presence of E17 caudal cells. E19 rostral cells were cultured in the presence of equal density E17 caudal cells. E17 caudal cultures were prepared in the morning from one pregnancy, and on the same day in the afternoon, E19 embryos were obtained to prepared E19 rostral cortical cultures. Cultures were examined each day. E19 rostral cells in co-cultures were compared to rostral cells cultured separately but obtained from the same embryonic age group. There were no apparent differences between the proportion of dying cells between rostral cells cultured alone or together with E17 caudal cells. The difference in the survival of E19 rostral and E19 caudal cells as observed in earlier experiments still remained strikingly obvious. The apparent ratio of dead cells in E17 caudal cells seemed not to be affected by co-culturing with E19 rostral cells.

To test the hypothesis that E17 caudal cells were rescuing E17 rostral cells from cell death via a diffusible signal, medium presumably containing the survival signal, was aspirated from caudal cells. The aspirated medium was subsequently used to treat E17 rostral cells.

Conditioned medium

Frozen caudal conditioned medium

Caudal cells were prepared from E17 rat cortices and cultured for 4 days. In a number of experiments the conditioned medium was withdrawn from caudal cells using a 1000 µl pipette and straightway (without the addition of protease enzyme inhibitors) stored in microcentrifuge tubes at –20°C. Fresh rostral cells were prepared from E17 brains and plated as described in the Methods section onto coverslips in 24 well plates. Pre-warmed caudal conditioned medium was added to freshly prepared rostral cells once they had settled onto the coverslips after plating (30-40 min). Rostral controls were also prepared which were incubated in 5% HIFCS and subsequently in 1% HIFCS. Fresh caudal controls were also prepared ensure that the differential cell survival was still present in the fresh cultures to (internal controls). The caudal cells from the first experiment (from which some of the conditioned medium was aspirated) were further cultured to monitor any change in their survival.

The experiment was performed only twice. In each case, the conditioned medium failed to change the survival of rostral cells examined at 3 DIV. That is in the presence of the conditioned medium obtained from caudal cells newly prepared rostral cells continued to die, with almost 100% of cells dead by 3 DIV, the time at which rostral untreated cultures had completely degenerated. Caudal cells prepared at the

same time as fresh rostral cultures continued to survive as expected. Caudal cells from which the medium was aspirated continued to proliferate and survive as expected.

Fresh caudal conditioned medium

Caudal cultures were prepared in from E17 rat neocortices. Caudal cells were cultured for 4 days. On the 4th day, fresh rostral cultures were prepared from another pregnant dame. Once the rostral cells had adhered to the substrate on the coverslip (30 min) the media from caudal cells was aspirated and added to the freshly prepared rostral cultures. Control rostral cultures were also prepared which were incubated in control culture medium. The survival of cells in control and caudal conditioned medium treated rostral cultures were compared at 3 DIV. No difference was observed in the survival. There was no cell survival in treated and untreated rostral cultures. Caudal cells prepared from the later pregnancy continued to survive and proliferate. All observations were made with the inverted microscope on unfixed material.

Summary 6

When E17 or E16 rostral cells were co-cultured in the presence of their respective caudal sister cells on the same coverslip, a significant reduction in the percentage of dead cells was seen. The control rostral cells (cultured alone) had completely degenerated by 3 DIV. Further, rostral cells co-cultured on separate coverslips but in the same environment as caudal sister cultures derived from the same age demonstrated a significant reduction in cell death and an increase in survival at 3 DIV. Caudal cells derived from E17 rat foetuses failed to maintain the survival of rostral cells prepared from E16 or E19 neocortices. The transferring of medium alone from caudal cells after 4 day conditioning failed to rescue E17 rostral cells.

In order to investigate what known isolated factors could promote the survival of rostral cells, and thus mimic the effects of caudal cells, a variety of neurotrophic factors and growth factors were applied to E17 rostral cells.

3.1.6. Treatment of E17 rostral cells with growth factors and neurotrophins

Rostral cells prepared from E17 rat brains were plated and incubated in 5% HIFCS. In addition to serum, cultures received differing concentrations of a variety of known growth factors and neurotrophins in an attempt to investigate factors which may promote the survival of rostral cells. Cultures were treated with the following factors added to the medium and replenished the subsequent day; NGF, BDNF, NT-3, bFGF and EGF (for concentrations, see methods). Cultures were fixed at 3 DIV and compared to untreated control rostral cultures (Fig. 18A).

All factors tested failed to rescue rostral cells from undergoing cell death when examined at 3 DIV, except bFGF and BDNF. The survival effects of bFGF were greater than those seen by BDNF. Only 23.7% cells had died in rostral cultures treated with bFGF (Fig. 18B) compared to near 100% death in NGF, NT-3 and EGF treated cultures. BDNF rescued an average of 13% of cells from cell death.

Previous experiments revealed that bFGF had the most profound effect on the survival of rostral cells derived from E17 cortices. The following experiment was employed to test whether rostral cells responded in a dose dependant manner to this growth factor.

Basic FGF demonstrates a concentration dependent cell survival effect

Cells isolated from rostral regions of the E17 cerebral cortex were treated with increasing concentrations of bFGF and assayed using the MTT cell survival assay at 1 DIV, 3 DIV and 5 DIV. Rostral cell survival was significantly promoted by bFGF when cultures were assayed at 3 and 5 DIV. Rostral cell survival was significantly affected by bFGF even after 1 day in culture (Fig. 19). Higher concentrations than 1 ng/ml bFGF an approximately 33% increase in survival at 1 DIV. Concentration of 10 ng/ml of bFGF appeared to have the greatest effect on cell survival, with an approximate 9 fold increase on the survival after 3 days.

Caudal cells exposed to bFGF, too, increased in survival and apparently proliferation. Caudal cells were not assayed.

Basic FGF often acts as a mitogen. Here the notion that bFGF might be promoting the proliferation of cells in rostral cultures and thereby effectively promoting their survival was tested by assaying BrdU uptake amongst bFGF treated cultures.

Proliferation in rostral cortical cultures treated with bFGF

The rate of proliferation was assessed using the thymidine analogue BrdU. Cultures of rostral and caudal cells were prepared from E17 cortices. Cultures of rostral regions were treated with 10 ng/ml of bFGF. BrdU pulses were administered at 1 and 3 DIV , 1 hr prior to fixation in 4% PFA (Fig. 18B). Immunocytochemistry was employed to detect cells that had incorporated BrdU. The percentage of BrdU immunopositive cells in cultures at 1 day was approximately 16.1% in rostral cultures,

14.4% in caudal cultures and 23.4% in rostral cultures treated with bFGF ($P<0.05$). At 3 DIV near 100% of rostral cultures had died. The BrdU immunoreactive population in caudal cells was reduced to 5.5%. A decrease was also observed in the proliferative population in rostral cells treated with bFGF (19.6%).

Summary 7

Rostral cells derived from E17 neocortices were treated with a variety of neurotrophins and growth factors to determine which factors could promote the survival of rostral cells. Neurotrophins, NT-3, NGF, BDNF, and growth factors, bFGF and EGF were used to treat cultures of rostral cells on the day of plating. Basic FGF was the only factor observed to significantly reduce the proportion of dead cells and promote the survival when examined at 3 DIV. For all other factors, no difference between the rostral untreated and rostral treated cell groups. Furthermore, rostral cells were rescued from cell death by bFGF in a dose dependant manner, with increasing concentrations of bFGF increasing the fraction of surviving cells over the time period examined (1, 3 and 5 DIV).

The rate of proliferation of rostral untreated cultures in comparison to rostral cells treated with bFGF (10 ng/ml) was increased. A mild increase in the proportion of cells undergoing division was noted at 1 and 3 DIV.

Caudal cells in previous experiments were noted to survive for longer periods than rostral sister cultures. Further, caudal cells were able to continue to proliferate in culture. In order to investigate whether proliferation was essential for the survival of caudal cells, caudal cells were exposed to a known inhibitor of proliferation, AraC.

Caudal cell treated with AraC

Cytosine arabinoside is a cell proliferation inhibitor. In order to investigate whether cell survival was independent of cell proliferation, AraC was added to caudal cultures derived from E17 rat cerebral cortices. To determine the optimal concentration, increasing concentrations of AraC were added to caudal cultures and cell survival was assayed using the MTT assay at 1 and 3 DIV. Increasing concentrations above $1x10^{-4}$ mg/ml of AraC resulted in all cells dying 1 day after administration. Concentrations between $1x10^{-6}$ and $1x10^{-5}$ resulted in 18% and 27% cell loss, respectively (Fig. 20). The cell loss was concluded not to be a result of toxicity of the reagent since cells were seen to survive till 3 DIV. However, inhibition of the cell cycle increased the susceptibility of the cells to cell death. AraC was used at a concentration of $1x10^{-4}$ mg/ml in future experiments. The readings seen for DMSO indicate the level in which all cells had died.

In an attempt to test whether the effects of bFGF on the survival of rostral cells was dependent or independent of its mitogenic effects, caudal cells were treated with a combination of cell cycle inhibitor and bFGF.

Caudal cells treated with AraC and bFGF

Elsewhere, caudal cells have been shown to rescue rostral cells by secreting a survival factor. As rostral cells responded well in terms of survival to bFGF, the hypothesis that caudal cells maybe producing and secreting bFGF was tested. It is proposed that bFGF maintains the survival of cells of its source (caudal cells) and 'inadvertently' promotes the survival of other bFGF responsive cells that happen to be in their vicinity. This was tested by utilising a pharmacological inhibitor (genistein) of the receptor tyrosine kinase pathway, the pathway by which bFGF acts in caudal cells.

Caudal cells treated with genistein

Caudal cells derived from E17 embryonic cortices were treated with a range of concentrations of genistein, a tyrosine kinase receptor blocker. Estimates of the optimal concentration were made by observing cells in culture following treatment. Concentrations used were 5 mM (1:10 dilution), 0.5 mM (1:100 dilution), and 50 μM(1:1000 dilution) of genistein. Stock genistein was prepared in DMSO and stored at –20°C. Caudal cells treated with 50 μM genistein showed no significant difference in cell death at 1 DIV compared to untreated caudal cultures. 50 μM was thereafter used as the experimental concentration of genistein.

Treated cultures of caudal cells were compared to untreated caudal cells at 1, 2 and 3 DIV (Fig. 21). The ratio of dead to living cells was approximately equal at 1 DIV in treated and untreated caudal cultures (12.3% and 13.7%). Two days following

treatment with genistein, caudal cell death had almost doubled to 22.2%. Cell death in untreated controls remained approximately the same, 11.3%. At 3 DIV, genistein treated cultures had nearly all (96.5%) undergone cell death whilst the caudal untreated control cultures had very low fraction of dying cells (19.5%). Thus, genistein treatment resulted in caudal cells undergoing cell death. Caudal cells were subsequently treated with bFGF (10 ng/ml) in addition to 50 μM genistein. Basic FGF appeared not to exert its effects on caudal cells (data not shown). That is, as demonstrated in earlier experiments, bFGF promoted the survival and proliferation of caudal cells, however, in the presence of genistein, the growth factor failed to promote the survival/proliferation of caudal cells. In the presence of genistein and bFGF, nearly all caudal cells underwent cell death by 3 DIV.

In one experiment in which E17 rostral cells had been treated with genistein, the loss of cells was more acute compared to rostral untreated cultures.

Summary 8

E17 caudal cells were treated on the day of plating with a pharmacological agent known to inhibit the proliferation of cells. Caudal cells treated with AraC underwent a significantly greater degree of cell death in the presence of this inhibitor when compared to untreated cultures, revealing that proliferation was to a degree important in their survival.

Caudal cells obtained from E17 neocortices were exposed to genistein, an inhibitor of receptor tyrosine kinases. A variety of concentrations were tested in order to determine the optimum concentration for use in culture. A concentration of 50 μM genistein added to caudal cultures. The percentage of dead cells was determined in caudal cultures in the presence and absence of the inhibitor. Caudal cells continued to

survive well after exposure for 2 days. However, by 3 DIV the vast majority of caudal cells underwent cell death in the presence of the inhibitor, whilst untreated caudal cells continued to survive and proliferate. Basic FGF failed to rescue caudal cells treated with genistein.

As genistein treatment of caudal cells successfully resulted in increased cell death amongst caudal cells, the presence and abundance of bFGF and FGFR1 were tested in the subsequent experiment.

3.2. Basic FGF and FGF receptor 1 immunoreactivity in rostral and caudal cultures

E17 rostral and caudal cells were cultured for one day and subsequently fixed in 4% PFA for 20-30 min. Basic FGF and FGFR1 were detected using immunocytochemical methods with mouse (monoclonal, Ab 118) and rabbit (polyclonal) antibodies respectively. As described in the methods, both the growth factor and receptor were detected by the avidin-biotin-horseradish peroxidase Vectastain kit, and visualised by the peroxidase substrate diaminobenzidine (DAB). The appearance of the immunoreactivity was described elsewhere. Here the finding of the fraction of immunopositive cells was presented.

Counts of the number of cells immunoreactive for bFGF were made on cultures fixed 1 day after plating in both rostral and caudal regions (Figs. 22 and 23). Of the rostral cells examined, 59.1% contained bFGF whilst 63.9% caudal cells were immunoreactive for bFGF. Although this difference was small ($p<0.05$).

Immunodetection of FGFR1 revealed that 51.5% of rostral cells possessed the receptor. Significantly, more caudal cells presented with FGFR1 receptor (60.8%, $p<0.05$).

In an attempt to co-localise bFGF immunoreactive population with those possessing the receptor, double immunocytochemistry was performed. No conclusive results were obtained as staining intensity was compromised by longer incubation times and reduction in the amplification steps as fluorochromes were used to detect proteins.

To investigate other potential differences that might explain differential survival of rostral and caudal cells, immunostaining for different cell types was carried out on acute preparations of rostral and caudal cells primarily at E17 and E19. In addition, we were aiming to determine which of the cell types were most vulnerable to cell death.

3.2.1 Cell types present in rostral and caudal cultures

Cell types present in rostral and caudal cells isolated from E17 neocortices

The cell types present in rostral and caudal cultures at 1 and 3 days after plating were analysed using antibodies raised to cell specific markers. The avidin-biotin conjugated to HRP was used as the immunodetection system in these cultures. Counts were made of immunoreactive cells against total live cell population present in the culture at each time point examined. Results from cultures prepared at 1 day were presented. Cultures grown for 3 days demonstrated a greater degree of variability since so few cells were present in rostral cells at this time.

Nestin immunoreactivity in E17 rostral and caudal cultures

Cells were immunostained for nestin, a marker of intermediate filament often associated with undifferentiated neuroepithelial cells. Comparing nestin immunoreactivity in E17 rostral cells to E17 caudal cells, rostral cultures had have fewer nestin labelled cells (44.1%) than caudal sister cultures which contained an approximate 50.2% nestin immunolabelled cells. This difference was not statistically significant. Nestin immunoreactive cells were the greatest proportion of cells examined in rostral and caudal cultures at E17 (Figs. 24A and 25).

MAP-2 immunoreactivity in E17 rostral and caudal cultures

Rostral and caudal cultures were prepared from E17 neocortex and cultured for 1 day in 5% HIFCS. Cultures were fixed in 4% PFA and processed for MAP-2 immunoreactivity. MAP-2, a microtubule associated protein was more often found in more differentiated neurons.

Counts to estimate proportions of MAP-2 labelled neurons were made in rostral and caudal cultures at 1 DIV. Of all cells present in rostral cultures, 44% showed MAP-2 immunoreactivity; the proportion of caudal cells containing MAP-2 was lower, 35.4%. The MAP-2 immunoreactive population was the second largest group in cultures of E17 neocortices (figs 26A and 27).

GABA immunoreactivity in E17 rostral and caudal cultures

Rostral and caudal cells isolated from E17 cortices were cultures for 1 day and fixed in 4% PFA. Using an antibody raised to GABA, a neurotransmitter utilised by inhibitory interneurons of the adult cortex, was used to analyse the differentiation of

neurons in cultures of rostral and caudal cortex. GABA immunoreactivity was detected using the ABC-HRP method.

A very small proportion of all cells in rostral and caudal cultures were detected with antibodies to GABA (Figs. 24B and 28). Interestingly, few (4.2%) rostral cells appeared to present GABA immunoreactivity, whilst significantly more GABA immunopositive cells were detected in caudal cultures (8.1%), almost double that seen in rostral cultures.

Only 9.5% of all neurons present in rostral cultures appeared to contain GABA, whilst 22.9% of neurons in caudal cultures were immunoreactive to GABA. This was assuming that all GABA cells were neurons.

GFAP immunoreactivity in E17 rostral and caudal cultures

Cultures obtained from rostral and caudal regions of E17 cortex fixed after 1 day in culture were examined for their glial population using a marker against GFAP. No cells were observed to be immunoreactive for GFAP in rostral cultures. Very few GFAP immunolabelled cells were seen in cultures prepared form caudal cortex, 0.7% (Fig. 26B).

Birth of neurons in rostral and caudal regions at E16

The proportion of neurons born at E16 was determined in an experiment in which a peritoneal injection of BrdU was made in a pregnant dame at 16 days gestation. The mother was sacrificed 24 hr later and rostral and caudal regions of the cerebral wall were obtained. Dissociated cultures were prepared and incubated for a few hours. Cells were fixed in 4% PFA and processed for BrdU immunocytochemistry. BrdU immunoreactive cells were counted and ratios of

labelled cells to unlabelled cells were determined in both rostral and caudal regions (Fig. 29). Rostral cells contained 46.1% BrdU labelled cells whilst caudal derived cells contained fewer BrdU immunoreactive cells, 40.5% ($p<0.05$).

3.2.2. Development of E17 rostral and caudal cells *in vitro*

Differentiation of MAP-2 neurons in rostral and caudal cultures

The generation and differentiation of neurons was examined in E17 rostral and caudal cultures over 3 days *in vitro*. Relative proportions of MAP-2 immunolabelled cells were estimated in cultures at 1, 2 and 3 DIV. 44% of rostral cells were labelled with MAP-2, whilst 35.4% of MAP-2 immunoreactive cells were observed in caudal cell groups. No real change in the fraction of MAP-2 immunoreactive cells was seen 2 DIV (44%). However, at 2 DIV, the percentage of caudal neurons occupying the culture had reduced to 24.2%. By 3 DIV, no rostral cells remained alive, and thus no MAP-2 immunoreactivity was detected (Fig. 26A).

Development of nestin immunoreactive cells in E17 rostral and caudal cultures

The changes in nestin immunoreactivity were monitored in cultures prepared from E17 rostral and caudal regions of the developing cerebral cortex. Of the cells in rostral cultures, 44.1% were immunostained for nestin, 50.2% of cells in caudal cultures were immunoreactive for nestin 1 day after plating. Nestin immunolabelled cell significantly reduced over the culture period examined. At 2 DIV, approximately 18.7% of cell in both rostral and caudal cultures contained the intermediate filament nestin, and almost 3 fold decrease. Nestin immunopositive cells continued to be

detected in caudal cultures at 3 DIV. A 100% cell loss in rostral cultures at this time point meant that no nestin immunoreactivity was observed (Fig. 24A).

Development of GABA immunoreactivity in E17 rostral and caudal cultures

GABA immunoreactivity was examine over 3 days in cultures of E17 rostral and caudal cortical cells. A marginal increase in the development of GABA immunoreactivity was observed in caudal cortical cell preparations from 8.1% GABA labelling at 1 DIV, to 9.9% and 11.3% at 2 and 3 DIV respectively (Fig. 24B).

Development of GFAP immunoreactivity in E17 rostral and caudal cultures

E17 rostral and caudal cell development was monitored in terms of differentiation of GFAP immunoreactive populations over 3 days in culture. No GFAP positive cells were detected at any time in rostral cultures. Very few GFAP immunolabelled cells were detected in caudal cultures (Fig. 26B); less than 1.2% cells were detected at 2 DIV. Even fewer cells were detected in caudal cultures at 3 DIV (0.51%).

Summary 9

Cortical cultures were prepared from rostral and caudal regions of the E17 developing cerebral cortex. Cells were fixed at 1 DIV and processed for immunocytochemistry for cell specific markers to which antibodies were available. Visualised by ABC-HRP, counts of immunopositive cells were compared to total cell population. Immunolabelling for nestin, and intermediate filament found in undifferentiated neuroepithelial cells, MAP-2, a protein found in mature neurons, GABA, an inhibitory neurotransmitter found in interneurons of the adult brain, and

GFAP, a protein primarily associated with astroglial cells were the markers used to identify subpopulations of cells. All cells were found to be present in rostral and caudal cultures, except GFAP immunolabelled cells which were absent in all rostral cultures. No significant differences were found in nestin immunoreactivity between rostral and caudal cells groups. The largest population of cells present in cortical cultures from both regions was nestin positive. A small difference was found between the percentage of neurons in rostral and caudal cultures, with the latter having few differentiated neurons. A relatively high fraction of cells in culture were MAP-2 positive. Of the differentiated neurons, some cells expressed GABA. This population was very small in both rostral and caudal groups. However, caudal cells demonstrated 2 fold greater GABA immunoreactivity than those in sister rostral cultures.

BrdU injections were made at E16 to determine differences in the generation of neurons in rostral and caudal regions by culturing cells for hours 24 hr after the injection. Less than 50% neurons were born at E16 and observations revealed that slightly more neurons were born in rostral regions than caudal regions, consistent with *in vivo* evidence for neuronal generation.

3.2.3. Cell types present in rostral and caudal cells isolated from E19 neocortices

The cell types present in rostral and caudal cultures 20 hr after plating were analysed using antibodies raised to cell specific markers. The ABC method was used as the immunodetection system in these cultures. Counts were made of immunoreactive cells against total live cell population present in the culture at each time point examined.

Nestin immunoreactivity in E19 rostral and caudal cultures

Cells were immunostained for nestin. Comparing nestin immunoreactivity in E19 rostral cells to E19 caudal cells, rostral cultures appeared to have more nestin labelled cells (33.5%) than caudal sister cultures which contained approximately 27.7% nestin immunolabelled cells. Nestin immunoreactive cells were no longer the largest population of cells present at this age (Fig. 30B).

Vimentin immunoreactivity in E19 rostral and caudal cultures

Vimentin was used as an alternative to nestin to determine the fraction of undifferentiated cell types in rostral and caudal cultures derived from E19 rat neocortices. Vimentin was present in undifferentiated cell types. Estimations of ratios of vimentin immunoreactive cells to total cell population revealed roughly equal proportions of vimentin immunolabelled cells in rostral and caudal cultures (50.5% and 51.4% respectively). Vimentin immunolabelled cells exceeded nestin immunolabelled cells suggesting that the two markers were present to some degree in different cells (Figs. 3B and 31).

MAP-2 immunoreactivity in E19 rostral and caudal cultures

Immunolabelling of rostral and caudal cultures of E19 cerebral cortices for MAP-2 were carried out on cells fixed after 20 hr incubation. A high proportion of E19 rostral and caudal cells were immunoreactive for MAP-2, revealing that the majority of cells in E19 cultures were indeed neurons, as might be expected at this age (Figs. 30B and 19). A marginal difference in the proportions of MAP-2 immunopositive cells was observed between the two cell groups, with rostral cells containing fractionally more neurons (66.2%) than caudal sister cells (63%).

Glutamate immunoreactivity in E19 rostral and caudal cultures

Cultures of E19 rostral and caudal cortical cells were examined with an antibody raised to glutamate, the neurotransmitter utilised by pyramidal cells of the cerebral cortex, to examine the differentiation of neurons *in vitro*. Glutamatergic cells made up a very large population of cells in both rostral and caudal cultures examined at 20 hr *in vitro* (Figs. 30B and 32). 56.4% of all cells present in rostral cultures contained glutamate, and 52.8% of all cells present in caudal cultures were immunoreactive for glutamate. Calculated, 85.2% of all neurons derived from rostral regions of the cortex (MAP-2 immunoreactive cells) contained glutamate, and 83.8% of caudal derived neurons were immunolabelled with glutamate. These proportions reflect immunoreactivity *in vivo*.

GABA immunoreactivity in E19 rostral and caudal cultures

GABA immunoreactivity was examined in rostral and caudal cell cultures derived from E19 embryonic rat cerebral cortices. The fraction of GABA immunoreactive population was small (Figs. 30B and 32). In rostral cell cultures, 2.3% of the cells were GABA positive, whilst caudal cells GABA positive cells made up 5.8% of all cells present. Thus 3.5% of all neurons (MAP-2 immunopositive cells) were GABAergic and 9.2% caudal neurons contained GABA.

GFAP immunoreactivity in E19 rostral and caudal cultures

Very few cells were positive for GFAP in either rostral or caudal cultures derived from E19 cortices 20 hr *in vitro*. No GFAP positive cells were detected in rostral cultures, and only 1% of GFAP immunolabelled cells were present in caudal cultures (Fig. 30B).

Summary 10

Cell types present in E19 rostral and caudal cultures were determined using antibodies raised to cell specific markers in cultures fixed 20 hr post plating. Cell types investigated were undifferentiated neuroepithelial cells (nestin or vimentin antibody), neurons (MAP-2 antibody), GABAergic neurons (GABA antibody), glutamatergic neurons (glutamate antibody) and astroglial cells (GFAP antibody). All cell types were present in cultures of both regions, except GFAP which was only seen in a few cells in caudal derived cortical cells. The greatest population of cells were neurons (MAP-2 immunoreactive cells), the majority of which expressed glutamate. Although rostral cells appeared to have more neurons, the difference was small. Very few GABAergic cells were present in culture, with rostral cells containing fewer GABA immunoreactive cells than caudal. The proportion of nestin and vimentin immunopositive cells was lower than all other cell types examined. Relatively equal proportions of vimentin cells were observed in rostral and caudal cultures whereas more nestin immunolabelled cells were found in rostral cultures compared to caudal cultures.

Nestin immunoreactivity in rostral and caudal cells prepared from different ages

Nestin immunoreactivity was examined at 1 DIV in rostral and caudal cortical cultures prepared from different embryonic ages, E15, E16, E17 and E19 (Fig. 33A). Nestin immunopositive cells made up the greatest fraction in E15 rostral and caudal cultures. 72.7% of E15 rostral cells contain nestin intermediate filament, and 75.5% of caudal cells were immunolabelled for nestin. The difference between rostral and caudal cells at E15 reflected to greater or lesser extent the difference expected when considering the rostro-caudal gradient of development of the cerebral cortex. With

growth and maturation of the cerebral cortex, cells derived from older embryos were observed to reduce their nestin immunoreactive populations, also closely resembling changes occurring during development *in vivo*. A 25.2% decrease in the population of cells immunopositive for nestin was seen in rostral cultures prepared from E16 neocortices, whilst a 33% reduction was seen in caudal cortical cultures resulting in net 54.4% and 50.6% of nestin immunopositive cells in E16 rostral and caudal cultures at 1 DIV.

Culturing of rostral and caudal cells from E17 cerebral cortices and subsequent nestin immunolabelling revealed a further reduction in cells containing nestin. Of all cells in rostral and caudal cultures, 44.1% and 50.2% respectively contained nestin, a reduction of 18.9% and 0.7% in the two respective regions from embryonic cultures derived from animals 1 day younger. Nestin immunoreactivity was lowest in E19 rostral and caudal cortical cultures. 33.5% of rostral cells contained nestin and 27.7% caudal cells labelled for nestin. Thus, a further decrease of 24% nestin immunoreactivity in rostral cultures compared to nestin labelling at E17 and even greater reduction in caudal cultures (44.9%).

MAP-2 immunoreactivity in rostral and caudal cells prepared from different ages

MAP-2 immunoreactive populations were estimated in rostral and caudal cortical cultures derived from different ages, E16, E17 and E19 (Fig. 33B). The fraction of neurons increased with age in both rostral and caudal cultures. The increase in MAP-2 positive cells was marginally greater in rostral cells than caudal with increasing age. In E16 derived cortical cultures, 21.9% and 22.8% of total population of cells were observed as MAP-2 immunopositive in rostral and caudal cortical cultures respectively. At E17, a 2 fold increase to 44% of MAP-2 positive

cells was observed. A smaller but significant increase (1.6 fold) in neurons was seen also in caudal cells compared to caudal cells staining positive for MAP-2 at E16, averaging to 35.4% MAP-2 positive cells. An approximate 20% difference in MAP-2 immunoreactive cells between rostral and caudal cells.

By E19, a massive increase in the proportion of MAP-2 labelled cells was seen relative to E17 cultures. Over 50% more neurons were being produced in E19 rostral cortical cultures compared to E17 rostral cultures. Furthermore, a 78.3% increase in cortical neurons in caudal regional cultures was observed compared to E19 caudal cultures. Only a very small difference in the relative percentages of neurons was observed in E19 rostral and caudal cultures.

Summary 11

The neuronal and neuroepithelial (MAP-2 and nestin immunoreactive) cells were compared in rostral and caudal cultures prepared from different ages, E15, E16, E17 and E19. The general trend observed was that with increasing age of embryos from which cultures were derived. There was an increase in the differentiation of neurons as seen by an increase in percentage MAP-2 labelling. At E16 and E19, the relative proportions of neurons were approximately the same in rostral and caudal cultures. A significant difference in the neuronal population in rostral and caudal cultures of E17 cortices was observed, with the former containing more neuronal cells than the latter. The differentiation *in vitro* over the different ages examined closely reflects the trend in differentiation seen *in vivo*.

Nestin immunoreactive cells decreased with increasing age. No large differences in the populations of nestin immunoreactive cells in rostral and caudal cells was observed in the different ages examined. E15 cortical cultures contained the

greatest proportion of undifferentiated cells types as seen by nestin immunolabelling. A graded reduction in the nestin positive population was seen with increasing age of embryos. This trend too reflected changes seen *in vivo* as neurogenesis proceeded.

A great deal of direct and indirect evidence from the above experiments implicates bFGF as the survival factor that maintains the differential survival of cells derived from the two regions examined. Homozygous bFGF -/- mouse embryos were used in the subsequent part of the study to determine if caudal cells did indeed produce bFGF. To test whether bFGF maintained caudal cell survival in an autocrine fashion rostral and caudal cultures were set up from mutant mice and cultures examined.

3.3. E14.5 basic FGF-/- bFGF mouse cultures

Rostral and caudal cultures were prepared from E14.5 (equivalent of E16.5 rat) mouse cerebral cortices as described elsewhere. Cells were plated on lam/PLL coated glass coverslips and incubated in 5% HIFCS cultures medium for 4 days. Cultures from each group were fixed at 1-4 DIV inclusive. Cells were stained with PI and dead cell counts made cultures from each region for each time point.

The proportion of dead cells in rostral bFGF-/- cortical cultures was relatively low over the first 3 days in culture (Fig. 34A). At 1 DIV, 6.1% cells were dead or dying and, at 2 DIV, 9.6% cells had undergone cell death. By the third day in culture, dying rostral cells had more than doubled (25.6%). However, 4 days after plating, an average of 86.1% cells had died reflecting closely what was observed in rat cultures from the equivalent age (Fig. 34A).

The percentage of cell death in caudal cultures was low to begin with and increased at a slow rate over the period examined. At 1 DIV, 11.2% of cells had died, 12.6% had died by 2 days, 12.1% had died by 3 days and, by 4 DIV, 29.1% of caudal cells had degenerated.

Comparing the survival in the two, shows that there was a difference; cells in the caudal survive for longer periods than cells derived from rostral regions of the embryonic cortex. This was not expected in the homozygous mutant mice. Caudal cells were expected to degenerate at approximately the same rate as rostral sister cultures.

E14.5 wild type (+/+) mouse rostral and caudal cortical cultures

Rostral and caudal cultures were prepared from E14.5 from wildtype mouse neocortices. Cells were plated as described and cultured in the same conditions as mutant mouse cultures. Rostral and caudal cultures were fixed at 1-4 DIV inclusive and stained for dead cells using PI.

The proportion of dead and dying cells was low in rostral (11.2%) and caudal cultures (6.4%) one day following plating. Note that almost 50% less cells die in caudal cultures compared to rostral cultures (Fig. 34A). The percentage of dead cells increased marginally in both rostral and caudal cultures when examined at 2 DIV (12.6% and 8.9% respectively). A significant increase (75%) in the fraction of dead cells was observed at 3 DIV amongst rostral cell groups with 22.1% cells having undergone cell death since day 2 *in vitro*. An approximate 50% increase in death of caudal cells was observed at 3 DIV giving a total of 16.4% dead cells in these cultures. The difference at this time point in the proportion of dying cells in rostral and caudal cultures was not considered to be significant (due to variation amongst

coverslips). Strikingly, however, by 4 DIV approximately 81% if rostral and 100% of caudal cells had undergone cell death.

The results from this experiment did not to follow the trend in differential survival of rostral and caudal cells observed in rat cultures prepared from equivalent aged embryonic cortices.

Comparison of E14.5 +/+ and bFGF-/- mouse rostral and caudal cultures

Wildtype (WT) mouse cultures were used as controls for examining changes in cell death amongst rostral and caudal cell cultures in bFGF homozygous mutant mice. Mutant rostral cells followed a similar trend with respect to the ratios of dying cells, as those observed in WT mouse cultures. Although at the outset and early period of the culture (1 and 2 DIV) WT rostral cultures had marginally greater proportions of dead cell by 3 DIV this difference was no longer apparent, both WT and mutant strains of mouse rostral cultures began with low levels of cell death but later in the culture period nearly all cells degenerated. This trend closely resembled embryonic rat cultures prepared in the same way.

Mutant caudal cells appeared to give contrasting and unpredicted results. Early in the culture period, low levels of cell death occurred in both mutant and WT caudal cultures (less than 20% dead cells,) by the 4^{th} day in culture all WT caudal cells had degenerated whilst only 29.1% cells had died in mutant caudal cortical cultures. The reverse was expected, that mutant caudal cells would all degenerate due to the lack of bFGF and that WT caudal cells continued to survive and proliferate.

E15.5 basic FGF homozygous for loss of function (-/-) bFGF mouse cultures

Rostral and caudal cultures of E15.5 bFGF homozygous for loss of function mouse neocortices were prepared and plated as described in the Methods section. Cells were fixed at 1-4 days inclusive and stained with PI to examine dead cells in cultures.

The proportion of dead cells in rostral bFGF-/- cortical cultures were relatively low over the first 2 days in culture. At 1 DIV 4.6% cells were dead or dying and at 2 DIV 14.4% cells had undergone cell death. By the 3rd day in culture, dying rostral cells had more than doubled (35.7%). By 4 days, all the cells had died (Fig. 34B). The percentage of cell death in caudal cultures was low to begin with and increased at a slow rate over the period examined. At 1 DIV, 4.6% of cells had died, 4.7% had died by 2 days, 19.1% had died by 3 days, and by 4 DIV, 100% cells had died.

Comparing the survival in the two regions showed that there was no difference in caudal and rostral -/- mouse cultures at 1 DIV. A marginal difference arises by 2 DIV with more cell death occurred in rostral than caudal cells. By 3 days, there was a difference between rostral and caudal cell death, but as predicted, no difference in the rostral and caudal cell death occurred by 4 days (Fig. 34B).

E15.5 wild type (+/+) mouse rostral and caudal cortical cultures

Rostral and caudal cultures were prepared from E15.5 WT mouse neocortices. Cells were plated as described earlier and cultured in the same conditions as mutant mouse cultures. Rostral and caudal cultures were fixed at 1-4 DIV inclusive and stained for dead cells using PI.

The proportion of dead and dying cells was low in rostral (9.8%) and caudal cultures (5.8%) one day following plating. The percentage of dead cells increased

dramatically in rostral cultures when examined at 2 DIV with all cells undergoing cell death (100%). Less than a 3 fold increase in the levels of cell death occurred in caudal cells (14.8%). A significant increase (75%) in the fraction of dead cells was observed at 3 DIV amongst rostral cell groups with 22.1% cells having undergone cell death. By 3 days in cultures, there was a 0% cell survival in caudal cell cultures.

The results from this experiment did not follow the trend in differential survival of rostral and caudal cells observed in rat cultures prepared from equivalent aged rat embryonic cortices.

Comparison of E14.5 +/+ and bFGF-/- mouse rostral and caudal cultures

At the outset (1 DIV) of the culture, WT rostral cultures had marginally greater proportions of dead cell by 2 DIV, death in WT rostral cells had far exceeded that seen in mutant rostral cells.

Early in the culture period low levels of cell death occurred in both mutant and WT caudal cultures, (less than 20% dead cells) but, by the 3rd day in culture, all WT caudal cells had degenerated, whilst only 19.1% cells had died in mutant caudal cortical cultures. The reverse was expected, that mutant caudal cells would all degenerate due to the lack of bFGF and that WT caudal cells would continue to survive and proliferate. However, by the 4th day, all mutant caudal cells had degenerated.

Summary 12

Rostral and caudal cortices were obtained from bFGF -/- transgenic E14.5 and E15.5 mice. Cultures of WT embryonic cortices were also prepared and used as

controls to compare changes in the ratio of cell death in rostral and caudal cultures in mutant mice.

E14.5 transgenic (bFGF-/-) mouse cultures had low levels of cell death occurring early in the culture period, but by the 4th day all mutant rostral cells had degenerated whist the majority of mutant caudal cells survived. WT rostral cells also had low levels of cell death occurring during the early period in culture, but complete cell loss was observed by the 4th day in culture. Thus, mutant rostral cells followed the trend of WT rostral mouse cultures and rat cultures prepared from the equivalent embryonic age. However, WT mouse caudal cells behaved differently to rat caudal cell cultures and mutant caudal cells. By the 4th day, 100% cell loss was experienced in WT caudal cells.

Comparable results were obtained from E15.5 mouse cultures. However, WT rostral cell death occurred sooner and to a greater extent with 100% cell loss by 2 DIV. Mutant rostral cell death increased over time with 100% cell death occurring by 4 DIV. Unexpectedly, all WT caudal cells underwent cell death by 3 DIV followed by complete cell loss of mutant caudal cells by 4 DIV.

In order to reproduce the differential survival/cell death observed in cells derived from rostral and caudal regions of the developing cortex, experiments were designed to remove the presence of caudal cells in vivo to observe the effects on cell death in vivo of rostrally placed cells. By creating lesions in newborn rat pups in rostral or caudal regions of the cortex, TUNEL labelling was employed to detect changed in cell death in various other regions of the brain.

A preliminary examination of results obtained from such experiments reveals that lesions in occipital cortex of newborn rats results in increased cell death in frontal cortices when examined with TUNEL labelling. In controls, in which frontal cortex was destroyed (by aspiration), no such cell death was observed in occipital cortex.

4.1. RESULTS II

In an effort to demonstrate the presence, localisation and pattern of distribution of basic fibroblast growth factor and its receptor, FGFR1, in E17 rat neocortex, immunohistochemistry, radioactive and non-radioactive in situ hybridisation was performed on cryosections and wax sections of brain tissue.

Immunohistochemistry on tissue sections

Basic fibroblast growth factor immunostaining in E17 cryostat sections with Ab106

Cryoprotected E17 rat brains were sectioned at 10 μm. Sagittal sections were obtained and collected on PLL coated microscope slides. Sections were then processed for immunohistochemistry for bFGF and visualised using the DAB reaction. Sections from several brains were obtained. Immersion fixed adult brains were also cryosectioned at a thickness of 10 μm and processed for bFGF immunocytochemistry.

Two different bFGF antibodies (Abs) were used in this study. Antibody 106 was kindly obtained from Dr. Anne Logan's laboratory (University of Birmingham). Western blot analysis in their laboratory indicate that antibody 106 recognises approximately 18, 21 and 23 kDa protein bands of which protein extracts were prepared from adult brain tissue (Gonzalez et al., 1995). The Ab 106 was raised against the amino-terminal peptide fragment 1-23 (PALPEDGGGAFPPGHFKDPKR) of rat bFGF. This was the sequence of the protein obtained for the peptide competition control immunostaining in tissue processed with Ab 106. Some sections of adult and embryonic brain were also counterstained with Meyer's Haemalum

(BDH) or toludine blue. The second antibody was obtained from (UBI). Western blot analysis revealed that this Ab recognised only the 18 kDa band (Fig. 35).

4.1.1. Basic FGF in E17 cortex

Basic FGF immunoreactivity was observed in embryonic (E17) para-sagittal sections of brain. Observations revealed bFGF immunoreactivity throughout the cortex (Fig. 36). Immunoreactivity was seen in presumptive neostriatum, hippocampus and particularly in the developing cerebral cortex. A subtle but quite apparent gradient was observed in the staining intensity was seen in the neocortex. Although no quantitative analysis was performed on sections, caudal areas demonstrated more intense immunostaining than more rostral areas. Examination at low power also reveals that bFGF was distributed in a layer specific manner. Cells in nearly all embryonic cortical layers were immunolabelled. Clearly, there was a graded labelling amongst cells. Labelling was, however, specific as non-immunoreactive cells were seen beside immunopositive cells. More detailed examination of immunostaining pattern revealed a distinct layering of cells containing bFGF in the neocortex. Specifically, neuroepithelial cells of the VZ were intensely stained. Cells traversing the IZ were also immunoreactive for bFGF. Intense immunoreactivity was observed in the CP. Cells in both CP and VZ were radially oriented. Cells in the MZ were also intensely labelled. Basic FGF immunolocalisation appeared to be throughout the cell cytosol and nucleus. Howeve, using the light microscope, it was not possible to determine whether processes also contained bFGF. More importantly, the overall intensity of immunoreactivity increased from rostral to caudal. No densometric analysis was performed to determine the ratio of packing density against intensity of immunolabelling.

Antibody omission control

No specific immunolabelling was observed in sections in which the primary antibody had been omitted (not shown). Incubating sections in goat anti-rabbit antibody alone did not produce any signal. In sections in which peroxidase activity had not been blocked with hydrogen peroxide, blood cell labelling was observed as a result of the endogenous peroxidases.

Pre-absorption control on E17 cryosections

A pre-absorption control was performed by incubating Ab 106 with excess peptide to which the antibody was raised as a negative control for Ab 106 immunoreactivity. Diffuse, non-specific labelling was seen. No labelling was seen specifically localised to any particular layer in the cortex, suggesting that the antibody with peptide had no binding sites available in the antiserum following pre-incubation.

Basic FGF immunoreactivity in control adult cryosections

Immunostaining for bFGF was seen in cryostat cut sections of adult tissue. Adult sections were used as positive controls for bFGF immunoreactivity. Stained cells were sparse. Basic FGF was seen to be present to a greater extent in the small rounded cells of the cortex. Many of these intensely labelled cells were arranged in pairs with no specific laminar pattern. The distribution of small rounded cells containing bFGF closely resembled that of glial cells of the cortex, the astrocytes. The small rounded cell bodies also suggest that these cells were astrocytes. Staining was largely specific to astrocytes, however some neuronal populations were seen to be immunoreactive for bFGF.

Pre-absorption control on adult cryosections

Ab 106 was pre-incubated with the peptide comprising the sequence to which the antibody it was raised to. In parallel to the positive adult control and E17 cryosections, pre-absorbed antibody was incubated on adult tissue sections. No immunolabelling was observed in any cells of the adult cortex.

Basic FGF immunostaining in E17 wax sections with Ab 106

Embryonic day 17 brains were dissected, fixed and dehydrated before embedding in wax. Sagittal sections, 6-8 μm thick, were cut and collected on PLL coated slides. Sections were allowed to dry overnight before de-waxing in histoclear. Sections were re-hydrated, washed in PBS and processed for immunocytochemistry. Ab 106 immunoreactivity was less discernible in wax sections compared to cryostat sections of the same tissue. Immunostaining was apparent in all layers of the developing cerebral cortex, but not localised to any one specific layer. Labelling was cytoplasmic rather than nuclear. No rostro-caudal gradient of immunostaining was observed in these sections.

Pre-absorption control in E17 wax sections

Ab 106 was pre-incubated with the protein comprising the peptide sequence to which it was raised before placing onto sections, for use as a negative controls. No immunolabelling was observed suggesting that the little binding seen in sections incubated with non-preabsorbed antibody was specific, although somewhat reduced. Tissue was counterstained with Meyer's Haemalum.

Basic FGF immunoreactivity in control adult cryosections

Adult brain tissue was embedded in wax. Coronal sections, 6-8 μm thick, of adult brain were cut and processed for immunohistochemistry for bFGF to use as positive controls for Ab106 (Fig. 37, 38). Intense immunolabelling was seen in some populations of cells of the adult cerebral cortex. These cells corresponded to those seen in adult cryosections. Immunopositive cells were small and rounded and distributed throughout the depths of the cortex. The distribution and size suggested that immunolabelling was largely confined to cortical astrocytes of the cortex. Neurons were also observed to be immunolabelled in some layers of the cortex. However, there was a lack of consistency amongst sections with regard to the latter. Tissue was counterstained with Meyer's Haemalum.

Pre-absorption control on adult wax sections

Ab 106 was pre-incubated with the protein comprising the peptide sequence to which it was raised before pipetting onto sections for use as a negative control. No immunolabelling was seen, demonstrating the specificity of the antibody. Tissue was counterstained with Meyer's Haemalum.

4.1.2. Immunostaining in section for FGFR1

FGFR1 immunostaining in E17 cryostat sections with anti-flg

Cryosections of E17 rat brains processed for immunolocalisation of FGFR1 and visualised using the DAB reaction. Sections from several brains were obtained. In addition to embryonic brain tissue, adult brain tissue was immersion fixed and frozen section were cut and processed for FGFR1 immunohistochemistry.

FGFR1 was present in the majority of cells in the cortex. A different intensity of FGFR1 immunolabelling was present in different laminae. The pattern of staining resembled the pattern observed in bFGF immunostained sections. VZ cells labelled intensely for FGFR1 (Fig. 39) as did neurons of the CP. Varying degrees of labelling were observed in cells traversing the IZ. Further, intensity of receptor staining appears to follow a similar rostro-caudal gradient as that observed in the immunolabelling for bFGF. That is, cells in caudal areas of the cortex presented were more intensely labelled than those in more rostral cortex. It is pertinent to note that receptor staining intensity began to decrease approximately at one third of the distance from the caudal point of the cortex. Interestingly, this was the approximate region at which caudal cortex was dissected and isolated for preparing dissociated primary cell cultures (see below). That is the caudal cortical region contained more receptor than more rostral regions.

Pre-absorption control for flg antibody

Antibody to FGFR1 was pre-incubated with the FGFR1 peptide to which it was raised before use on sections, as a negative control for anti-FGFR1. Some staining was apparent. This was due to either incomplete pre-absorption of the antibody to the antigen or some non-specific binding. However, the staining was mild and prevalent in the VZ. Less intense staining was observed in the CP. No rostro-caudal gradient of immunoreactivity was observed.

FGFR1 immunostaining in E17 wax sections with anti-flg

Embryonic day 17 brains were fixed, dehydrated and embedded in wax. Sections 8-10 μm thick were cut and mounted on slides, subsequently processed for

Basic FGF immunoreactivity using Ab 118 on adult cryosections

Adult brains were removed, sliced coronally into 5 mm thick slices, and immersed in 4% PFA for fixation overnight. Slices were frozen, and 10 μm thick sections cut and mounted on PLL coated slides. Sections were largely collected from more fronto-parietal regions. Slides were allowed to come to room temperature and air dried before processing for immunocytochemistry. Sections were used as positive controls for Ab 118, and so were processed alongside E17 brain sections.

Basic FGF was detected specifically in what appeared to be astrocytes similar to antibody detection of bFGF using Ab 106. Cell bodies were rounded. Occasionally pairs of stained cells were found distributed uniformly throughout the thickness of the cerebral cortex. No neurons were seen to be immunopositive for bFGF in adult cortex.

Summary 1

Basic FGF was present in E17 cortices. There were disparities in the pattern of distribution when examined with 2 different antibodies. Antibody 106 shows bFGF to be present in all embryonic laminae of the cortex. More intense labelling was observed in CP and VZ. Importantly, there was a rostral to caudal increase in intensity of staining. FGFR1 as detected by anti-flg antibody, showed a similar pattern in terms of distinct staining in the CP and VZ and a rostral to caudal increase in immunoreactivity. Immunostaining was clear in cryosections than wax sections of embryonic tissue. However, both wax and cryosections of adult tissue demonstrated clear bFGF localisation to small rounded cell bodies considered to be astrocytes of the cortex. Some pyramidal neurons of the adult cortex contained bFGF and upper layer neurons were also labelled for FGF R1.

Ab 118 shows a different pattern of protein distribution. Basic FGF as detected by Ab 118 was shown distinctly to be present in the CP and MZ. VZ was absent of bFGF staining. No rostral to caudal gradient of immunoreactivity was apparent. Basic FGF in the adult cortex was localised to small rounded cell bodies considered to be astrocytes.

4.1.3. Non- radioactive in situ hybridisation

Expression of basic FGF m RNA

In situ hybridisation using DIG labelled cRNA probes were used and showed the site of synthesis of bFGF in E17 brain tissue. Probes were synthesised from cDNA containing the cloned fragment of bFGF.

The protocol applied was designed for mRNA detection in fresh frozen tissue. E17 brains were dissected and mounted on blocks and rapidly frozen in pre-chilled isopentane on dry ice. Sagittal sections, 20-25 μm thick were cut and 10-14 sections mounted on silane coated slides. Sections were subsequently processed for in situ hybridisation.

Digoxigenin labelled anti-sense probes successfully hybridised at 60°C. Using anti-DIG alkaline phosphatase to visualise the RNA:RNA hybrids, labelling was observed throughout the cortex, but specifically in the CP and VZ. No signal was detected in what was assumed to be the SP. Although not quite as clear as immunolocalisation of bFGF, the pattern of messenger expression followed a similar rostro-caudal gradient discussed above. The intensity of labelling for mRNA in caudal areas of the cortex was greater than more rostral regions. The intensity throughout the VZ and CP remained uniform from rostral to caudal.

Examination of sense hybridisation reveals some non-specific binding of cRNA to in situ mRNA. However, the degree of labelling observed in control tissue was considerably less than with anti-sense localisation of messenger (Fig. 40) .

Expression of FGFR1 mRNA

In situ hybridisation used DIG labelled cRNA probes to show the site of synthesis of bFGF in E17 brain tissue. E17 brains were dissected and mounted on blocks and rapidly frozen in pre-chilled isopentane on dry ice. Sagittal section, 20-25 μm thick were cut and 10-14 sections mounted on silane coated slides. Sections were subsequently processed for in situ hybridisation.

Successful hybridisation was achieved at 60°C. Alkaline phosphatase conjugated to anti-DIG was used to visualise the cRNA:mRNA hybrids. Messenger FGFR1 was seen to be present throughout the embryonic cortex. Specifically, intense labelling was seen in CP and VZ. With the thickness of the CP being greater anteriorly than posteriorly, more labelling was therefore seen in anterior CP than posterior. Labelling in the VZ remained consistent throughout. Although not strikingly obvious, more messenger RNA was be synthesised in more caudal regions than rostral regions of the cerebral cortex. Cells of the IZ synthesised mRNA, some more intensely than others. Interestingly cells just ventral to the CP had reduced signalling intensity. This area corresponded to SP (Fig. 41).

4.1.4 Radioactive in situ hybridisation

Oligonucleotide sequence for bFGF and FGFR1 were purchased (see methods). Oligonucleotides were radioactively labelled with ^{35}S and subsequently processed for in situ hybridisation.

Autoradiographs revealed the presence of bFGF mRNA in the cortex. The laminar distribution of the growth factor mRNA was difficult to determine. No distinct labelling was observed specifically within the VZ or CP either. Autoradiographs revealed the presence of FGFR1 messenger in the cortex. Specifically radioactive signal was present in the VZ and more intermediate laminae of the cortex. No quantitative analysis was performed to calculate the degree of labelling in the rostral and caudal axis. No obvious gradient was discernible (Fig. 59).

Summary 2

The presence of bFGF and FGFR1 mRNA was detected using radioactive and non-radioactive probes. In the former case, oligonucleotide sequences were generated (see methods) and labelled with ^{35}S. In the latter, non-radioactive DIG was used to label cRNA probes synthesised from cDNA cloned into a plasmid vector.

DIG labelled bFGF probe revealed that bFGF mRNA was synthesised to a greater extent in the VZ, whilst the receptor mRNA was present in all layers but a greater signal was observed in CP and VZ. A mild gradient in mRNA expression was observed in the synthesis of mRNA for bFGF, caudal cortical regions tended to have more intense labelling than more rostral regions. The FGFR1 mRNA demonstrated a similar pattern of distribution, with caudal regions having marginally more intense signal than rostral cortical regions.

4.1.5. Immunocytochemistry on cultured cells

To examine the proportions of cells containing bFGF and FGFR1 rostral and caudal cultures from E17 brains were prepared as described in methods. Cell were

incubated in 5% HIFCS containing culture medium for 24 hr and fixed in 4% PFA and subsequently stored in PBS before processing for immunocytochemistry.

Ab 118 was used to analyse the bFGF content of cells in rostral and caudal cultures. For quantitative results, refer to Results Chapter 1. Briefly, 59.1 ± 1.3% cells were immunoreacitve for bFGF detected by Ab 118 in rostral cortical cultures. Marginally more cells were immunopositive for bFGF detected by the same antibody in caudal cultures (63.9 ± 2.6%). Antibody to flg was used to analyse the receptor (FGFR1) content of cells in rostral and caudal cultures. FGFR1 immunocytochemical analysis in rostral and caudal cultures revealed a difference in the proportions of cells containing the protein. An almost 10% difference in the proportion of cells immunoreactive for the receptor was observed, with rostral containing less (51.5 ± 2.98%) than caudal cultures (60.8 ± 4.8%). These data reflect observations made in immunostained sections of E17 cortex.

Basic FGF immunocytochemistry on E17 rostral and caudal cortical cultures

Basic FGF was present in rostral and caudal cultures derived from E17 cortices. The staining intensity was low, and marginally discernible from unstained cells. Basic FGF immunoreactivity was present throughout the cell soma. No distinctions could be made with regard to its presence in nuclei or cytoplasm. Staining appeared to be uniform throughout the cell body. Rarely was staining seen in fibres or neurites. Dead cells in culture did not appear to label with bFGF. However, some cells distinctly contained central 'inclusion' like areas in which the intensity of bFGF increased significantly. It was believed to be an artefact of staining (Fig. 23).

FGFR1 immunocytochemistry on E17 rostral and caudal cortical cultures

Immunostaining for FGFR1 was striking in cortical cells obtained from E17 rostral and caudal cortical regions. FGFR1 was observed to be present mainly in the cytoplasm demonstrating a halo like appearance in culture in which the nucleus contained no detectable receptor (Fig. 21 and 42). Staining was intense and specific. Immunoreactivity was not seen to extend beyond the cell soma, although at X 40 it was difficult to discern detail of individual fibres that emanate from the cell body. Dead cells in culture did not appear to label with the antibody, further demonstrating its specificity.

4.1.6. SDS-Polyacrylamide gel electrophoresis and Western blotting

Presence of bFGF in E17 rostral and caudal cortical regions

Embryonic day 17 rostral and caudal areas of the cerebral cortex were dissected on ice. Tissue was pooled from 2 pregnancies, approximately 32 embryos. Crude cell lysates were obtained using a mild buffering solution (methods) containing a cocktail of protease inhibitors. The cell cytosol was separated from the remainder of the cell following homogenisation and centrifugation. The crude cytosolic fraction was suspended in approximately equal volume of gel loading buffer containing beta-mercaptoethanol. After a brief period of denaturation of proteins by boiling, samples were loaded into a gel. Approximately equal volumes of sample from rostral and caudal cortex were loaded into the wells. Control recombinant bFGF protein was also diluted in β-mercaptoethanol and denatured and 100 ng of human recombinant bFGF was loaded in the third lane (Fig. 35). Following immunohistochemical detection and visualisation with electro chemiluminescence, the blot was scanned and examined. The antibody used in this section of the investigation was Ab 118 (UBI).

Clearly there were bands of protein in each lane. In the third lane in which the recombinant protein was loaded there were 2 distinctly large bands. One protein band at above 16.5 kDa was observed (upper band) and a lower band was a smaller protein, recognised by the Ab 118, resides at approximately 10 kDa molecular weight. There were no other bands in this lane demonstrating that Ab 118 was able to specifically recognise a protein that was marginally greater than 16.5 kDa. The recombinant form is 18 kDa. Ab 118 was, therefore, considered to recognise one isoform of bFGF.

Lane 1 was loaded with rostral cortical cytosolic extract. Two distinct protein bands of molecular weight the same as those detected in lane 3 in which the

recombinant version of bFGF protein was loaded. This strongly suggests that bFGF was detected in rostral cortex. Lane 2 contained proteins loaded from the cytosolic extract of caudal cortex. The bands detected closely resembled those seen in the other 2 lanes. Therefore, bFGF was present also in the caudal cortex. There was a notable difference in the density of the bands close to 16.5 kDa in rostral and caudal preparations. That is, rostral cytosolic fraction was less dense and therefore contained less bFGF than caudal cortex. However no densometric analysis was performed, nor was the protein concentration determined before loading. Notably, no other specific bands were detected by Ab 118.

Presence of FGFR1 in E17 rostral and caudal cortical regions

Embryonic day 17 rostral and caudal areas of the cerebral cortex were dissected on ice. Tissue was pooled from 2 pregnancies, (32 embryos). Crude cell lysates were obtained using a mild buffering solution (methods) containing a cocktail of protease inhibitors. The membrane and cytosolic fractions were separated following homogenisation and centrifugation. The crude cytosolic fraction was suspended in approximately equal volume of gel loading buffer containing β-mercaptoethanol. The membrane fractions was homogenised further and a crude protein extract made. After a brief period of denaturation of proteins by boiling, samples were loaded onto the gel. Approximately equal volumes of sample from rostral and caudal cortex were loaded into the wells. Membrane and cytosolic extracts were prepared from 3T3 cells as controls. After electrophoresis and protein transfer the filter paper was processed for detection of the receptor. Following immunohistochemical detection and visualisation with ECL, the blot was scanned.

The results were inconclusive and have not been included in the present study (Fig. 35).

5.1 RESULTS III

5.1.1. Examination of the appearance of cells in culture

Immediately after dissociation, cells took on a spherical and uniform appearance. They were small and usually possessed a distinct halo. Cells attached almost immediately following plating on an adherent surface. Adherent surfaces tested (data not shown) were collagen type IV, fibronectin and laminin with PLL. The surface most conducive to growth and differentiation of cells was laminin. Cultures plated on collagen type IV and fibronectin aggregated within 24 hr of plating. Laminin supported the adherence and 'spread' of cells over the surface of the coverslip.

Phase contrast appearance of E15 cortical cultures

Rostral and caudal cultures derived from E15 neocortices were a mixed population of cells. In particular, the adhesive nature of the meninges made removing them difficult, the cultures therefore consisted of meningeal cells as well cortical cells. Meninges comprise largely fibroblast and endothelial cells. The blood cells however do not adhere to the substratum. The large fibroblast like cells were distinguished easily and seldom stain for markers used in this study.

Several hours after plating in the presence of serum, E15 cortical cells began to loose their rounded appearance and developed processes. The appearance of cells ranged from unipolar, bipolar to multipolar. The confluency of the culture increased to almost double, 24 hr after plating. The intercellular space had reduced a great deal with cells spreading widely over the coverslip; individual cell bodies appeared to demonstrate extensive spreading. With increasing time in culture, continued proliferation resulted in cell clumping. Often long processes extended from one cell

aggregate to another. No striking difference in morphology at the light microscope was observed in rostral and caudal cultures.

Phase contrast appearance of E17 cortical cultures

Cultures of rostral and caudal cortex prepared from E17 brains, shortly after dissociation appeared rounded with processes began to extend from their surface. The rate of process outgrowth was slow since extensive networks of neurites had not formed even after 24 hr in culture in the presence of serum. Morphologies of cells ranged from unipolar, bipolar to multipolar. However, cell bodies remained relatively rounded and processes were finer, and few cells appeared to have multiple neurites. Some detail on the neurites could be identified, with branching occurring after 24 hr. With the increase in the confluency of the culture, neuritic branching was no longer discernible. Although no measurements were taken, the spread of caudal cells over the coverslip was more marked than rostral cells. Caudal cells tended to appear larger than rostral cells. Caudal cells did not cluster as easily as rostral cells. As results demonstrated, rostral cultures had degenerated by the 3rd day. No obvious changes were seen in caudal cells at the light microscope except for more extensive neurite outgrowth and compaction of cells on the coverslip (Fig. 6).

Phase contrast appearance of E19 cortical cultures

Immediately after dissociation not all E19 cells took on a rounded appearance. Some cells maintained at least one neurite. Cell somata had a triangular to rounded morphology, and some cells a bipolar morphology. Cell degeneration was marked in rostral cultures 24 hr after culture. Neurite outgrowth was considerably slower than

that seen in cultures prepared from younger embryos. With time, cultures became confluent (Fig. 5).

Phase contrast appearance of dying cells in culture

Dead cells were easily distinguished from living cells. Cells undergoing cell death retract their neurites. Cell bodies were rounded and changes in the integrity of the membrane cause distortion in the appearance of cells in culture. The nuclear material appears condensed into several fragments, more often 2 nuclear condensates were formed as a single cell degenerated. Occasionally multiple condensed nuclear bodies were observed. At later stages of cell death (secondary necrosis), the cell/nuclear bodies appeared condensed and approximately half of the size of live cells in culture (Figs. 5 and 6).

PI labelling

Cultures were stained with a non-specific DNA binding, and stained with PI after fixation. Coverslips were mounted in Citifluor and observed under the fluorescence filter. The cell nuclei appeared red (Fig. 10). All cells present in the culture took up the dye. Nuclei of healthy living cells appeared to be uniformly labelled with the dye. The intensity of labelling was considerably less than cells which had undergone cell death. Healthy cell nuclei appeared large and oval shaped. Some cells were seen to be undergoing mitosis, in which the chromosomes had lined up along the spindles and were migrating towards opposing poles of the cell. Dead and dying cells could be readily distinguished as the intensity of labelling of such cells was greater than surrounding healthy cells. As the nuclear material was broken down and incorporated into apoptotic bodies the staining reflected the stages of cell death.

That is, intensely stained small rounded clusters of 2-3 (and sometimes more) apoptotic bodies were observed. After programmed cell death was completed, nuclear material formed one complete intensely stained red body.

TUNEL labelling

TUNEL labelling involves the enzyme mediated addition of biotinylated nucleotide (dUTP) to fragmented ends of DNA. The break down of nuclear material in cells undergoing cell death is different to that which occurs during necrosis, such that labelling observed using TUNEL is specific to apoptotic cells, and not cells that have undergone secondary necrosis in culture (Fig. 43). TUNEL labelling in the present study was visualised with streptavidin conjugated chromophore (FITC). The resulting cells were seen as green under the fluorescence microscope. The classical appearance of apoptotic cells was observed, that of small rounded fragments of condensed nuclear material (apoptotic bodies). Often multiple apoptotic bodies were seen within one dying cell whilst other times only 2-3 apoptotic bodies were seen.

Acridine orange was also used to determine amongst living (un-fixed) cultures which cells were undergoing active cell death. Acridine orange, a vital dye, when taken up by living cells appears green and when taken up by dead cells appeared red under the fluorescence microscope (Fig. 43).

Double labelling of PI and TUNEL in cortical cultures

It was important to determine whether the population of dying cells labelled with TUNEL was different to the population of dead and dying cells observed when stained with PI alone. Specifically, since PI was the marker used to identify dead cells

in the majority of the experiments in this study, it was imperative to be sure that they were indeed undergoing apoptosis (Fig. 43).

A difference was observed in the cells that labelled for TUNEL and with PI. All dead cells labelled with TUNEL were also labelled with PI. However, a small proportion of cells (not quantified) were labelled with PI but were TUNEL negative. This suggests that TUNEL did not identify all cells that were apparently undergoing apoptosis (late stages). Thus, any estimates of proportions of cells undergoing PCD made with TUNEL labelled material would be an underestimate of the true population of cells that had died.

It is noteworthy that estimates made of dying cells using PI in culture, too, were underestimates of dying cells, since dying cells finally round up and some become detached from the adherent surface and become suspended in the culture medium, thus not amenable to quantitation.

5.1.2. Immunocytochemistry of cells in culture

Nestin immunolabelling

The neuroepithelial cells of the VZ contain the intermediate neurofilament nestin. Anti-nestin polyclonal antibody was used to identify undifferentiated neuroepithelial cells in cultures of cerebral cortex derived from E15-E19 embryos (Figs. 25 and 44). Nestin expression was observed in both rostral and caudal cultures at all ages examined. Nestin expression reduces with time in culture as cells differentiate. Quantatative measurements of proportions of nestin immunoreactive cells were made and were discussed in Chapter 1. The morphology of nestin immunoreactive cells varied considerably. Nestin filaments were located in the processes of cells and predominantly in the cytoplasm. The morphology of nestin

immunoreactive cells varied considerably. Some cells were rounded with few if any processes, other cells had extremely long and extensive processes extending from the cell body, resembling radial glial cells in the intact brains. Cells demonstrating a bipolar morphology were often seen to have these long processes extending from both poles of the cell body. Cell bodies were comparatively smaller and more elongated than cells whose process lengths were considerably shorter. Protruding processes were often seen to possess 'varicosity' like bulges at or near the end of the process. These possibly resemble the 'end feet' of the radial glia cells in the cortex. With increasing confluency the size of individual cells decreased.

Basic FGF treated rostral cultures were also immunostained for nestin. By 3 DIV, a qualitative analysis revealed there was an increase in the proportion nestin immunoreactive cells. Many cells had a rounded appearance but most maintained one or 2 processes (Fig. 44).

Vimentin immunolabelling

Neuroepithelial cells of the VZ of the cerebral cortex contain another intermediate filament that is used as a marker for undifferentiated cells, vimentin (Figs. 45 and 18). The morphology of cells detected using anti-vimentin antibody resembled closely cells immunoreactive for nestin. Large cell bodies localised vimentin specifically in the cytosol, the vimentin filaments extended out in fine processes that emanated from the cell soma. However, vimentin immunolabelled cells did not posses long fine fibres that nestin immunolabelling demonstrate. Fibres were also smooth and display not irregularities such as 'varicosity' like swellings.

Microtubule associated protein-2 immunolabelling

MAP-2 is present in differentiated neurons of the cerebral cortex. In culture, MAP-2 was observed in an increasing fraction of cells with increasing embryonic age of cells from which the culture was prepared. Over time in culture, as differentiation proceeded and the neuronal pool was expanded more cells became immunoreactive for MAP-2 (Figs. 27, 46, 18 and 47). Soon after plating cells from older embryos, cells demonstrated MAP-2 immunolabelling. The cell bodies of neurons vary from being rounded in appearance, bipolar and pyramidal. The MAP-2 immunoreactivity was observed in the cytoplasm and extends into the neural filaments extending from the cell somata. As differentiation proceeded and with time in culture, extensive networks of neurites formed. Fine long neurites varied in thickness throughout their length, with swellings apparent at different points along their length. Some neurons possess one, two or three neurites that emerged from the cell body. With on going differentiation more and more cells emerge with multiple neurites. Neuritic length also appeared to increase. However no quantatative measurements were made.

γ-aminobutyric acid immunolabelling

The neuronal population of cells was further subdivided according to the neurotransmitter they contained. GABA is the neurotransmitter classically associated with interneurons, the inhibitory neurons, of the cerebral cortex. Antibodies raised to GABA allow the detection of interneurons of the cerebral cortex. GABA immunoreactivity was distinct (Figs. 28 and 32), labelling only a small proportion of neurons in the whole culture. The variation in the proportion of GABAergic neurons over cultures derived from different ages was not discussed here. GABA containing cells early in E17 cultures appeared to have multiple neurites extending from the cell

body. Often one large neurite was observed with many small neurites emerging from other points on the cell soma. Many GABA immunoreactive neurons appeared to be bipolar, whilst most were multipolar. Over time in culture differentiation of GABA containing neurons became evident with the increase in the length and branching of neurites. GABA containing neurites also demonstrated swellings throughout their extent. A large number of GABAergic cells acquire a pyramidal morphology. A number of GABAergic cells had a thick neurites emerging from the apical surface, of what appeared to be pyramidal shaped cell bodies, which divided immediately.

Glutamate immunolabelling

The neurons containing glutamate, the excitatory neurotransmitter of pyramidal cells in the cortex, form another subpopulation of neurons in the cortex. E19 cultures were examined for their glutamatergic cell population using anti-glutamate antibody. Glutamate staining was less specific (Fig. 32). Cell bodies as well as neurites that extended from the cell soma were immunoreactive for glutamate. A large population of neurons presented themselves as gluatamatergic.

Glial fibrillary acidic protein immunolabelling

GFAP was a protein found within astroglial cells of the cerebral cortex. In embryonic cultures in this study, very few astroglial cells were observed. Rostral cortical cultures did not demonstrated any immunoreactivity to GFAP (Fig. 48). Few cell were seen in caudal cortical cultures. The GFAP immunopositive cells resembled some cells that were seen to be immunoreactive for nestin, particularly those cells with elongated bipolar cell bodies and long filamentous extensions emerging form both poles. These cells were considered to be the radial glial cells of the cortex. Other

cells were more of an astrocytic morphology in which the cell body was large and many processes emerged in a star like manner from the soma. The GFAP was present in the cell cytoplasm and in the processes which extended out of the body.

Basic FGF treated rostral cultures derived from E17 cortices were immunostained for GFAP at 3 DIV. A dramatic increase in the GFAP positive cells was observed, although fewer than expected in cultures treated with a potent mitogen such as bFGF (not quantified).

Bromodeoxyuridine immunolabelling

To identify the proliferative population of cells in cultures derived from embryonic cortices, pulses of BrdU were given to cells in rostral and caudal cortical cultures. After fixation cells were processed for immunocytochemistry to visualise the cells which had incorporated BrdU into their DNA. One hour pulse resulted in cells being labelled to different extents. Cells that had just entered the S-phase of the mitotic cycle at the end of the pulse period and cells that had just exited the cell cycle at the beginning of the pulse period were lightly labelled to differing degrees. However, cells that remained in the S-phase throughout the period of exposure to BrdU were intensely labelled (Fig. 13). Immunolabelling was seen only in the nuclei of cells.

Immunocytochemical labelling in tissue cryosections

To examine differences in the different cell populations in rostral and caudal cerebral cortical regions immunohistochemistry for different cell types was employed on E17 frozen sagittal sections.

Nestin immunolabelling in E17 para-sagittal cryosections

Fixed E17 brains were frozen and 10-12 μm thick sagittal sections cut. Immunohistochemistry for the neuroepithelial marker, nestin was performed. Nestin immunoreactivity was not clear in sections. Fibres were immunoreactive throughout the thickness of the cerebral wall. Particularly at the pial and ventricular surface, radially oriented processes were observed. The plane of section did not allow a complete cell to be observed. There were no striking differences in nestin immunoreactivity in rostral or caudal regions of the cortex.

TuJ1 immunolabelling in E17 para-sagittal cryosections

TuJ1 was a neurofilament present in new born neurons of the cerebral cortex. Antibodies raised to TUJ1 allow the identification of newly generated neurons in rostral and caudal cortical regions. TuJ1 immunoreactivity was detected in the cerebral cortex. Immunostaining appears to be specific to the IZ, CP and MZ. Marginal zone cells appeared to be the most intensely stained. A gradient in labelling was apparent along the rostro-caudal axis i.e. more immunoreactive cells were detected in more rostral than caudal regions. This pattern was a reflection of the neurogenetic gradient of development of the cortex with differentiation initiated more rostrally than caudally. Some TUJ1 positive cells were identifiable in the VZ, but were few in number.

MAP-2 immunolabelling in E17 para-sagittal cryosections

MAP-2 labelling was performed in sagittal sections of fixed frozen E17 brains. MAP-2 immunohistochemistry was used to determine the differences in neuronal populations in rostral and caudal cortical regions.

MAP-2 immunoreactive cells were predominantly present in the MZ throughout the rostro-caudal axis. MAP-2 immunoreactivity was largely restricted to the CP in both rostral and caudal regions of the developing cerebral cortex. However IZ was also seen to be lightly labelled with some intensely labelled cells in the VZ and SVZ. No striking differences in rostral and caudal regions were discernible.

Immunohistochemistry for MAP-2 was also performed in adult cryostat cut sections as positive control for MAP-2 labelling. Pyramidal cells of all layers appeared to be positive for MAP-2. The apical dendrite was also labelled.

BAX immunolabelling in E17 para-sagittal cryosections

Among the proteins important in the programmed cell death cascade are the members of the Bax family. BAX is known to be involved in the promoting the programmed death of neurons. Here immunohistochemistry utilising a rabbit polyclonal antibody to BAX was used to ascertain differences in the expression of this protein in the rostro-caudal axis.

Immunolabelling for BAX revealed that this protein was present largely in neurons of the cerebral cortex located in the CP and SP (data not shown). Some immunoreactivity was detected in the MZ. Notably more labelling for BAX was observed in rostral than caudal regions of the cortex. This however could be as a result of the gradient of differentiation of CP neurons, despite the lack of a MAP-2 gradient. A thin layer, possibly of single cells was observed beneath the pial surface in more caudal regions containing cells immunoreactive for BAX.

6.1 RESULTS IV

6.1.1. Cell types in culture

Morphology of cells using the scanning electron microscope

Cortical cultures were prepared from E17 brains and incubated for 24 hr in the presence of serum before fixation in 2% glutaraldehyde. Coverslips were further processed before coating with gold for observation at the SEM. Several low power scans were photographed to enable the observation of the different morphologies of cells present in the culture (Fig. 49).

Notable was the extensive network of neurites covering the surface of the coverslip not seen at the light microscope. Many artificial breaks in the network of neurites were present, thought to occur as a result of the method of fixation. Often fixing in glutaraldehyde followed by dehydration results in shrinkage of the laminin substrate and neurites.

Categories based on morphological appearance of cells revealed several broad cell types; 1) very dense rounded cells, 2) less dense rounded cells, 3) cells with corrugated surfaces, 4) flat cells with unipolar, bipolar and mutlipolar cell bodies and 5) elongated cell with unipolar or bipolar cell bodies.

Very dense rounded cells

Cells that appeared to be intensely white and rounded were observed in all cultures. There was some degree of variation in the size of such cells but were almost always smaller than surrounded less dense cells. These cells never had neurites emanating from their surface. It was possible that the neurites were broken off in the processing of the material. High power magnification revealed that the surface membrane properties were distinctly different to surrounded cell somata. The

membrane appeared porous, many elevations and depression had developed. In some instances, there was one or two depressions giving the cell a raspberry like appearance. Other cells that were classed in this category had a coral appearance in which the cell appeared to have disintegrated considerably. Cells in this category were therefore regarded as having undergone programmed cell death and were in the secondary necrotic phase; often observed in monolayer cultures when macrophages were no longer available to remove dead and dying cells. In some instances, a nuclear body was seen, whilst in other cases multiple bodies possibly apoptotic bodies were observabed, suggesting that the cells were in the early part of the late phase of cell death. Often the space around these dead cells was clear of neurites and other adhesions. In some instances a pair of such cells was observed (Figs. 50-53).

Less dense rounded cells

A number of cells appeared to have a spherical morphology, and were slightly larger than the very dense round cells. These cells sometimes appeared as pairs of cells, suggesting that they were cells that had just undergone cytokinesis following mitosis (Figs. 50 and 51). High power magnification revealed that the minor irregularities in the membrane existed but overall the integrity of the membrane appeared not to have been compromised. Often these cells did not have long processes extending from their cell soma, suggesting that they had retracted neurites before division. It was difficult to determine in cases in which a neurite was proximal to the cell was actually connected to another cells or simply passing over another neurite. Alternatively, these cells have rounded up to enter early stages of programmed cell death. Occasionally, blebbing was seen from the surface, however blebbing of the membrane was not always associated with apoptosis and can be produced by ionic

constituents in the surrounding media. Other cells in this category appeared to have relatively smooth cell surfaces but appeared to bulge out of the coverslip more so than surrounding cells. It was difficult to determine at the level of the scanning electron microscope what cell type these were. It was possible that these cells were the progenitor cells of the neuroepithelium rounding up ready for mitosis or neurons or neuroepithelial cells at the very early stages of cell death.

Cells with a corrugated surface

A small proportion of cells were identified according to the changes that had taken place on the surface of the membrane. Clearly these cells were rounded and hemispherical, and maintained processes on occasions, whilst some cells lost all neuritic processes. The degree of convolution of the membrane varied from mildly convoluted to extremely convoluted (Figs. 52 and 53). These membrane irregularities were considered to be similar to membrane blebbing often associated with apoptotic cells. These cells had not acquired the porous membrane structure observed in the first cell types described above, suggesting that these cells had possibly entered the early to middle phase of cell death. In some instances, cells appeared to demonstrated the classical blebbed morphology. However, it was questionable whether these cells were associated with debris from other cells or actually had membranous blebs protruding from their membrane surface. The size of cells with corrugations was approximately similar to cells that appeared 'healthy'. However, no statistical analysis or measurements were made.

Flat unipolar, bipolar and mutlipolar cells

Cells that appeared to be flatter amongst the cultured population were classed under this category. Although an arbitrary distinction, cells resembled neuronal cells seen at the light microscope (Fig. 50). The cell soma was either almond shape, unipolar cells, elongated, bipolar cells or pyramidal shape largely in the case of multipolar cells. The cells appeared darker in scanning micrographs and were often seen associated with one, unipolar, 2, bipolar or 3, multipolar, neurites. Frequently cells with more than 3 neurites were observed. Occasionally cells with fibroblast like cytoplasmic extensions or pseudopodia, were observed. It was possible the latter were actually fibroblasts that have entered the cultures as a result of inefficient removal of the meninges from the cerebral cortex during dissection. Unipolar cells often had only one process extending from their pole, bipolar cells had two neurites growing out from both poles and pyramidal shaped cells were seen to have 3 neurites extending from the apices of the pyramidal body. Other multipolar cells had more neurites extending from their surface. It was possible to speculate that pyramidal and bipolar flat cells were neuronal populations, and that possible the former were glutamate containing. However evidence from SEM alone was insufficient to support this. Cells that had a star shaped morphology could be GABA containing cells or astrocytes. These cells were unlikely to be astrocytes as cortical cultures have been demonstrated to have very few astroglial cells, and it was difficult to ascertain that they were GABAergic based on morphology in culture alone.

Elongated cell with unipolar or bipolar cell bodies

Another population of cells was identified in cultures were cells that appeared more 3 dimensional than the flat cells described above. Two types of cells were

observed in this class, the elongated unipolar and elongated bipolar cells. Often both cell types would have long thick neurites that could be traced for a considerable distance, almost resembling the radial glia or undifferentiated neuroepithelial cells seen at the light microscope. These cell appeared to be more dense than the flatter cells, appearing relatively white under the SEM. The surface of the cells appeared scaly but relatively smooth. The unipolar cells often appeared almond shaped with one end being extremely rounded with a process extending from the opposite end. Occasionally a process would be seen emerging from the rounded end of the cell. It was difficult to estimate what these cell types might be. It was possible these cells were the neuroepithelial cells in culture. The bipolar cells appeared to be rounded at the centre of the cell body and from the two poles emerge relatively thick neurites. The surface of the cell appeared also to be scaly but relatively smooth. Thick neurites were seen to emerge from either pole and often extended for considerable distances. Full lengths of neurites were not visible due to the complexity of the network of neurites arising from all the cells in the culture. It was possible to speculate that the bipolar cells might be the radial glial cells of the cerebral cortex. The cell bodies of both unipolar and bipolar cells in this category appeared to be relatively smaller than the flatter cells described earlier. However no quantitative data was collected.

6.1.2. Morphology of cells at the level of the transmission electron microscope

Cultures of cortex were prepared from E17 brains on marinex and incubated in the presence of serum for 24 hr. Cells were fixed in 2% glutaraldehyde before processing for transmission electron microscopy. After embedding in Araldite ultra thin sections were prepared beginning at the base of the cells. Sections were collected

on copper coated grids and stained with uranyl acetate as described in methods. Sections were examined and photographed at the TEM.

The aim of this section of the study was to identify the type of cell death occurring in cultures. TUNEL labelling, PI and SEM strongly suggest that dying cells were undergoing programmed cell death. TEM data supports this.

The recognition of apoptosis and necrosis was based primarily on the distinctive changes that take place within the affected cells. When these processes occur *in vivo* they also differ in their distribution and in the tissue regions that were associated with them. These latter features maybe subsidiary use in identification. However in culture this was not the case. The cell can be considered to be undergoing apoptosis according to ultra structural correlates.

According to Kerr et al (1984, 1987), the earliest unequivocal morphological evidence of the onset of apoptosis was found in the nucleus (Figs. 54-58). The chromatin condenses and becomes aggregated in the sharply delineated, fine granular masses of uniform textures that abut the inner surface of the nuclear envelope. The proportion of the nucleus occupied by the condensed chromatin varies with cell type. The early chromatin changes were often, but not always, accompanied by convolution of the nuclear outline. The nuclear convolution can become extreme resulting in budding to produce discrete nuclear fragments of varying size and chromatin content, which were still surrounded by double membranes. Condensed chromatin can occupy the whole of the cross sectional area of the apoptotic body or form a crescent in which the nuclear material was confined to one side. Since nuclear convolution and budding tend to be restricted in cells with a high nucelus:cytoplasm ratio it was not always possible to see such ultra structural changes in all cell types.

Cytoplasmic changes occur at the same time as nuclear changes. Overall condensations were often observed as protrusions or blebbing of the cell surface. However as in the case with the convolution of the nuclear outline the cell surface protrusions were not always identifiable in all cell types. Clear vacuoles may be numerous in the condensing cytoplasm and can sometimes be found discharging the contents of the cell by exocytosis. At the scanning electron microscopic level this was observable as surface craters. Cytoplasmic condensations were less observable in cell culture. Organelles remain well preserved at early stages of apoptosis. The protuberances that can be observed on the surface of the cell often bud off and produce membrane bound apoptotic bodied of varying size and composition. In some cases the cell was converted into many small bodies, whereas in others, one or two bodies were conspicuously larger than the rest. The number of nuclear fragments varies widely and the presence of a nuclear component was not consistently related to the size of the cell. The fate of apoptotic cells in culture differs from such bodies in tissue. In the latter phagocytosis and lysosomal degradation occurs to remove the dead cells by resident marcophages. However, in the former situation cells escape phagocytosis and spontaneously degenerate within a few hours. Their bounding membrane ruptures and swelling and dissolution of their organelles occurs; these changes resemble those observed in necrosis. Such degenerate apoptotic bodies can however e distinguished from cells that have undergone necrosis if at the beginning they contain apoptotic type nuclear fragments. The frequency with which such observations were made varies from cell to cell and cell type.

Necrotic features observed in cells differ in that the integrity of cell organelles was compromised as a result of disruption of the plasmallema. Features included swelling, possible surface blebbing, not to be confused with changes of membrane in

apoptosis, dilation of cisterna of the endoplasmic reticulum, dispersion of ribosomes and mild clumping of the nuclear chromatin (Trump *et al.,* 1981).

Some of the features described have been demonstrated in electron micrographs taken from cultures of cortex. In most instances the stage of apoptosis has advanced such that the beginning of the necrotic phase was observed.

7.1. DISCUSSION I

Although the functional specificity of cortical areas depends primarily on afferent and efferent connections, local area and lamina specific features also contribute to the functional and structural heterogeneity of the cerebral cortex. The developing neocortex is seemingly homogenous. However, evidence is mounting to suggest that there are differences between areas of the cortical analage. Here, we have shown that the homogenous appearance of the VZ masks underlying 'behavioural' differences amongst cells in different regions of the developing cerebral cortex. Furthermore, the differences in survival that we have described between cortical regions are considerably larger than those that can be attributed to neurogenetic gradients alone, and that bFGF plays an important role in the evolution of such differences.

The present study elucidates some of the differences present amongst cells within different regions of the developing cortex. Cells taken from two distant regions, rostral and caudal, of the embryonic cortex demonstrate a striking difference in their ability to survive when isolated from the remainder of the cortex. This difference in survival was maintained and notable throughout the period of neurogenesis examined. Rostral cells survived for significantly shorter periods than cells derived from caudal regions. Further, rostral cells were rescued when cultured in the presence of caudal cells at E17 and were able to survive for longer periods than controls. To examine whether this rescue effect could be mimicked, a range of neurotrophins and growth factors were tested for their ability to rescue rostral cells from cell death. Basic FGF had the greatest rescue effect, and did so, in a dose dependent manner. Caudal cells in the presence of a pharmacological blocker of the action of bFGF resulted in complete cell loss. Caudal cells lacking bFGF derived from

cortices of loss-of-function FGF2 -/- mutant mice underwent rapid cell degeneration shortly after culturing. Further, immunocytochemical, radioactive and non-radioactive in situ hybridisation histochemistry and Western blotting were used to detect the presence and pattern of expression of bFGF and its high affinity receptor, FGFR1, in embryonic sections of cortex and in dissociated cell cultures.

Using immunocytochemistry for known cell specific markers, differences in cell types present in rostral and caudal regions of the developing neocortex are described. Evidence is also presented to show the mode of cell death to be apoptotic and not necrotic cell death.

The hypothesis that certain features of cortical areas are specified within the VZ is supported by the finding that regions of the VZ supplying cytoarchitectural areas with a high cell density in the adult brain, proliferate over a longer period of time to give rise to a larger numbers of neurons in comparison to VZ regions which supply cortical areas with fewer cells (Rakic, 1982). This is evidence that VZ is divided into proliferative regions that form a protomap of the developing CNS (Rakic, 1988). Cell-cell and cell-environment interactions appear to regulate the formation of the mature cortex. Early in development, cell interactions can drive the expression of area-specific fates (Barbe and Levitt, 1991; 1992, 1995; Arimatsu *et al.*, 1992; Cohen–Tannoudji *et al.*, 1994) while at later stages the appropriate sensory stimulation is necessary for the proper development of precise cortical maps (Weise and Hubel 1963; O'Leary *et al.*, 1994). Our study highlights the potential role of bFGF in the creation of early regional differences and the formation of the protomap.

Methodological considerations

Cell culture

Relative to *in vivo*, *in vitro* systems offer a comparative advantage in two major points: accessibility and reduction of variables. The latter allows for a simpler interpretation of the effects of varying conditions, however the drawback is that the set up is significantly different form the *in vivo* situation, so that conclusions drawn can be wide and varied, and extrapolations should be made with this in mind.

Primary dissociated cell culture allows the investigator to determine the 'behaviour' of cells in isolation. Of particular interest is how the neural cell behaves in the absence of cells that would otherwise be close neighbours *in vivo*. This allows investigators to determine the dependence and independence of a cell on interactions with other cells, the extracellular matrix and growth factors that may have been in their local environment. Further, various parameters of a cell's development can be monitored. Cells might exhibit phenotypes and features artificial to their nature when challenged in a particular environment. Appropriate and sufficient controls can remove some of these drawbacks.

Treatment with growth factors and pharmacological agents

The benefit in using primary dissociated cultures is partly in the ability to test the reaction of cells to different agents added to the culture medium. However, often unrealistic quantities are required to induce a desired reaction. Often growth factors, neurotrophins, the substrates that a neural cell is exposed to *in vivo* differ a great deal in concentration, form and composition than when applied in culture. However, such conditions can be closely monitored and manipulated as required. The set up can intimately effect the behaviour of the neural cell. For example, the reaction of a cell to

a particular agent can vary according to the culture conditions, the cell plating density, the duration of exposure as well as the concentration of the agent. In attempting to understand the behaviour, various parameters have to be investigated for optimal results.

Rostral cells survive for shorter periods than caudal cells over the period of neurogenesis

One striking feature in the present study is the enormous difference in the survival capacity of cells from different regions of the developing cortex. Cells isolated from rostral cortex underwent rapid degeneration and cell death by apoptosis compared to caudal sister cultures. Using an assay that involves the incorporation and metabolism of MTT to an insoluble precipitate, an estimation of the extent of cell death and cell survival was obtained. The difference in cell survival was apparent in all ages examined, except E14. Interestingly, there was some variation in the ability of rostral cells to survive, and this was comparable across the different embryonic ages from which the cultures were derived. No difference was observed in the rate of survival of rostral and caudal cells in E14 culture. At E15 a difference was detected after 6 days in culture. At E16, in the majority of experiments all rostral cells had undergone PCD by the fourth day in culture. At E17, the time taken for rostral cells to degenerate was shorter by approximately 1 day, that is a wave of cell death had caused complete loss of rostral cells by 3 DIV. And at E19 the difference in the survival of rostral and caudal cells was more acute, that is rostral cells degenerated within 20-24 hrs, this phase of degeneration was closely shadowed by the occurrence of cell death throughout the caudal cultures too, although delayed and at a slower rate.

To our knowledge such cell cultures have not been set up before, neither have they been used to test differences in the rostro-caudal axis in the developing cerebral cortex. What is interesting is that the preparation and culture conditions for rostral and caudal cells were exactly the same. Both cell groups were isolated over the same period of time and plated at equal densities and incubated in the same culture media.

A number of issues arise here. Why is there a difference in the survival of cells in the two different isolated regions of the cerebral cortex? Why is there no difference or mild difference in survival of cultures prepared from young embryos compared to older embryonic ages? Why does the rate at which cells from rostral regions die change with increasing embryonic age? And finally, what are the possible reasons for such differences with respect to the development of the mature cerebral cortex.

The differences observed in culture can be perceived in a number of ways. That is, rostral cells may be more vulnerable, and have a lower capacity to survive in response to perturbation in the environment (culturing), or that they are in some way dependent on cell-cell interactions or cell-matrix interactions to maintain their survival. That is, they are in some way pre-programmed to undergo cell death in the absence of survival signals from their environment. Rostral cells may have a more restricted phenotype that prevents them from being able to adapt to changing environments. In the first instance, vulnerability maybe of benefit to the developing brain, since perturbations in the environment of a cell, in this case by contact with an artificial surface, maybe a signal that the cell is in the wrong environment (ectopic). As the correct positioning and wiring of cells in the cortex is essential to the correct functioning of the brain, the induction for cells to undergo cell death when ectopically placed would reduce the harm to the animal. Often cell death is associated with the

removal of unwanted or mutated cells. A more plausible reason is that cells of the rostral cortex undergo cell death in response to removal from their normal environment. The removal of possible survival signals from the environment in the form of cell-cell interaction or cell-matrix or cell-growth factors may be the cause of the cells to undergo cell death. Survival signals can take the form of gap junction mediated communication, afferent input, growth factors present in the extracellular matrix (ECM) or the ECM itself. Furthermore, rostral cells may have the capacity to produce their own survival signals, but insufficient receptors to be able to acquire the desired threshold to achieve the survival effect, and that *in vivo* the multiplicity of signals renders this dependence on threshold obsolete. Compare to the neurotrophic hypothesis, in which target derived signals promote survival of neurons endowed with sufficient receptor density (Levi-Montalchini, 1987).

Alternatively, caudal cells may have a better capacity to survive, or be less dependent on non-self signals. Caudal cells may be more malleable under altered conditions. That is, they may have a less restricted phenotype that allows them to respond in a non-self destructive manner, to changing signals in their environment. Caudal cells may have the capacity to provide their own survival signals which act in an autocrine manner. Caudal cells might, therefore, be endowed with receptive mechanisms to allow them to detect such self produced factors. Also the level of receptor expression might be greater in caudal cells than rostral cells; their sensitivity might also be set at a different threshold for activation by survival signals.

This differential survival phenomenon appears to be, in some way, age dependent. Neurogenesis in the cortex of the rat begins around E14 with extensive proliferation of the ventricular neuroepithelium. Since no difference in the survival of cells is apparent, this suggests that the differences in rostral and caudal cells that

mediate the differential survival have not yet been fully expressed. Alternatively, the differential survival of cells from rostral and caudal regions may be determined by the number of cycles the cell has undergone. At the onset of corticoneurogenesis, the difference in the number of cycles passed through by cells is minimal, between rostral cells and caudal cells, therefore it is expected that there would be difference in their survival. Thus, the hypothesis predicts that with increasing the number of cycles through which a cells transcends a concomitant increase in vulnerability and loss in survival capacity to the altered environment, occurs. This seems a plausible explanation for the difference in the survival, as neurogenesis proceeds from rostral to caudal (Bayer and Altman, 1991). That is, cells rostrally placed have undergone more cell cycles and thus are more 'worn' than less mature cousins residing in more caudal regions (Caviness *et al.*, 1995). That is cells in more rostral regions possibly channel their efforts into differentiating than simply 'trying to stay alive'. However, a number of investigators have previously reported that the vulnerability resides more with immature proliferating cells (presumably in the caudal regions) than more mature well differentiated cells (presumably in more rostral regions). That is, neurons survive better than undifferentiated neuroepithelial cells (Voyvodic, 1996). This contrasts with reports that, in fact, vulnerability lies in the opposite direction, that neurons are more susceptible to cell death than are undifferentiated uncommitted neural precursor cells (Oppenheim, 1991). Ionising radiation experiments conducted by Bayer and Altman (1991) demonstrated that young cortices exposed to X-irradiation show differential vulnerability. Caudal cells collapse and demonstrate pyknosis when compared with those in more rostral regions. However, with increasing embryonic age the patchy collapse of the cerebral wall is more notable in more rostral cortex than caudal. However, the difficulty in culturing postnatal embryonic cells suggests that

the vulnerability lies with the differentiated postmitotic cell (the neuron). In some ways, this appears to be counter intuitive, as a great deal of energy goes into producing a neuron. To make it vulnerable and possess a diminished capacity to survive appears on the surface to be a 'waste' of resources. However, the complex nature of the cortex, the complex connectivity and cytoarchitecture, and complexity of function makes it imperative that neurons connect up specifically, and that ectopic or incorrectly connected cells are removed, and thus removable. However, in the present study, the difference in survival cannot be attributed only to the neurogenetic gradient, that is rostral cells possess more neurons than caudal regions. Cells when dissociated and plated on coverslips tend to differentiate and, as the immunocytochemical data describes below, a proportion of neurons form in caudal cultures too. Further, the differential cell death cannot be attributed solely to the cycling history of the cells, as caudal cells undergo a number of cell divisions in culture and at no time are there levels of cell death comparable to those in rostral regions. Also, by E17 it is estimated that cells in the caudal regions of the cortex would have equalled the cycling history of that of rostral cells at E16. Thus if cell cycling was the determinant of cell vulnerability, caudal cells at E17 should have shown signs of apoptosis equivalent to the rostral cultures from E16 rostral cultures. However, with increasing age the vulnerability of rostral cells did increase because at peak neurogenesis, E16, substantial numbers of rostral cells were degenerating by the third day *in vitro*, and by the fourth day all cells had degenerated. One explanation would be the growing dependence with maturity on external influences. With increasing age, changes in connexin expression, and therefore gap junctional communication (cell-cell interaction; Nadarajah *et al.*, 1999), may alter the dependence of cells on each other, and thus in isolation cells do not communicate and subsequently die. However, no

known reports describe any differences in rostro-caudal expression pattern of such proteins that are required for gap junction channels. Alternatively, with increasing age, restriction of phenotype, changes in molecular expression patterns might alter, rendering cells of the rostral region less able to cope with deprivation of previous (*in vivo*) existing interactions with extracellular matrix and growth factors within or from adjacent cells, whilst caudal cells remain less restricted and maintain the capacity to survive independently of the environment in which they were resident. However, this notion continues to refer to the neurogenetic gradient, that rostral cells are more mature than caudal cells. Although the changing survival capacity ensues with age such that caudal cells reduce their capacity to survive by E19, the neurogenetic gradient alone is insufficient to account for this difference, as E17 caudal cells can survive for as long as 7 days. This in effect would allow caudal cells to attain the level of maturity attained by cells from older embryos. However, cells may develop the capacity to change their dependence to the new environment by conditioning themselves to it. Often, cultures which have been growing for some period rapidly degenerate when all the culture medium in which they have been incubated is replaced. At no age, except E19, did cultures of caudal cells have proportions of dying cells that approximately matched those in rostral cultures.

It is pertinent to note that one important difference in the cells in rostral and caudal regions is their geographic location within the rostro-caudal axis. Many activities of cells are influenced by the concentration of external molecules that they encounter. Physiological responses to agents can alter according to the concentration of that agent, such as in a dose dependent manner. Other concentration related responses include the cell's ability to sense position in a concentration gradient and adjust the resultant physiological response accordingly (Ghosh and Greenberg, 1995).

A considerable number of signalling events in the early development of flies, nematodes, frogs and chick appear to involve diffusible factors that determine cell fates in a concentration dependent manner. In many of these cases, responding cells switch from one kind of gene activity or differentiation pathway to another, as the local concentration of the signal increases. Therefore, cells at different positions in a concentration gradient of a signalling molecule respond in qualitatively different ways (Gurdon *et al.*, 1998, 1999). Such is the effect observed by Gosh and Greenberg (1995) in which different concentrations of bFGF determined different developmental fates in cortical neural precursors, switching between neuronal and glial phenotype. The research of Caviness characterised lateral to medial differences and demonstrated the changing length of the G_1 phase of the cell cycle across the proliferative epithelium. The gradient in T_{G1} is a morphogenetic gradient in the sense that it would be suited 'to serve those histogenetic processes which require the cell to 'know its position' within the proliferative developmental field' (Miyama *et al.*, 1997; Goodwin and Cohen 1969; Wolpert 1969, 1981). That the unique change in the cell cycle parameter encodes a histogenetic role for specification of the topology of the neocortical protomap (Miyama *et al.*, 1997).

On the issue of differences in proliferative behaviour of cells in the different regions determining their vulnerability, evidence from Polleux *et al.* (1997) in which quantitative areal comparisons of laminar production of cells over the period of neurogenesis between neighbouring areas was assessed. They found that marked areal differences in the timetable of laminar histogenesis with relative homogeneity within areas are not merely the expression of known neurogenetic gradients. Perhaps underlying differences such as these alter the susceptibility to PCD in rostral and caudal cells.

It is noteworthy that with increasing age, progression through neurogenesis continues, with the precursor population restricting, and thus altering their fate with respect to their early cousins which come to settle in the CP. Progressive cycling gives rise to cohorts of neurons destined to different laminar fates, thus changes in the radial dimension are determined by epigenetic or genetic cues in the ventricular epithelium that govern the production of subsequent layers of neurons. Frantz and McConnell (1996) propose that altering the cells competence to respond to environmental cues present in younger brains that signal the production of the deep layer neurons instead display more restricted potential, thereby generating upper layer neurons. Such radial and tangential cues combined may provide co-ordinated development of different regions of the cortex.

Evidence that environmental signals are key determinants of cortical regionalisation have been extensively presented by Ferri and Levitt, (1993). The notion that there are differences in the ventricular epithelium that determine areas specific fate is supported by the pattern of expression of limbic system associated membrane protein (LAMP; Levitt 1984; Pimenta *et al.*, 1995; Zacco *et al.*, 1990) in progenitors of presumptive perirhinal regions and not in presumptive sensory motor cortex (Ferri and Levitt, 1993). Ferri and Levitt (1993, 1995, 1996) described the influence of choice of molecular phenotype of neural precursors by TGFα in the presence of collagen type IV. Thus, specific signals affect choice of area phenotype during the cell cycle (Ferri and Levitt, 1996).

Molecular approaches and the use of classical experimental manipulations provide evidence that initial parcellation of the cerebral cortex into areas may in fact be achieved early in histogenesis, similar to other regions of the developing CNS (Krumlauf, 1994). Generation of distinct areas within the cerebral cortex in the

telencephalon is comparable to the formation of specific nuclei within rhombomeric segments in the hindbrain. The unique spatial distribution of *sonic hedgehog* results in the induction of specific ventral phenotypes (Echelard *et al.*, 1993). Patterning of the early cortex is thought to occur through interactions among cells of the progenitor pool in the VZ. Gradients of selective inductive and supportive signals lead to the cellular diversity underlying early cortical regionalisation (Ferri *et al.*, 1997).

Recent support for early regionalisation of the cortex comes from descriptive studies of molecular expression patterns. Several molecules have been described. An enhancer-trap transgenic mouse has been identified in which the *lacZ* reporter gene is expressed specifically in somatosensory cortex (Cohen-Tannoudji *et al.*, 1994; 1992; Gitton *et al.*, 1999), suggesting area specific promoter activity that distinguishes cellular forerunners of somatosensory cortex. Latexin expression first identified by examining the expression pattern of an antibody raised to a protein specific to the lateral cortical region, PC3.1 (Arimatsu *et al.*, 1992; 1994; 1999; Hatanka *et al.*, 1994). *Tbr-1* exhibits a complex expression pattern that is not confined to specific areas. Although uniformly distributed in the cortical ventricular epithelium, *Tbr1* displays a rostral dense to caudal sparse gradient in superficial layers (Bulfone *et al.*, 1995). FGF-7 expression in the forebrain is observed in the VZ (Mason *et al.*, 1994). Strikingly its expression is restricted to the presumptive parietal and frontal regions. Heregulins are also expressed in a unique pattern within the cortex (Marchioni, 1993). Further examination of neurotransmitter receptor expression reveals specific patterns between functional domains during development. (Lidow and Rakic 1994; Broide *et al.*, 1995). Later in cortical development, barrel formations characterise the somatosensory cortex in the rodent. The vibrissae structures are discrete aggregates of layer IV neurons (Woolesey and Van der Loos, 1970). More recently Donoghue and

Rakic identified a group of molecules that play a role in the initiation of cerebral cortical area formation, the Eph receptors and their ligands, the ephrins in primate cortex. Examination of these receptors reveals distinct gradients in laminar and areal domains in the embryonic cortex, before the formation of the thalamocortical connections. Thus Eph expression may be regulated by programmes intrinsic to cortical cells. Specifically EphA 3, 6 and 7 area all selectively expressed within the presumptive visual cortex (Donoghue and Rakic 1999). Interestingly, therefore, Eph3 Eph5, Eph6 and Eph7 and the ligand ephrin 5 are expressed in posterior regions, whereas Eph4 and ephrin A2 and eprhin A3 are either uniformly distributed or anteriorly biased (Donoghue and Rakic, 1999). Further, the SP has chemical tags which navigate incoming thalamocortical axons to the appropriate target (Bolz *et al.*, 1996).

Cortical regions may be defined by early expression of unique gene combinations, reflected in the subsequent development of anatomical and functional specialisations (compare to Hox code). Early genes that broadly define the entire cerebral cortex include Emx1 and Emx2 (Simeone *et al.*, 1992). Emx-1 in the mouse cerebral cortex at E12.5 is found to be restricted to a more rostral part of the cortex. Pax6 a paired box gene shows a rostral-to-caudal gradient like expression pattern in the VZ (For a review see O'Leary *et al.*, 1994).

A number of investigators studying roles of growth factors in the regulation of events during corticogenesis have described the distribution of such proteins and their receptors. Examination of FGFR1 in the rat reveals the presence of the receptor at high levels in more caudal regions of the E17 cortex than rostral (Wanaka *et al.*, 1991). EGFR is present in selected regions of the embryonic and adult brain, in particular, the frontal cortex (Gomez-Pinilla *et al.*, 1988; Werner *et al.*, 1988;

Kornblum *et al.*, 1997). Particularly striking are the results from EGFR mutant mice lacking the EGFR. Examination of early postnatal brains of mutants reveals a dramatic reduction with loss of a major part of the frontal cortex with a consistent up-regulation of c-fos expression in the degenerating cortex (Sibilia *et al.*, 1998), indicative PCD. In the spinal cord a graded decline in an antibody that detects a 65 kDa protein defines the rostrocaudal gradient (Suzue *et al.*, 1990). Luskin (1993) demonstrated that progenitor cells situated in a discrete part of the neonatal SVZ generate immense numbers of neurons destined for the olfactory bulb different from more caudal SVZ cells which give rise to the majority of the glial cells of the cerebral cortex (Luskin 1993).

The recent cloning of a transcription factor lead Drutel *et al.* (1999) to investigate its role in the potential survival of neurons. ARNT2 revealed high levels of expression in the occipital cortex compared with more rostral cortex.

Conclusions

Experimental evidence suggests that early influences on the progenitor pool in the cerebral wall, through perhaps transcriptional regulators, growth factors, growth factor receptors, may define basic regionalisation of the cortex. Thus differences observed in our cultures may reflect underlying masked differences actually present in the rostral and caudal cerebral wall.

Rate of proliferation is the same in rostral and caudal cells

BrdU pulse labelling experiments were carried out to determine if survival differences in rostral and caudal cultures could be attributable to differences in their capacity to proliferate. In other words, to test whether the survival of caudal cells was

a result of more cells (as might be predicted by the neurogenetic gradient) were undergoing cell division and thus replenishing 'stocks' of cells, making dead cells a minimal fraction in the culture.

Cultures derived from rostral and caudal regions from all ages over the period of neurogenesis were incubated with BrdU for 1 hr before fixation at 1 DIV. Cells were not pulsed immediately following dissociation, as it was believed that the sudden removal from their intact environment disrupts the cell cycle process, and would not truly reflect the state of mitotic cells in culture.

The finding that the proliferative fraction was greater in younger cultures than older cultures comes as no surprise and consistent with the notion that during progression through neurogenesis, the proliferative population diminishes as young neuroblasts attain maturity and exit the cycle to make their way to the CP. This clearly demonstrates that the culture set up reflects events occurring during neurogenesis *in vivo* and thus is a suitable set up for further examination of rostral and caudal cultures. At E15 less than one third of the total cell population had entered S-phase at 1 DIV. This suggests that the remainder of the population had either just exited the cell cycle while being pulsed, were quiescent or were postmitotic. Dead cells were not considered. BrdU counts were made only on living cells. Incidentally, no dead cells labelled with BrdU suggesting that progression through S-phase is necessary before making the decision to continue to divide, differentiate or die. This opens up avenues for influences that are imposed on the cell during DNA synthesis that might determine their fate (McConnell, 1991). Interestingly, a difference in the proliferative capacity of cells in the rostral and caudal regions was noted. E15 caudal cells had a greater fraction of proliferating cells than rostral cells from the same age. About 9% more caudal cells were dividing than rostral cells. Although no real difference was observed

during E15 in the survival of rostral and caudal cells in culture, this could be the potential means by which a difference in the rostral and caudal regions of the developing cortex could be set up. However, no differences in the proportion of proliferating cells was observed at E16 and E17, at the peak of neurogenesis. It would be expected at these ages that the maximal difference between rostral and caudal cells existed in terms of the neurogenetic gradient, as more newly differentiating cells were likely to be present in rostral regions than caudal cortical regions. It is noteworthy that at 1 DIV similar fractions of cells are observed to be dead in culture, so changes in cell survival alone cannot predict the absence of difference in the rostral and caudal cortical regions. If anything, it might be predicted that fewer rostral cells would be proliferating since rostral cells might be getting 'ready' to undergo PCD. Alternatively, since cell death and cell proliferation are closely related in terms of machinery and mechanisms that regulate these events (Thomaidou *et al.*, 1997; Fang and Newport, 1991), it might be suggested that more cells in rostral regions would be seen to be BrdU positive since these are the populations of cells with the highest probability of undergoing subsequent cell death. Although no studies to date have examined changes in cell cycle parameters in the rostro-caudal axis, predicted by the medial to lateral observation that G_1 phase increases, that is fewer cells might be dividing in lateral regions compared to medial, it might be expected that the length of G_1 increases from caudal to rostral. This would however contradict the findings of this current study as no significant difference in proportion of cells cycling were observed, particularly since the numbers of cells examined are relatively equal (Nowakowski *et al.*, 1989; Caviness *et al.*, 1996).

It is pertinent to note the presence of serum in the medium did not dramatically affect the culturs. Cultures, as discussed elsewhere, do not contain

significant proportions of glia, which are known to have a high proliferative capacity and to produce mitogenic growth factors that enhance proliferation of neural precursors (Loughlin and Fallon, 1993). The few glial cells seen in caudal cultures cannot alone account for the survival of cells in caudal cultures.

The significance of cell proliferation bears a definitive relationship on cell death. The outcome of regulation of such events is critical to corticohistogenesis. (Voyvodic, 1996). The finding that significantly large populations of proliferating cells undergo cell death (Blaschke *et al.*, 1996; 1998) raises the question as to why the cortex produces such large quantities of cells only to programme them to commit 'cell suicide' afterwards? It is understandable to assume that normal cell death in the nervous system regulated the eradication of extra cells after size matching following synaptogenesis, but this does not explain why proliferating cells might apoptose. Voyvodic, (1996) discusses three possible reason as to why proliferating cells might die. According to Darwinian natural selection for weeding out unfit cells, cells with error in DNA replication or errors in cell differentiation are potentially detrimental to the normal function. Thus, the embryo has a mechanism to rid itself of such cells. Cells undergoing rapid proliferation are more prone to error than non-dividing cells. Alternatively cell death may be a method employed to be rid of cells which have already served their purpose in development (SP cells for example). Finally, the third possibility proposed is that cell death in the proliferative zone is not cell specific, but is a result of inadequate supply of exogenous survival factors. Similar to the notion of target derived factor dependence believed to mediate the later wave of cell death associated with synapse formation (Voyvodic, 1996).

Cell death is an important issue, especially when considering the implications on interpreting results where, until recently, it has been considered to have a minimal

role in development. Lineage studies for example assume that relatively little cell death occurs. Not accounting for cell death when examining clones could create a bias, especially if there is a reason for particular cell types to be programmed to undergo cell death, apparently leaving behind homogenous clones, which then are interpreted as cells having derived from a pre-committed precursor, when in fact it might have been multipotent (McConnell, 1995). Interestingly, Blaschke *et al.* (1998) confirmed that a positive correlation exists between the start of differentiation and onset of PCD and that indeed these two mechanisms are linked. One interpretation would be that cell death does follow a neurogenetic gradient, in some way providing further support for the results of the current study. Linking cell death closely to proliferation and differentiation is a useful mechanism to control and co-ordinate events in neurogenesis.

In addition, the BrdU pulse labelling results demonstrate a steady decline in the proportion of cells undergoing proliferation with increasing age, with fewest cells undergoing mitosis in E19 cortices. The difference in rostral and caudal cultures observed with respect to proliferation at E19, although statistically significant was relatively small, and thus not discussed here.

E17 and E15 cultures were further examined for rate of proliferation over time in culture. Understandably, the fraction of dividing cells diminished overtime. With increasing differentiation, increasing proportions of cells exited the cell cycle in both E 15 and E17 cultures (only caudal cells were examined in E17). This parallels the continued neurogenesis *in vitro* observed *in vivo* (Bayer and Altman 1991). However, this diminution could be a result of simply increasing the number of cells on the coverslip, thus directing the balance towards post proliferative cells rather than proliferative. This might be so, but the current study reveals that the dynamic nature

A caudal derived diffusible factor rescues rostral cells from cell death

To further understand the mechanisms controlling the differential survival of rostral and caudal cortical neural cell co-cultures were set up. Such cultures were used to test whether interactions between caudal and rostral cells could alter the survival of either group.

Equal proportions of rostral and caudal cells taken from E17 embryos when mixed and plated on the same coverslip resulted in a reduced proportion of cells dying when compared to rostral cultures incubated alone. When compared with caudal cultures, no significant difference was observed in the fraction of dead cells. This is a very interesting observation as it reveals something of the 'communication' between the two cells groups. If no interactions between caudal and rostral cells were occurring then approximately 50% of cells would be expected to be dead by the second or third day. However, previous results demonstrate that caudal cells are able to survive and proliferate. Taking this into account the expected figure would be lower. However, consideration must also be given to the fact that caudal cells demonstrate a small but significant proportion of dying cells. Taking all factors into account it may be expected that a greater proportion of dead cells are present than that observed (average 15.6%) in mixed cultures. This strongly suggests that rostral cells are rescued by caudal cells, as rostral cells cultured in the absence of caudal cells begin to show extensive PCD by 2 DIV. Mixed cultures were observed over 6 days and no significant increase in PCD was observed. Incidentally, no significant decrease occurred either. With respect to the latter, if the altered fraction of dying cells was in fact simply a reduction through an increase in the surviving caudal population, a decrease over time in culture of dead cells would be observed.

How do caudal cells rescue rostral cells in these cultures? What is the significance of such a rescue? In the first, caudal cells may establish intercellular communications with neighbouring cells *in vitro*, these by chance are equally likely to be between rostral and caudal in mixed cultures. If caudal cells promote their survival in isolation by developing intercellular communication channels, perhaps they do this with rostral cells, and thus co-ordinate survival. Alternatively, caudal cells as discussed above, may produce growth factors or other trophic factors that they release and act in an autocrine manner. Such a factor could also diffuse in the medium to affect neighbouring (rostral cells). The significance of enhancing the survival of adjacent cells maybe the mechanism by which neurogenesis is co-ordinated *in vivo*. That is, factors that are released may not function solely as trophic factors, but may be pleiotropic in nature such as basic FGF. The inadvertent rescue of rostral cells by caudal cells does not necessarily mean that these two cell groups, which *in vivo* are some distance apart, actually communicate directly, although it does suggest this. This communication *in vitro* is purposely set up and thus not necessarily a true reflection of events that occur *in vivo*. Importantly though, this experiment demonstrates that both cell groups are able to respond to similar cues. In terms of co-ordinating activity with respect to cortical development, this is a beneficial mechanism. This ability to respond to similar cues could form the basis of responses to concentration gradients that might be set up *in vivo*, since it is clear that rostral cells can respond to such a signal but fail to manifest the signal itself. Alternatively, such a signal from caudal cells could induce the production of the signal within rostral cells and thereby rescue them from cell death. This could be tested by removal of caudal cells following exposure. However, this experiment provides evidence for the presence of a diffusible signal which originates in caudal cells and that both rostral and caudal cells can respond to.

Evidence for cortically derived growth factors was investigated by Lotto *et al.* (1995) and Price *et al.* (2000).

To test if this rescue effect could be reproduced in older embryos, mixed E19 rostral and caudal cortical cultures were prepared. No such rescue was seen. Cell tracker labelling of rostral cells was used to confirm that caudal cells were specifically rescuing rostral cells, however leaching of the dye caused ambiguity in interpretation of the results.

If caudal cells rescued rostral cells via a diffusible factor then in the absence of potential cell to cell communication, rostral cells should still survive in the presence of caudal cells. To test this hypothesis, cultures of rostral and caudal cells were prepared on separate coverslips instead of mixing cells. Then coverslips plated with cells from each region were cultured together in a well in a minimal volume of medium. Upon examination at 3 DIV (the time at which rostral cells cultured alone had died), the majority of rostral cells in the presence of caudal cells had survived, confirming that a diffusible factor had been supplied by caudal cells. No significant change in the proportion of dying cells in caudal cultures maintained in the same well as rostral cells was observed. Although in theory, fibres could potentially extend from caudal cells across to rostral cells, and thereby provide direct cell to cell contact. However, in general, few if any cells grew at the periphery of the coverslip, secondly, no fibres were visible at the edges of the coverslip. Thirdly, it is not conceivable that sufficient contacts were made between caudal cells and rostral cells which lay at some distance from the former cells. Thus, it is unlikely that such connections caused the rescue of rostral cells. This experiment also allays the suspicion that 'death factors' that might be released by rostral cells which might cause them to die as caudal cells did not show any increase in cell death.

To determine if the survival factor produced by E17 caudal cells was able rescue E16 and E19 rostral cells and to determine whether E16 and E19 rostral cells could indeed be rescued, and therefore be responsive, further co-cultures were set up. In these latter experiments E17 caudal cells were cultured with either freshly prepared E16 rostral or E19 rostral cells. No significant rescue was observed. This can be interpreted to mean a number of things. Firstly, E16 rostral cells may not be mature (naive) enough to respond to such signal, that is ill equipped. Secondly, that if they have the receptors, the factor is produced in insufficient concentration to achieve the threshold to produce a response. Otherwise, a response maybe elicited, but is short lived and thus not detected at the time points examined (3 DIV). E19 rostral cells may have lost the capacity to respond to such signals, or that other overriding factors disable their response as discussed earlier for older embryonic cultures (greater dependence on other parameters for survival, rather than just growth factor signalling). The failure of E19 caudal cells to sustain themselves for longer than 30-48 hr suggests that perhaps caudal cells lose the capacity to produce this signal and or respond to it if they do continue to produce it. As E16 caudal cells do sustain themselves, it can be inferred that they too might be producing the same signal.

In conclusion, during early neurogenesis, all cells produce the 'survival factor', and with increasing age, rostral cells become restricted in their potential and no longer produce significant quantities to maintain survival, and by E19 even caudal cells fail to produce it. The production of this factor may reflect the neurogenetic gradient, or it maybe a factor that it is expressed distinctly in the two regions.

If indeed a diffusible survival factor is produced by caudal cells then it must be present in the medium of such cultures. To test this caudal conditioned media (CCM) was removed at various time points after culturing caudal cells and transferred

to existing rostral cultures after storage or following fresh aspiration. The survival response was not elicited in such experiments. This could be due to a number of reasons. Assuming that the factor is a protein or peptide, the factor may have been digested during aspiration since some cells would be torn, releasing proteases. Further, as all of the medium from caudal cells was removed, a possibility that levels of nutrients had diminished (after 4 days in culture this is expected), so that the failure of rostral cells to survive was largely due to insufficient nourishment rather than failure of the factor to promote survival. In addition, freezing the factor may have altered its tertiary structure thus disabling it to induce the desired physiological response. Alternatively, the factor may have to be continuously exposed to the rostral cells, and that a 'one off' exposure was not sufficient to elicit the desired response.

Basic FGF rescues rostral cortical cells from PCD

In an effort to investigate the survival promoting factor, rostral cortical cultures were treated with a range of neurotrophins and growth factors known to influence cortical neural cells. Of the growth factors and neurotrophins tested, basic FGF had the greatest and most significant rescue effect. This is by no means surprising. What was surprising was that other factors failed to elicit (except BDNF) a survival. In addition, rostral cells show a dose dependent response bFGF even at 0.1ng/ml. This highlights a number of points. First and foremost, rostral cells are equipped with the capacity to respond to bFGF. Possibly possessing the high affinity tyrosine kinase receptor FGFR1, or FGFR2. Secondly, if rostral cells produce bFGF then it is in insufficient concentrations to act in an autocrine manner above threshold levels required for survival, or that bFGF is not produced by these cells at all.

Alternatively levels of receptor expression are extremely low and comparatively high concentration of bFGF are required to invoke a survival response.

Basic FGF has been reported to promote the survival of fetal hippocampal neurons (Ray *et al.*, 1993); to protect cortical neurons from Ca^{2+} ionophore induced cell death (Takei *et al.*, 1995); and to promote the survival of cerebral cortical neurons (Morrison *et al.*, 1986). Further, *in vitro* studies suggested that bFGF functions in a concentration dependent manner to regulate other events including cell survival, differentiation, proliferation and adhesion (Petroski *et al.*, 1991; Ghosh and Greenberg, 1995; Qian *et al.*, 1997; Gensburger *et al.*, 1987; Murphy *et al.*, 1990; Dreyer *et al.*, 1989; Walicke 1988; Kinoshita *et al.*, 1993). Neurotrophins have also long been implicated in the survival of central neurons (for review see Skaper and Walsh 1998; Jacobson 1991). In particular NT-3 and BDNF are expressed in the embryonic cortex (Maisonpierre *et al.*, 1990) and their receptors are present in the VZ (Tessarollo *et al.*, 1993). Experimental manipulation of cultures with anti-BDNF (Ghosh *et al.*, 1994) results in severe diminution in survival of cortical neurons, but this is not the case for NT-3. Thus, the results of the present study are in line with previous reports.

The effects of bFGF on the proliferation of rostral cortical cells

Basic FGF is known to promote the *in vitro* survival of large numbers of different central neuronal populations, as described above (Morrison *et al.*, 1986; Walicke *et al.*, 1986; Murphy *et al.*, 1990). The present study too, demonstrates the survival promoting ability of bFGF. However, does bFGF promote the survival directly or by mediating another response whose net effect results in survival. For example does bFGF promote the proliferation of rostral cells, thus increasing the

number of cells present in the culture? Basic FGF has been shown to promote proliferation of neural cells (Kilpatrick and Bartlett 1993; 1995;Ghosh and Greenberg 1995). However, evidence is available that bFGF can promote the survival of cells without affecting proliferation, that is, in the presence of a mitotic inhibitor (Morrison *et al.*, 1986). However, recent evidence from FGF2 loss of function transgenic mice reveals that bFGF is not essential for cell proliferation during cerebral cortex development (Dono *et al.*, 1998) suggesting another role for bFGF *in vivo*.

In this investigation, BrdU pulse labelling of bFGF treated and untreated rostral cultures revealed that proliferation is affected, but only a marginal increase was detected at 1 and 3 DIV. It is hard to believe that a 7% increase in proliferation can result in almost 80% rescue of rostral cells. Alternatively, as bFGF is known to promote the differentiation and proliferation of glia (Ali *et al.*, 1998), rostral cells could be rescued by growth factors secreted by newly generated glia. A recent report suggests that cortical neurons die after separation from glial cells as a result of neurotrophic deprivation, implying that glial cells produce neurotrophic substances. Or that the only cells surviving are the newly and largely expanded glial cell population. In considering this result in terms of the effect of bFGF *in vivo*, it is more likely that bFGF in the cerebral cortex (Vaccarino *et al.*, 1999) effects the neuronal survival and less so the proliferation (Dono *et al.*, 1998) or differentiation of glial cells in early development (Vaccarino *et al.*, 1998) since gliogenesis does not occur until postnatal development (McConnell, 1988).

The effect of bFGF on the survival of cells in the presence of a mitotic inhibitor

To eliminate the possibility that the mediation of survival of rostral cells by bFGF was via the effect on proliferating cells, AraC, a mitotic inhibitor (Morrison *et*

al., 1986) was applied to rostral cultures. Initially the optimal concentration was determined using the MTT assay. Primary cultures of caudal cells were used, in the presence of AraC, to determine the optimal concentration. High concentrations of AraC above $1X10^{-4}$ mg/ml resulted in toxicity, below $1X10^{-5}$ mg/ml of AraC delayed cell death amongst caudal cells. This suggests that proliferation is important for the independent survival of caudal cells, but obviously not essential since not all caudal cells are cycling. However, as cell plating density is an important factor in the survival of cells in culture, death of cycling cells, thereby reducing the apparent cell density, may initiate a cascade of cell death throughout. This may be a result of diminished growth factor production.

AraC applied to rostral cells in the presence of bFGF resulted in survival of cells. This strongly suggests that bFGF acts to a greater or lesser extent directly on the neural cells in promoting their survival rather than simply promoting the differentiation of neurotrophic factor producing cells (glia). This is contiguous with the mechanism that caudal cells might employ to promote their survival. That is, a direct effect of producing a factor which acts in an autocrine manner to promote their survival and not dependent on other cell populations to promote their survival.

Basic FGF is necessary for the survival of caudal cells

Results thus far indicate that a factor is produced in caudal cells that promotes their survival and that is able to promote the survival of rostral cells even in the absence of cell to cell contact. Further, of the factors tested, bFGF is able to rescue rostral cell from cell death. Perhaps bFGF is produced by caudal cells and is the factor that promotes their survival and in co-cultures the survival of rostral cells. To test this hypothesis the action of bFGF was inhibited using genistein (Gu *et al.*, 1996). When

applied to caudal cortical cells immediate cell death was not induced, suggesting that application of this agent did not induce toxicity. However, at 3 DIV nearly all caudal cells underwent cell death whilst untreated caudal cells continued to survive. This implies that bFGF is important for the survival of caudal cells, and in the absence of exogenous bFGF, genistien mimicked a 'neurotrophic deprivation' effect. Addition of exogenous bFGF to caudal cultures was used to test if genestein was truly blocking the effect of bFGF. Caudal cultures in the presence of bFGF alone (data not shown) promoted proliferation and altered the morphology of the cells somewhat (Kinoshita *et al.*, 1993). Caudal cultures failed to be rescued by bFGF in the presence of genestein, providing strong evidence that genestein inhibited the action of bFGF on caudal cells. In sum, these experiments strongly suggest that bFGF is the factor produced by caudal cells to promote their survival. In an effort to confirm this, neutralising antibody (Ab 117 UBI) was applied in the presence and absence of bFGF in caudal cultures at increasing concentrations. This antibody has previously been reported to inhibit activity of bFGF in cerebellar cultures. Even 100 μg/ml failed to reproduce the effect observed in genestein treated cultures. The neutralising antibody also failed to block the bFGF induced proliferation. These experiments were thus disregarded and the results considered to be ambiguous due to insufficient neutralisation of bFGF by the antibody. Incidentally, caudal cells have a more spread morphology. Kinoshita *et al.* (1993) proposed that bFGF affects the cell spreading which they speculate provides better support for cells and thus improving their survival without compromising their ability to cycle.

Basic FGF and FGFR1 are present in rostral and caudal cultures

The hypothesis that caudal cells are producing bFGF and that this is the survival factor discussed earlier, then it must be present in caudal cells. Indeed caudal cells must also possess the receptor. As earlier experiments have demonstrated that rostral cells are rescued by a diffusible signal emanating from caudal cells, and since rostral cells respond to bFGF they, too, must possess the receptor. The exact distribution of bFGF in the brain has been a point of great controversy. In order for bFGF to affect a cell directly, the cell must be equipped with receptors to bFGF. Basic FGF has been found to bind with high affinity to FGFR1 (III c isoform) (Johnson and Williams 1993). Binding results in receptor dimerisation (Bellot *et al.*, 1991) and tyrosine kinase activity (Neufeld and Gospodarowicz, 1986). The distribution of FGFR 1 is still an issue of debate. The present study describes conclusively the distribution of bFGF and FGFR1 using immunohistochemistry, radioactive and non-radioactive insitu hybridisation and Western blotting. Further, the presence of the receptor and protein are described in E17 rostral and caudal cultures.

Immunocytochemistry for bFGF and FGFR1 reveals the presence of bFGF and FGFR1 in a significant proportion of cells in both E17 rostral and caudal derived cortical cultures. Approximately 59.1% rostral cells and 63.9% caudal cells were immunoreactive for bFGF. The fact that almost the same proportion of cells contained bFGF in both rostral and caudal cultures suggests that bFGF is not the factor responsible for promoting the survival of cells as it is present in both cultures at the approximate same ratio. Alternatively the difference in survival may be as a result of difference in responsiveness to the released factor, or differences in the release from cells. Speculation has it that the mechanism by which bFGF might be released is by dead or dying cells, and as more cells appear to die in rostral cultures then caudal

cultures, it might be expected that the initial sacrificed population would rescue the remaining cells in culture from cell death. Obviously this is not the case. It has already been demonstrated that rostral cells survive in response to bFGF, therefore it maybe inferred that bFGF release is mediated by some other mechanism and not cell death. It maybe that bFGF is not secreted at all, and is utilised intracellularly (Logan et al., 1990) or that the amount released is insufficient to attain the physiological survival response. The rostral cells may not possess the receptor to detect released bFGF.

FGFR1 immunoreactivity reveals that it is present in rostral and caudal cells. This confirms the responsiveness of rostral and caudal cells to exogeneously applied bFGF seen in earlier experiments. Approximately half (51.5%) rostral cells have the potential to respond to bFGF. Significantly more caudal cells possess the receptor (60.8%). It is noteworthy that not all cells possess the receptor. This implies that, perhaps, more than simply bFGF alone is required for caudal survival. If the mechanism proposed here is true, that is bFGF acts in an autocrine manner to promote the survival then only 60.8% cells can respond. Incidentally, this proportion is very close to the proportion of cells producing bFGF in these cultures, providing further evidence for the notion of an autocrine mechanism. Although the bFGF blocking experiment suggests that all cells degenerated due to lack of bFGF , it could be that the approximately 60% cells that required bFGF died and as a result of diminished cell density, the remainder followed suit. Alternatively, another receptor may be important in mediating responsiveness to bFGF. FGFR 2 and FGFR 3 have also been shown to dimerise following bFGF binding (Johnson and Williams, 1993). It would be interesting to know if co-localisation between receptor and growth factor exists. This would confirm the proposed autocrine mechanism of action of bFGF.

Conclusion

These experiments confirm that bFGF is present in caudal cells. That caudal and rostral cells possess the receptor able to mediate the survival response. Further, inhibiting the action bFGF on caudal cells results in severe cell loss, providing further evidence that these cells specifically require bFGF for survival. However, it is possible that aFGF, very similar to bFGF, mediates these response, or maybe even other FGFs.

Cell types present in rostral and caudal cultures

To examine the cell types constituting rostral and caudal cultures, and to determine if differences in cell types existed between the two culture groups, immunocytochemistry for known cell specific markers was employed in cultures derived from different embryonic ages. Furthermore, insights into which cell populations were most vulnerable to death, and most likely to be responsive to bFGF were obtained.

E17 rostral and caudal cultures contained nestin, MAP-2, GABA immunoreactive cells, caudal cultures also contained GFAP immunopositive cells. Thus, the cultures consisted of undifferentiated precursor cells, GABAergic neurons, and caudal cells also contained a few astroglial cells. As cultures were examined only 24 hr after plating, it was assumed that proportions of cells present in cultures reflect closely those proportions observed *in vivo*. Just less than half the cells in both rostral and caudal cultures were undifferentiated neuroepithelial cells. *In vivo*, the ventricular epithelium occupies a large portion of the radial depth of the cortex. However, an abnormally large portion of rostral and caudal cells have differentiated and become neurons (MAP-2+), not reflecting the situation *in vivo*, in which the few neurons

express this protein, a marker for well differentiated neurons. However, Qian *et al.* (1997) noted that upon dissociation, a large proportion of cells exit the cell cycle and become postmitotic neurons. A small fraction of GABAergic neurons is present in these cultures, more in caudal than in rostral, a paradoxical result when considering rostral regions tend to be more differentiated (Bayer and Altman, 1991). However, in line with the neurogenetic gradient, fewer neurons are present in caudal than rostral cultures. Therefore, no striking differences are present in the cell types at E17. Thus, in terms of cellular vulnerability, if any one group of cells is more prone to PCD in isolation than any other, the probability of cell death occurring in either rostral or caudal cultures would be predicted to be the same. It maybe suggested that differential cell survival can not be solely attributed to the constitution of the regional cortical cultures. It maybe argued that the difference in the neuronal population accounts for the difference in survival between caudal and rostral cortical cultures. If neuronal content of cultures determines the survival, then with increasing time in culture, increase in differentiation is observed, but caudal cells continue to survive. However, the ratio does diminish as proliferation exceeds differentiation. Paradoxically, nestin positive cells also diminish over time in culture (caudal cultures examined). This suggests that the mature neuronal phenotype has not been expressed and thus MAP-2 immunoreactivity is an underestimate of true neuronal cells in culture. This is however consistent with diminishing VZ epithelia *in vivo* with increasing age of the embryo (Bayer and Altman, 1991).

To understand the process of differentiation a little more, injections of BrdU were made at E16 to label newborn neurons. Cultures of rostral and caudal cells were prepared and BrdU positive cells counted. An approximately 5% difference in BrdU labelled cells was observed, suggesting that the generation of neurons was

approximately equal in rostral and caudal regions, and thus the differential survival was not simply due to the neurogenetic gradient.

Similarly, immunocytochemistry for cell type composition of rostral and caudal cultures derived from E19 embryos was used to elucidate the mechanisms underlying the differential cell death, and whether there was a direct correlation with cell type and cell death. Preliminary results reveal that all cell types are present, that is undifferentiated neuroepithelial cells, differentiated neurons, GABAergic and glutamatergic neurons and astroglial cells (in caudal cultures only). As cultures were derived from embryonic brains in which neurogenesis is coming to an end, relative proportions of these different cell types were expected to have changed. *In vivo* a reduction in proliferating neuroblasts with a consequential reduction in the thickness of the VZ is observed whilst neuronal, moreover differentiated neuronal cells, increase as more cell exit the cells cycle and migrate to the CP. The present cultures reflect these events *in vitro*. Furthermore, the ratios of GABAergic and glutamatergic cells represents the true ratio observed *in vivo*. It is apparent that the greatest proportion of cells are the mature neurons in both rostral and caudal cultures. Only a negligible difference in the proportion of neurons in rostral and caudal cultures is observable. Indeed, these cells maybe vulnerable, and hence explain the exaggerated rate of cell death in cultures derived from both regions. However, the delayed cell death of caudal cells cannot be explained by neuronal composition, since this is almost equivalent.

To determine which cells are undergoing cell death it would be necessary to monitor which cell types remain in the culture. However, due to the dynamic nature of the culture, that is continued proliferation, differentiation and cell death, this was not possible.

In addition, qualitative examination of E17 rostral cultures treated with bFGF and immunostained for GFAP and nestin reveal a significant increase by 3 DIV. However, although an increase in astroglial cells is observed, these cells still only occupy a minor proportion of the culture, whilst nestin immunopostive cells appear to have increased many fold. Thus, consistent with previous reports (Ali *et al.*, 1998, Cavanagh et al., 1997), bFGF increases the neuroblast population, and since proliferation was only marginally affected, it may be concluded that differentiation to a postmitotic state is delayed. It may be considered that the addition of bFGF to the culture caused an increase in the GFAP immunoreactive population which in turn promotes the survival. In line with the explanation given earlier, this is not necessarily the case, as only few astroglial cells in bFGF treated cultures are observed early on (1DIV). Thus, bFGF seems to be acting non-specifically on neuronal and astroglial precursors, as well as directly on neurons. Double labelling for bFGF or FGFR1 with cell specific markers would provide a better understanding of the interaction of bFGF and specific cell types.

Basic FGF mutant experiments

Preliminary results from cultures prepared from loss-of-function FGF2 mutant mice reveal some interesting insights into the differential regulation of survival of rostral and caudal cells *in vitro*, and provide further support for the hypothesis that the diffusible survival signal originating in caudal cell is bFGF. In mutant cultures, both rostral and caudal cells degenerate, suggesting that bFGF is essential to the survival of cortical cells. Further, these experiments provide strong evidence that caudal cells indeed produce bFGF that effects their survival, and thereby the survival of neighbouring cells, even cells at some distance. However, as control experiments

were unsuccessful, extrapolation of results is hindered. In support of the present findings, two separate investigators (Dono *et al.*, 1998; Ortega *et al.*, 1998) have described the abnormal cortical phenotype in embryonic neocortex in mutant animals. FGF2 deficient litter mates display defects in organisation and differentiation of cerebral cortex. In particular the thickness of the cerebral cortex in homozygous mutants was reduced by ~10% when compared to wildtype controls (Dono *et al.*, 1998). Moreover, pronounced abnormalities in the cytoarchitecture of the frontal motor sensory area were detected by immunohistochemistry and histological examination. This supports the current hypothesis that differential physiological requirements by rostral and caudal cells of bFGF exist. The authors speculate that the diminished cell density and differential effect on frontal sensory motor cortex maybe due to cell death (Ortega *et al.*, 1998). In addition, both studies indicate that such phenotypes are as a result of interaction that bFGF would have with neurons as opposed to astroglial cells, consistent with the current investigation. Furthermore, microinjections of bFGF into the ventricles of developing cerebral cortex reveals an increase in the volume and total number of neurons in the cortex. Interestingly, adult treated rats showed cortical enlargement particularly in the antero-posterior dimension (Vaccarino *et al.*, 1999). Considering the massive amount of cell death that occurs in developing cerebral cortex (Blaschke *et al.*, 1996, 1998; Thomaidou *et al.*, 1997) injected bFGF maybe rescuing such cells from cell death, resulting in an increase in the cell number in addition to the increase in BrdU uptake observed (Vaccarino *et al.*, 1999). Additional evidence for differential survival comes from mice lacking EGFR, in which massive cell death is observed in frontal cortex. And finally, insitu hybridisation for FGFR1 mRNA transcripts has been reported by Wanaka *et al.* (1991). Examination of sagittal sections of labelled cortex reveals a distinct elevated

expression in caudal cortex. Recently ARNT2 a transcription factor regulating the survival of neurons displays higher expression pattern in caudal cortex of the adult rat brain (Drutel *et al.*, 1999).

Dorsal lateral geniculate (DLG) body undergoes cell death following ablation of the visual cortex. The DLG is entirely trophically dependent on the integrity of the cortex. Evidence has been presented that such a trophic factor is a diffusible agent (Cunningham *et al.*, 1987). Previous research in the peripheral nervous system has shown that soluble growth factors regulate developmental events in an autocrine and paracrine fashion. The work of Price and colleagues (Lotto and Price, 1995) has revealed new and exciting insights into diffusible signals originating the cortex (Lotto *et al.,* 1994). *In vitro* studies have shown that neocortical explants promote the growth of neurites from embryonic thalamic explants (Lotto and Price, 1994, 1995). Further, Hisanaga and Sharp (1990) have demonstrated a trophic dependence of some dissociated thalamic neurons on diffusible factors released by the cortex *in vitro.* In contrast to the present study, prenatal slices of anterior or posterior cortex fail to stimulate outgrowth from co-cultured thalamic slices, arguing against the notion that diffusible cortex-derived growth factors, in particular the influence on thalamocortical development (Lotto and Price, 1995; Rennie *et al.*, 1994). Postnatal cortical slices rescue dissociated thalamic cells from cell death in co-cultures. Further, investigation reveals that levels of mRNA for FGF are increased in the postnatal brain (Maisonpierre *et al.*, 1990). E14 occipital cortex conditioned media is able to prolong the survival of DLG nucleus neurons following ablation of the entire posterior one third of the neocortex (Cunnigham *et al.*, 1987).

7.2. DISCUSSION II

The results presented here describe the localisation of bFGF and one of its high affinity receptors in the embryonic brain at E17. This investigation demonstrates the widespread distribution of bFGF in the cerebral cortex and of FGFR1. Moreover the pattern of distribution of these proteins suggests early regionalisation in the cortex. A multidisciplinary approach was used to identify these proteins and their transcripts.

Basic FGF immunoreactivity in the developing cerebral cortex

The results reported here were obtained with 2 antibodies raised to bFGF. Ab 106 (Gonzalez *et al.*, 1995) is raised against a peptide fragment of rat bFGF. It recognises all three isoforms of bFGF. The second antibody, Ab 118 (Matsuzuki *et al.*, 1989) was raised to purified bovine brain bFGF, and found not to cross react with bovine aFGF. Ab 118 recognises the 18 kDa form of bFGF.

Ab 106 immunoreactivity is observed in CP, SP , VZ and SVZ and scattered amongst the migrating neuroblasts of the IZ. Thus, all three isoforms, 18 kDa, 21 kDa and 23 kDa are present within the embryonic cortex. Of particular interest is that caudally positioned cells are more intensely stained than more rostral regions in contrast to findings of Vaccarino and colleagues (1999) of uniform immunoreactivity. However, they comment that down regulation in the VZ follows the neurogenetic gradient (Bayer and Altman, 1991). It is proposed that the difference observed in staining could be due to differences in packing density, but in line with the present results, it is proposed that more caudal regions of the cortex possess more protein. This confirms previous reports that bFGF is present in the cortex of embryonic brain (Gonzalez *et al.*, 1990; Caday *et al.*, 1990; Gomez-Pinilla1994; Powell *et al.*, 1991;

Weise *et al.*, 1993; Matsuyama et a 1992; Vaccarino *et al.*, 1999; Dono *et al.*, 1998). However, Kuzis *et al.* (1995), failed to detect bFGF immunoreactivity in embryonic cortex. There is some discrepancy between reports with regard to the exact distribution of this protein in the embryonic cortical laminae. Immunohistochemistry for bFGF detected using Ab 118 reveals that only deep CP and SP cells are immunoreactive. This suggests that different isoforms have different functions in the cortex and that 18 kDa isoform of bFGF is involved in the differentiation and perhaps survival of cells of the SP. Further, bFGF may act as a chemoattractant for corticothalamic fibres. Thus, 21 kDa and 23 kDa isoforms may specifically regulate proliferation and differentiation of ventricular neuroepithelium. E16/17 is considered to be the time of strongest bFGF staining (Weise *et al.*, 1993). At this stage, neurogenesis is at its peak. The labelling in the proliferative zone suggests that the physiological function of bFGF could be mitogenic. Dono and colleagues report that bFGF is not essential for cell proliferation (Dono *et al.*, 1998). In contrast, exogeneously applied bFGF *in vivo* increases cell number. It is proposed that this increase is as a result of bFGF acting on progenitor cell proliferation (Vaccarino *et al.*, 1999). However, expression in the CP suggests a more trophic and differentiating role. This conclusion is supported by the current *in vitro* experiments of exogeneously applied bFGF (Walicke, 1988) and blocking of bFGF function using genestein. bFGF *in vivo* may act to attenuate the natural cell death (apoptosis). Massive cell death occurs in the proliferative epithelium as well as in the CP (Blaschke *et al.*, 1996, 1998). Basic FGF might be the mechanism by which vast numbers of cells are replaced, by proliferation of VZ cells and/or promoting their survival. Baisc FGF in the IZ may support neuronal migration. The significance of more intense labelling in the caudal region, or presumptive visual cortex is that of differences in the resultant

cell numbers in this region compared to other regions, particularly frontoparietal cortex. Specifically, Finlay and others have described increased cell numbers in the occipital cortex (Finlay and Slattery, 1983) and Polleux *et al.* (1997) described differences in the timetable of neuronal generation in presumptive area17 and 18. Such regional specific phenomena maybe under the control of factors such as bFGF.

Immunoblotting data using Ab 118 detects the presence of 18 kDa protein in both rostral and caudal cortices. It appears that there is more protein in caudal regions than rostral, although total protein content was not determined in this study. Specificity for 18 kDa isoform of bFGF is demonstrated by Ab 118 as no other bands are detected above this molecular weight. A possible breakdown product could be the nature of the ~ 10 kDa band observed in all three lanes, rostral cortex, caudal cortex and recombinant human bFGF. bFGF has a short half life and is susceptible to proteases. In addition only a crude cytoplasmic extract was obtained which is known to contain largely 18 kDa isoform. Appropriate controls demonstrated specificity of binding of antibodies. Adult cortex contains bFGF primarily in glial cells (Logan *et al.*, 1995)

Investigators have occasionally reported different patterns of distribution (Folkman *et al.*, 1988; Kardami *et al.*, 1990). What is apparent is that the method of fixation and processing affects the immunolocalisation of the protein, and the antibody specificity. Here, although no large differences are observed in preparations for this study between paraffin embedded and cryostat cut sections, different results were obtained between the two antibodies used in the study.

FGF2 deficient mice display neurological defects, in particular cell density is reduced (Dono *et al.*, 1998) and differential effects on frontal sensory motor cortex (Ortega *et al.*, 1998) as well as altered migratory behaviour (Dono *et al.*, 1998).

Consistent with these findings is that bFGF is found in IZ of wild type. The expression of bFGF in the VZ indicates that it participates in defining both cell fate (Ghosh and Greenberg 1995) and migration. It may be proposed that as bFGF is present *in vivo* in a variety of cells, culture cells containing bFGF largely originated in the VZ and are thus neuroepithelial cells, and some CP and SP neurons. As not all IZ cells were labelled, the unlabelled population observed *in vitro* described here are likely to be some of these migrating neurons.

In situ hybridisation for bFGF

There is some disparity amongst the results from radioactively labelled oligonucleotide probes and the DIG labelled antisense riboprobes. Little if any label was observed in autoradiographs of bFGF. Although above background levels bFGF transcripts appear to be diffusely present throughout the cortex of the embryo, no specific lamination could be observed. However, DIG labelling reveals that the messenger expression resembles closely that observed in immunohistochemistry for the protein when examine using Ab 106, but overlaps with Ab 118. The CP, SP, IZ and VZ cells demonstrate labelling. Intense labelling is seen primarily in the CP and VZ. Again a gradient-like expression is observed with rostral light and caudal dense labelling. Providing conclusive evidence that bFGF is actually present within these layers. Low sense signalling was detected, indicating specificity of the probe.

Giordano *et al.* (1992) described the developmental regulation of different molecular weight forms of bFGF in the CNS in an effort to determine how one growth factor can mediated the multitude of effects observed by bFGF. In agreement with the present finding that different molecular weight forms are present and therefore can mediate different effects, particularly , the 18 kDa form present in the

deep CP/SP cells possibly mediating survival and signals for in coming afferent. Since higher molecular weight forms are predominantly located in the nucleus (Renko *et al.*, 1990, Bugler *et al.*, 1991) differences in function can be inferred. Basic FGF can potentially act at the level of DNA as well. This is consistent data presented here, that 21kDa and 23 kDa forms are expressed in the VZ, where DNA synthesis and cell fate decisions are taking place

Immunostaining and in situ hybridisation for FGFR1

To understand how bFGF exerts its effects, the FGFR1 distribution was mapped using immunohistochemistry and in situ hybridisation. For the immunolocalisation of FGFR, an antibody raised against a specific peptide sequence of the C-terminal domain of human FGFR1 (Gonzalez *et al.*, 1995). The results from this study showed that the FGFR1 antibody is specific, as pre-absorption controls showed no or little receptor localisation. Immunoreactivity was detected in paraffin embedded and frozen sections. FGFR1 positive cells were present in all layers, with greater intensity observed in VZ. This is consistent with the distribution of bFGF, and also consistent with previous reports of FGFR1 localisation (Vaccarino *et al.*, 1999, Weise *et al.*, 1993; Wanaka *et al.*, 1991). Further, cryostat sections revealed a distinct laminar distribution with specific immunoreactivity in the CP and VZ, and a distinct difference in staining in more rostral and caudal cortex, the latter being more intensely labelled (Wanaka *et al.*, 1991) consistent with previous reports that there is asymmetry in FGFR1 distribution in the antero-posterior axis. These findings suggest that regional co-expression of growth factor and receptor allow bFGF to mediate its effects in an autocrine or paracrine manner. The consistent finding of FGFR1 immunoreactivity in the neuronal and neuroepithelial layers adds support to the notion

that bFGF can serve more general trophic functions in neuronal maturation as well as regulate proliferation and cell fate decisions. In situ hybridisation demonstrates the expression of transcripts of the receptor consistent with FGFR1 immunoreactivity, conclusively confirming the distribution.

7.3. DISCUSSION III

Immuohistochemical localisation of known cell specific markers was performed on sagittal section of E17 cortices to determine if any striking differences were present in the rostro-caudal axis of development. MAP-2 immunoreactivity followed the natural neurogenetic gradient, as did TUJ1 staining for newly generated neurons, and so did nestin immunolabelling (Bayer and Altman 1991).

The final part of this present study investigated the mode of cell death employed by rostral (and some caudal cells) in culture using TUNEL labelling, scanning and transmission electron microscopical methods. Insights into the molecular mechanisms underlying follicular B cell lymphoma and genetic studies of the stereotypic cell death in nematode lead to the identification of death repressor (Bcl2) and apoptosis promoting molecules (e.g. Bax). Neuronal death but trophic factor deprivation has been shown to be modulated by levels of such molecules (Greenlund *et al.*, 1995). Here, Bax immunohistochemistry was performed to determine (data not shown) whether any regional distribution existed that may further explain the mechanism behind the differential cell death observed in culture. Bax immunoreactivity was located primarily in the CP but no regionalised expression was observed. Bax immunoreactivity in the CP coincides with the notion that neuronal cells are more susceptible to cell death, and that neurotrophic factors including bFGF may act to regulate the balance between cell death promoting and repressing genes.

Morphology of dying cells

To understand the mode of cell death employed, largely, by rostral cells in culture TUNEL histochemistry combine with vital dye labelling and analysis at the light microscope revealed that a large proportion of cells (not quantified) were TUNEL + (Gavrielli *et al.*, 1992). PCD is a selective process of physiological cell deletion (Wyllie 1981). Its execution plays a major role in the control of shape and size in normal and abnormal processes (Kerr *et al.*, 1972). It seems that chromatin cleavages is the most characteristic biochemical feature of the process (Wyllie *et al.*, 1980).

Morphologically, PCD or apoptosis shows certain gross features identified at the EM. Ultrastructural features observed indicating apoptosis were the appearance of a shrunken nucleus, condensed chromatin and relatively intact cytoplasmic organelles and plasma membrane in an early stage. Necrotic neurons found demonstrated severe membrane disruption and loss or distortion of cytoplasmic organelles. This latter type of cell was considered to be the secondary necrosis often observed after cells complete apoptosis and are not cleared away by macrophages. The most striking change was the margination of the condensed chromatin within the nucleus. Other features observed were cytoplasmic condensation, membrane bound apoptotic bodies, Sometime the condensed chromatin covered the cross sectional area of an apoptotic body whilst in other cells the condensed chromatin formed a crescent shape on the inside of the membrane. Blebbing was not obvious at the electron microscope. At later stages of cell death, perhaps at the early stages of secondary necrosis, cells became very rounded. Having escaped phagocytosis apoptotic cells spontaneously

degenerated. The binding membranes appeared to have ruptured and swelling and dissolution of their organelles was observed.

A characteristic of cells undergoing apoptosis is extensive membrane blebbing of the plasma membrane (Wyllie *et al.,* 1980). Whereas neurons that had not undergone cell death showed variation in neurite out growth, with a range of unipolar, bipolar and multipolar cells observable in culture and relatively uniform membranes displaying a mild scaly appearance, apoptotic cells displayed changes on the membrane surface. Early apoptotic cells appeared to have ripple formation on the membrane, these convolutions became extensive. Some cells had marked membrane blebbing whilst other cells it was questionable as to whether membrane blebbing was occurring or the spontaneous degeneration of the cell and compromised integrity of the membrane. Nevertheless, the scanning transmission electron microscopical evidence conclusively confirms that cells in culture were undergoing cell death by apoptosis and not necrosis. The exact nature of the cell undergoing PCD was not discernible.

Further, light microscopical findings that DNA fragmentation and compartmentation into apoptotic bodies was occurring as it does in apoptosis was revealed by TUNEL histochemistry and PI labelling. Bright fluorescent spot inside cells were observable, correlating to those described by Gavrielli *et al.* (1992) and to the electron microscope findings. PI labelling appeared to pick up more cells. Further, phase contrast the long well delineated neurite network reduced in cultures undergoing cell death. Nuclear fragmentation was also visible at the light microscopical level.

Thus, it may be concluded that rostral cells and some caudal cells in culture undergo PCD when isolated *in vitro* and that bFGF is able to rescue these cells from actively undergoing cell death.

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Figure 1

A montage of a section of the cerebral cortex at E17. The vast majority of the cells of the ventricular zone (VZ) were radially oriented, with a few mitotic figures apparent at the ventricular surface. The subventricular zone (SVZ) shows a more varied orientation of the progenitor cells. The intermediate zone (IZ) occupies a greater area of the cerebral mantle, with cells more sparsely distributed and tangentially orientated. No mitotic figures were present in the IZ. The cortical plate (CP) at E17 was several cells thick. These also have a marked radial orientation. The marginal zone (MZ) occupies the subpial space, with few horizontally oriented cells. Lateral ventricle (LV). Scale bar 150 μm.

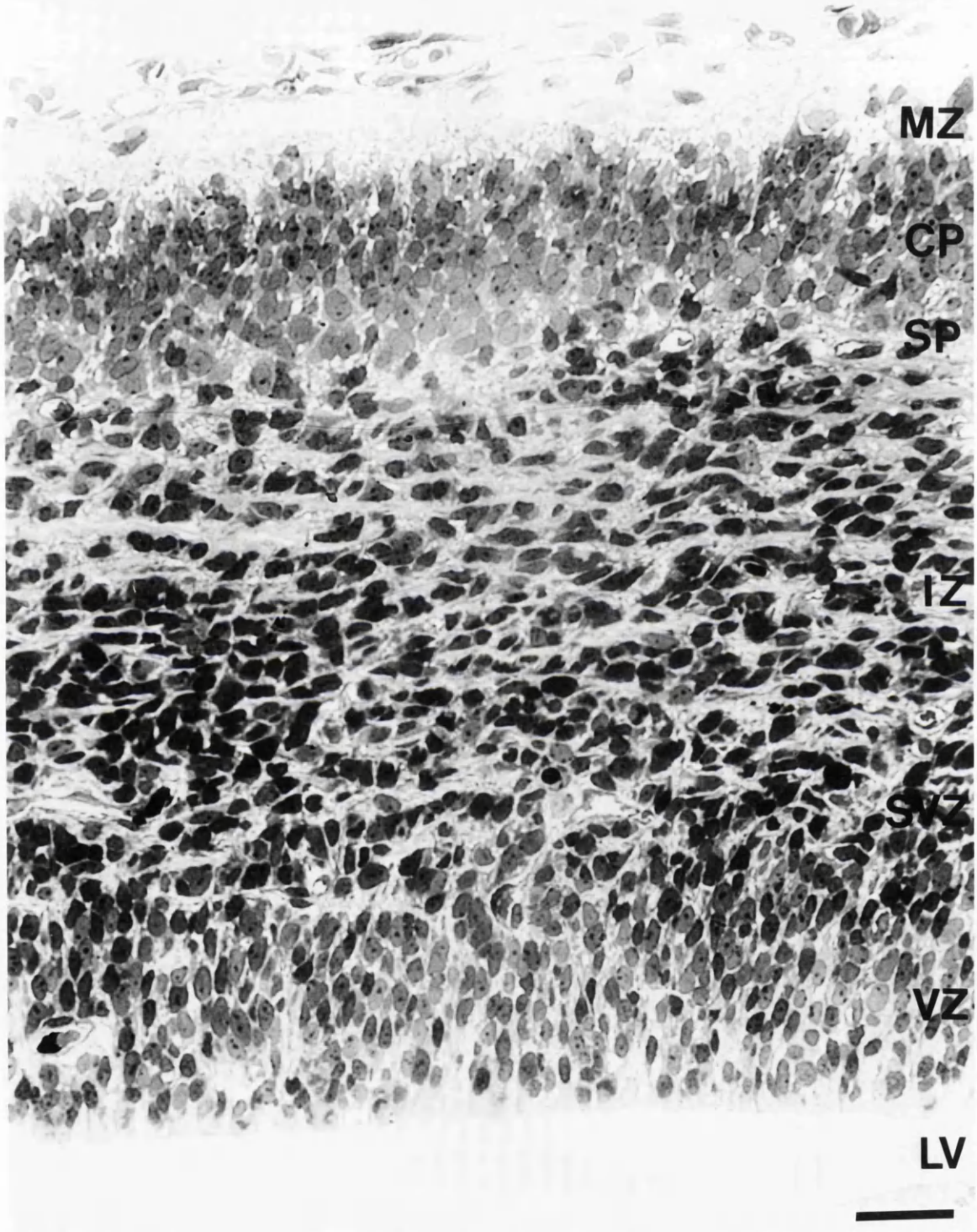

MZ
CP
SP
IZ
SVZ
VZ
LV

Figure 2

The progressive development of the cerebral cortex was demonstrated in sagittal sections cut from paraffin embedded embryonic cortices at E14 (A), E17 (B) and E19 (C). Note the reduction in the ventricular space with expansion of the cerebral cortex. The difference in thickness of the cerebral cortex rostrally (right) compared with caudally (left) was more apparent at E17 than E14 and E19. Scale bar = 800 μm.

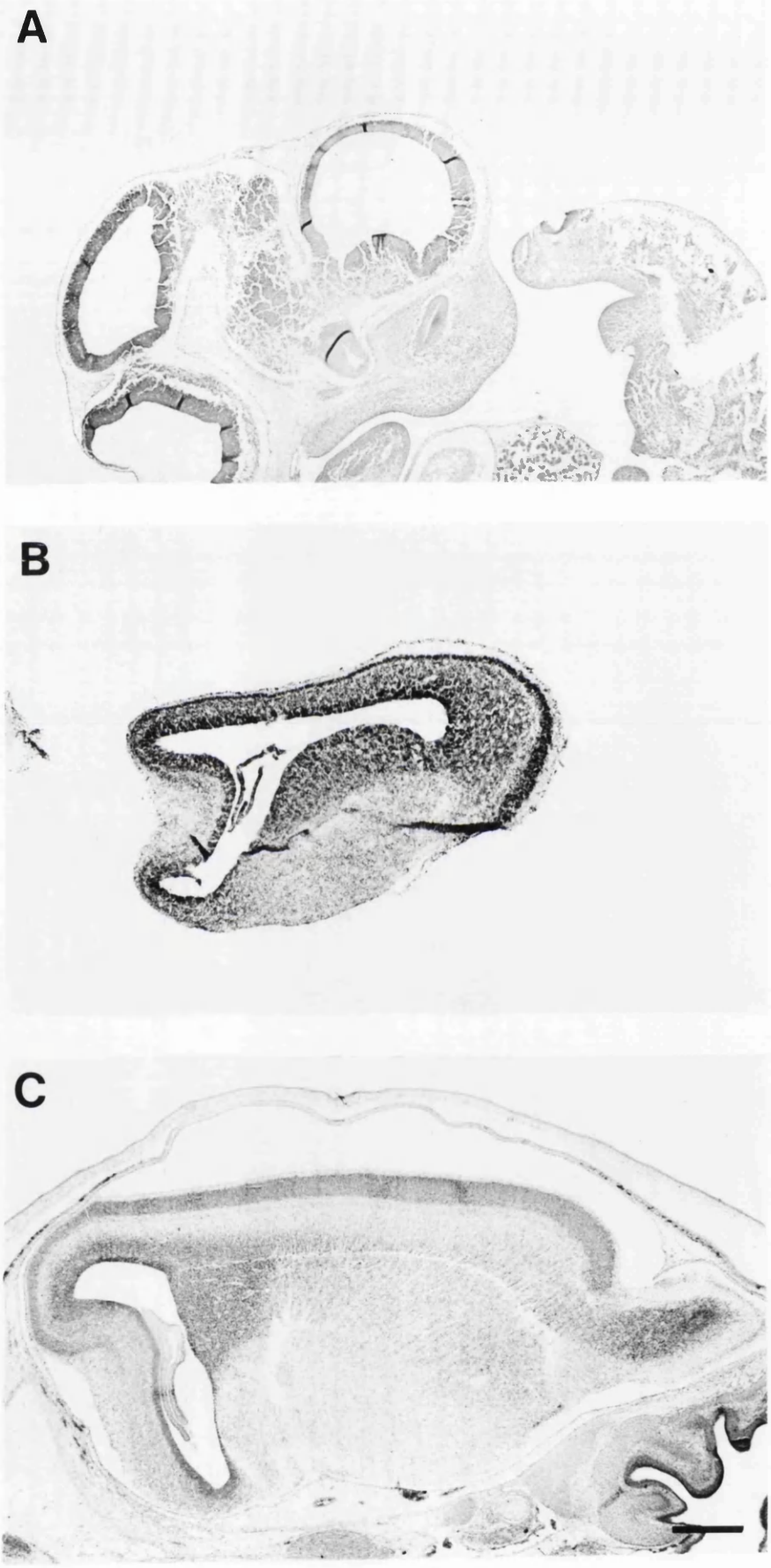

A
B
C

Figure 3

Sagittal section of E17 rat brain, courtesy of Bayer and Altman, 1991. The boxes mark the approximate regions taken for culture; rostral (Rost), caudal (Caud). Abbreviations; lateral ventricle (lv), septum (sept), pallidum (pal),olfactory bulb (ob), hippocampus (hpc), thalamus (thal), hypothalamus (hypothal), pituitary (pit) and cerebral cortex (cx).

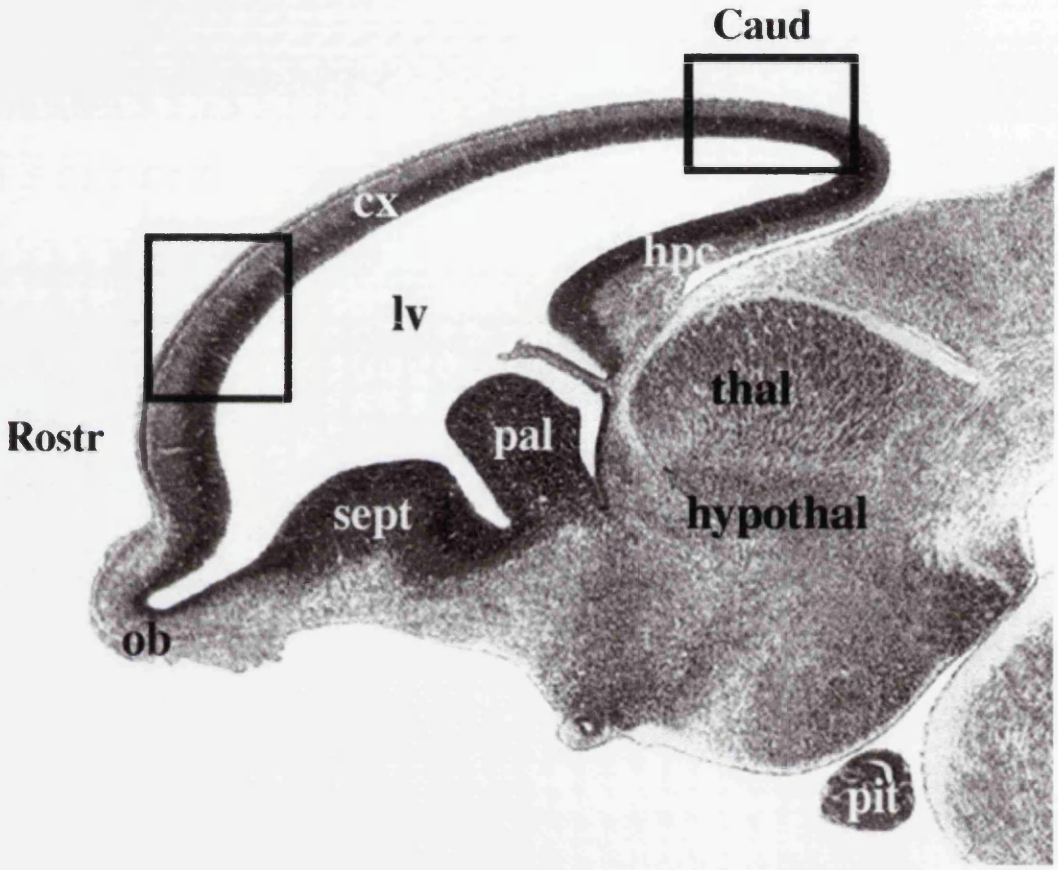
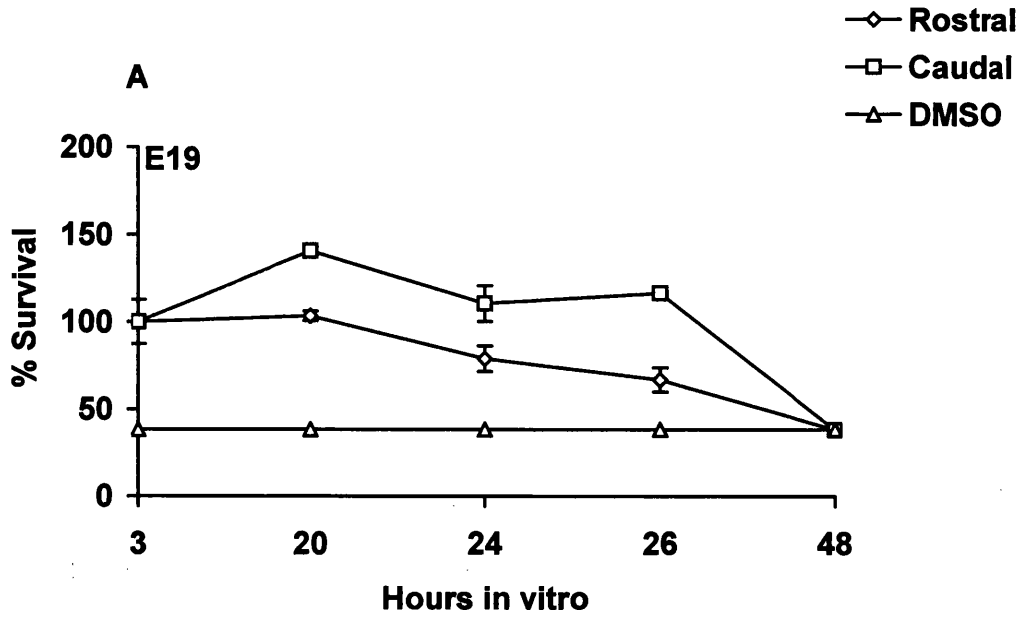

Caud
cx
hpc
lv
thal
Rostr
pal
sept
hypothal
ob
pit

Figure 4

A MTT cell survival assay of cultures derived from E19 embryos. Note that the cell survival in caudal cultures was greater than rostral cultures. By 48 hr in culture both rostral and caudal cells no longer survive.

B MTT cell survival assay of cultures derived from E17 embryos. Note that caudal cells survive for significantly longer periods, and to a great extent than rostral cells. The vast majority of rostral cells had undergone cell death by 3 DIV.

The percentage survival was expressed as a percentage of the initial 3 hr reading. Error bars represent SEM, n=9.


A
Rostral
Caudal
DMSO
E19
200
150
100
50
0
% Survival
3
20
24
26
48
Hours in vitro

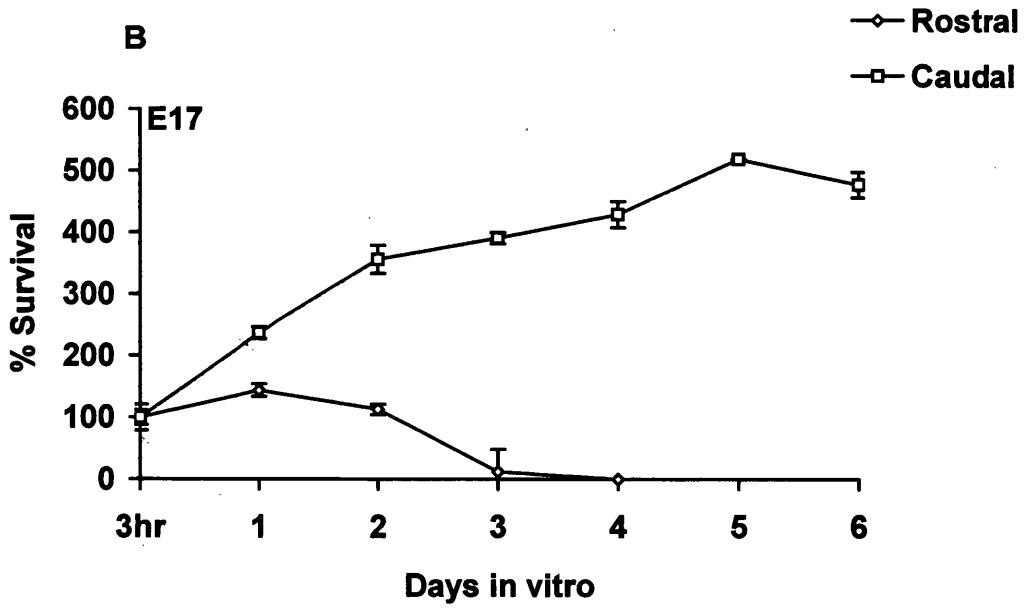

B
Rostral
Caudal
E17
600
500
400
300
200
100
0
% Survival
3hr
1
2
3
4
5
6
Days in vitro

Figure 5

Phase contrast image of E19 cortical cultures. Rostral cultures at 20 hr, 24, 28 and 36 hr *in vitro* (A, C, E, G) and caudal cells at these times *in vitro* (B, D, F and H respectively). Note that by 24-28 hr rostral cells have already undergone an considerable degree of cell death and by 36 hr few if any cells remain alive, whilst caudal cultures continue to survive until 28-36 hr when cultured under the same conditions. Scale bar 30 μm.

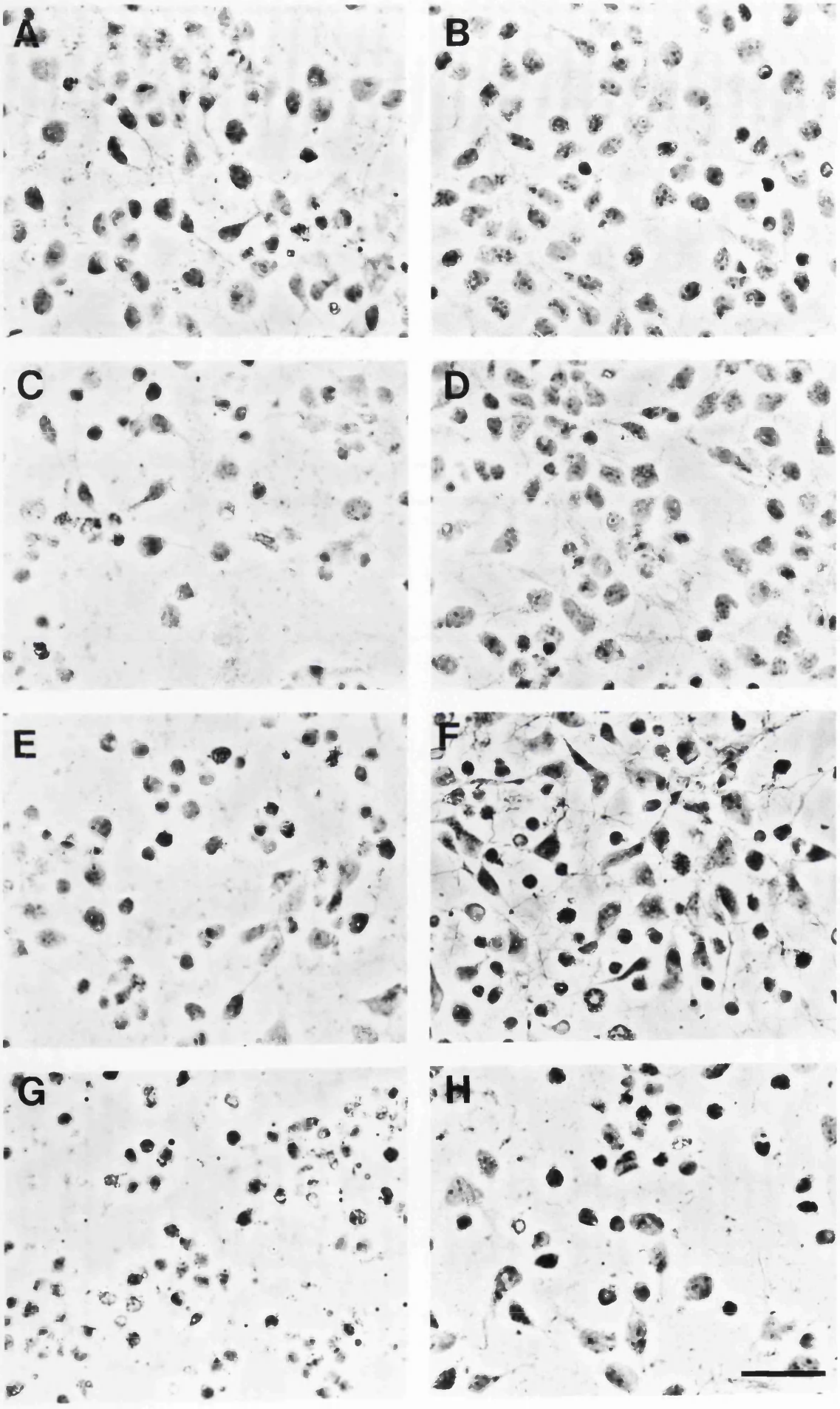

A
B
C
D
E
F
G
H

Figure 6

Phase contrast images of E17 cortical cultures. Rostral cultures at 1, 2 and 3 DIV (A, C, and E) and caudal cells at these times *in vitro* (B, D and F respectively). Note that at 2 DIV rostral cells do not have large numbers of dead cells. However a wave of cell death occurs between 2 and 3 DIV in rostral cultures which results in the death of the entire culture, whereas the vast majority of caudal cells continue to survive under the same conditions to later than 3 DIV. Scale bar 30 μm.

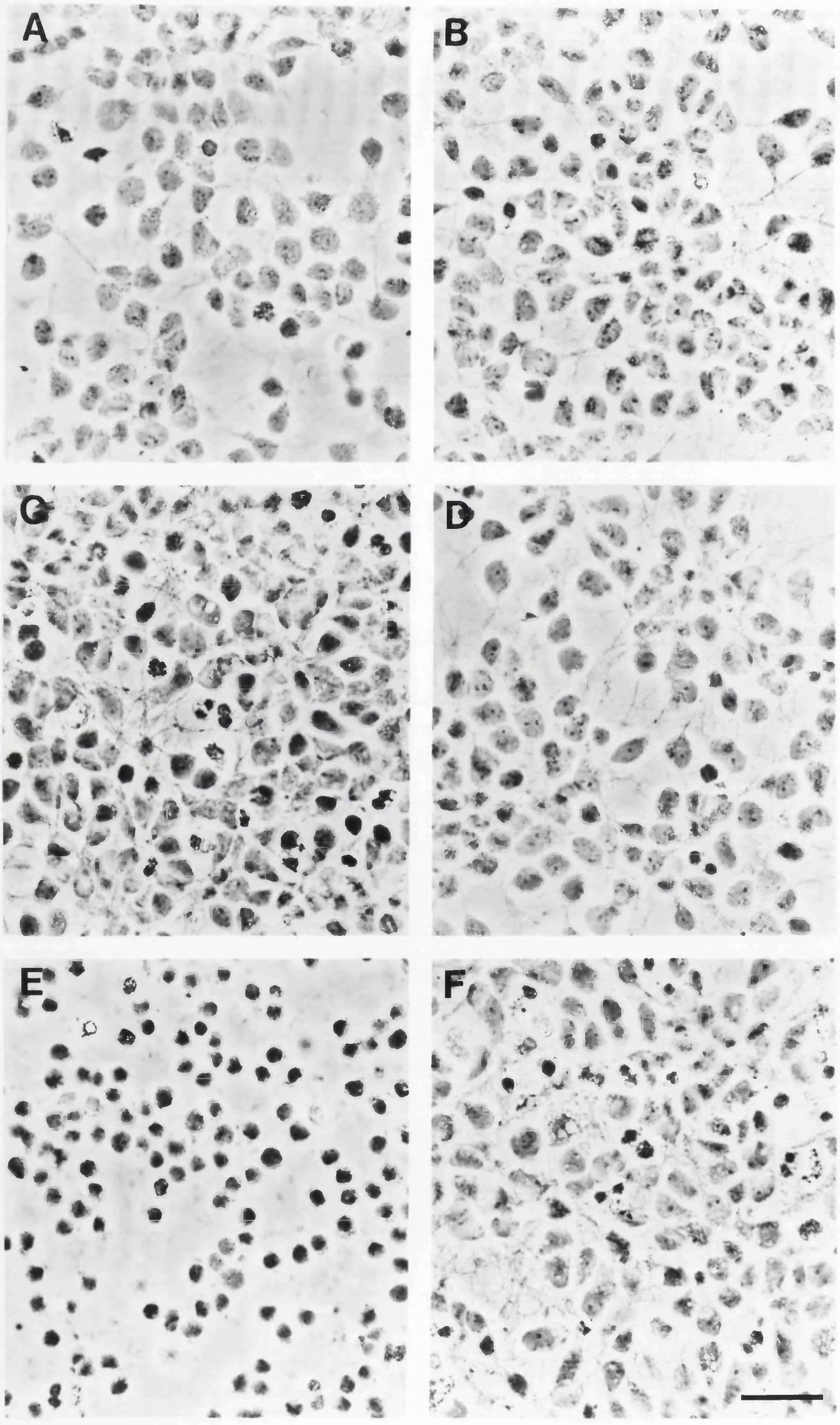
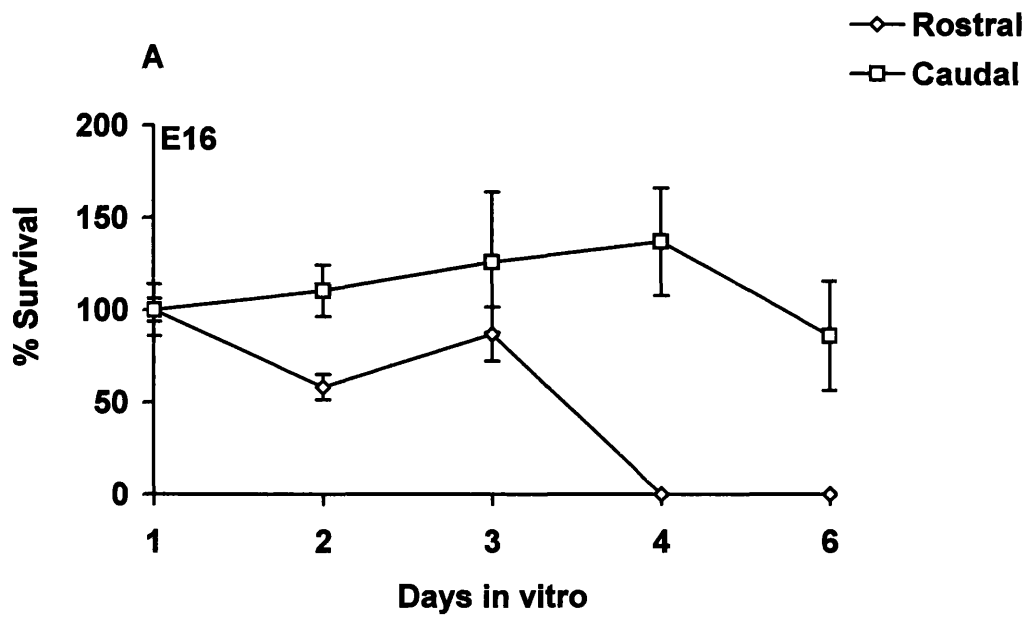

A
B
C
D
E
F

Figure 7

A MTT cell survival assay of cultures derived from E16 embryos. Note that the cell survival in caudal cultures was greater than rostral cultures with the latter surviving for only 4 days.

B MTT cell survival assay of cultures derived from E15 embryos. Only 2 time points were observed. Note that at 6 DIV the proportion of surviving rostral cells was significantly less than caudal cells ($P<0.05$).

The percentage survival was expressed as a percentage of initial reading. Error bars represent SEM, n=3-9.


A
Rostral
Caudal
E16
200
150
100
50
0
% Survival
1
2
3
4
6
Days in vitro

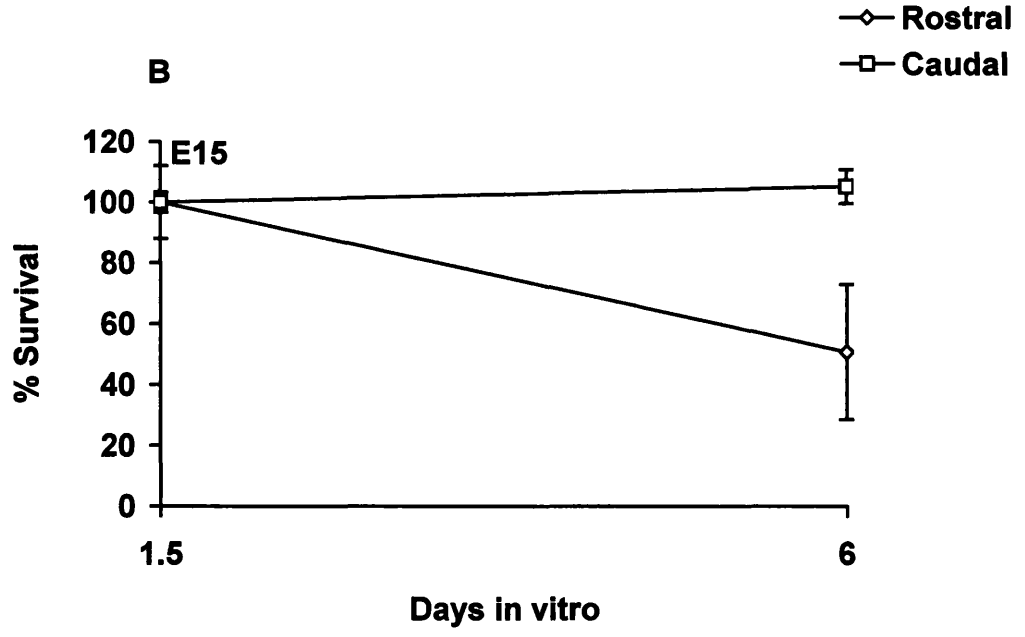

B
Rostral
Caudal
E15
120
100
80
60
40
20
0
% Survival
1.5
6
Days in vitro

Figure 8

Phase contrast image of E15 rostral cells at 1 and 3 DIV (A and C) and caudal cells at 1 and 3 DIV (B and D). Note the extensive neuritic connections and the confluence of the cultures after 6 days in culture. Relatively few dead cells were present in either rostral or caudal cultures at this time point. Scale bar 30 μm.

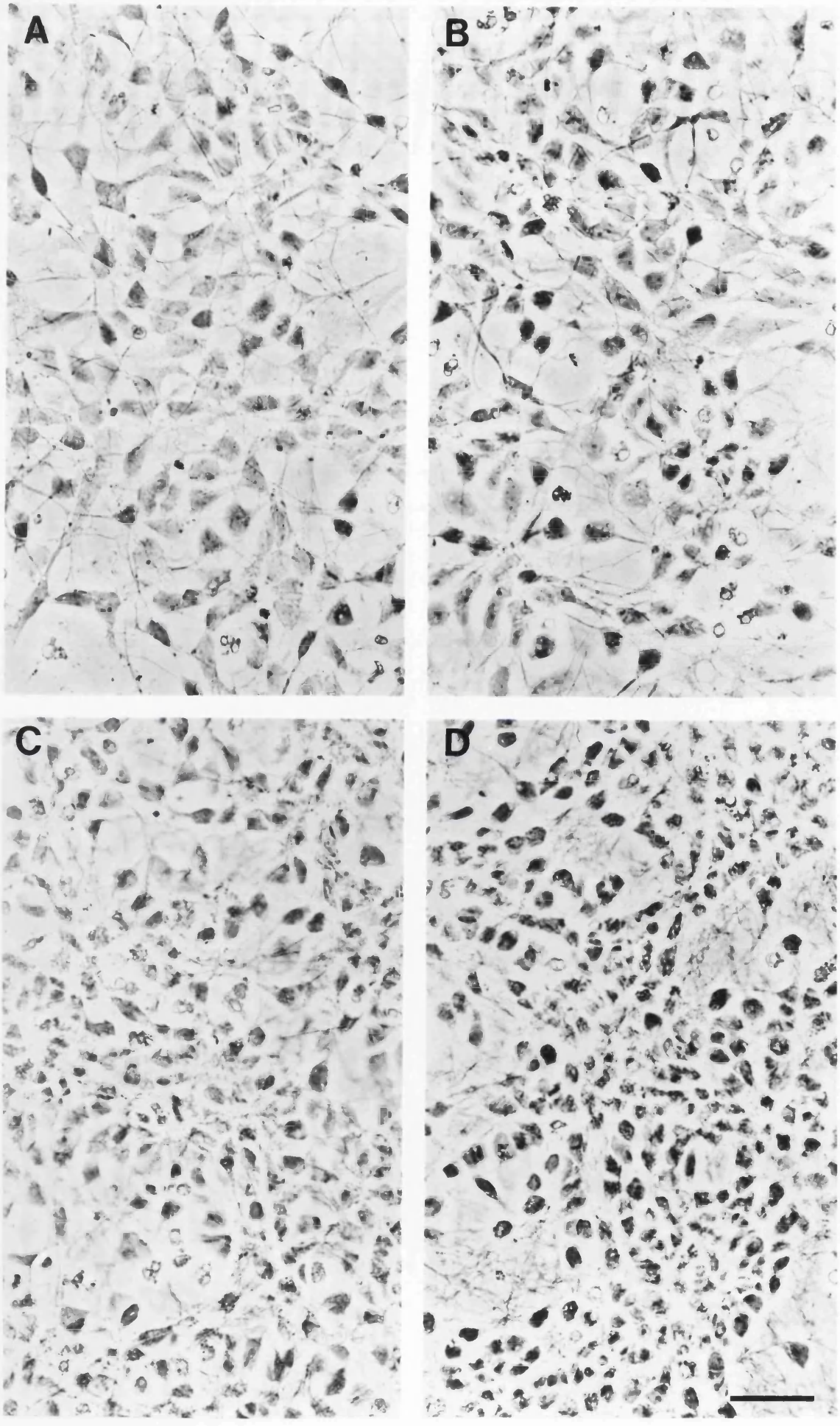
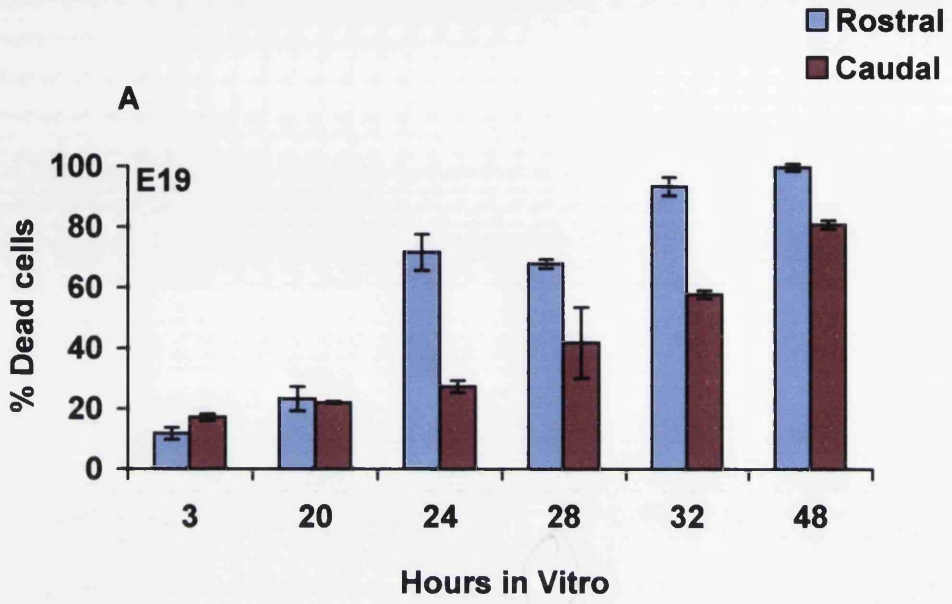

A
B
C
D

Figure 9

A The percentage of E19 cells undergoing cell death in rostral cultures exceeded that of dying cells in caudal cultures. Greater than 50% of cells in rostral cultures had died by 28 hr *in vitro* compared with caudal cultures.

B At E17, rostral, intermediate and caudal cells were compared for dying cells. Few dying cells were observed in caudal cultures. The first, cells to undergo cell death were rostral, followed by the intermediate group. By 3 days, nearly all rostral cells had died.

Counts of dead cells were made and expressed as a fraction of total number of cells. Error bars represent SEM, n=9.


Rostral
Caudal
A
E19
100
80
60
40
20
0
% Dead cells
3
20
24
28
32
48
Hours in Vitro

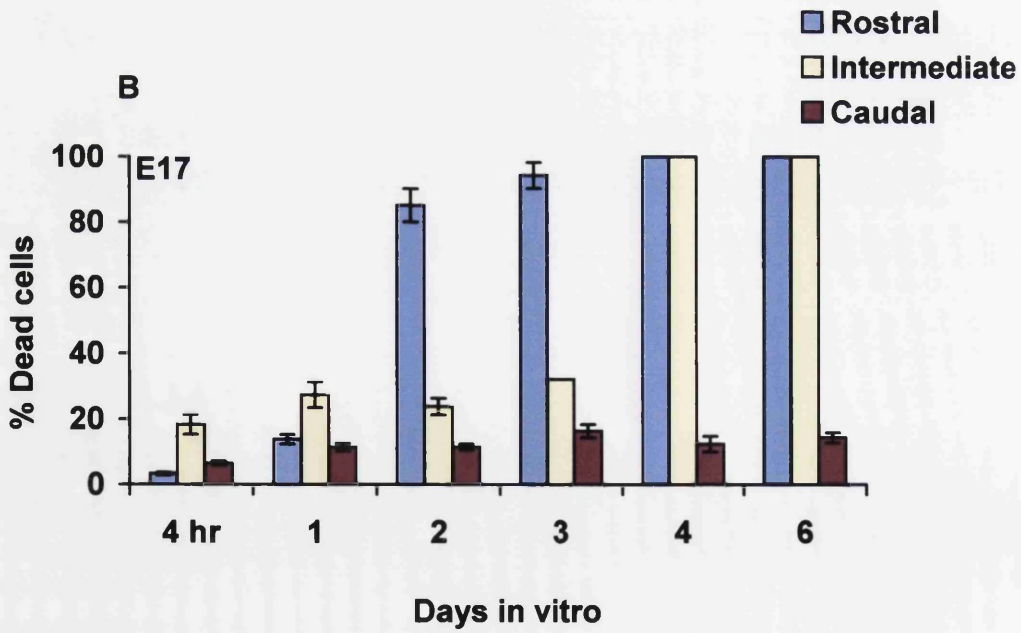

Rostral
Intermediate
Caudal
B
E17
100
80
60
40
20
0
% Dead cells
4 hr
1
2
3
4
6
Days in vitro

Figure 10

Cultures prepared from E17 rostral cortex, incubated for 1, 2 and 3 DIV shown in panels A, C and E. Sister cultures prepared from caudal cortex cultured for 1, 2, 3 and 4 DIV shown in panels B, D, F and G. Cells were stained with PI. Healthy cells (arrow heads) were visualised by the presence of a large rounded nucleus with uniform intensity of labelling. Cells undergoing cell death appeared to be rounded and forming blebs (arrows). Note that by 3 DIV most of rostral cells had undergone cell death whilst, at the same time in culture, the majority of cells have survived. Scale bar 30 μm.

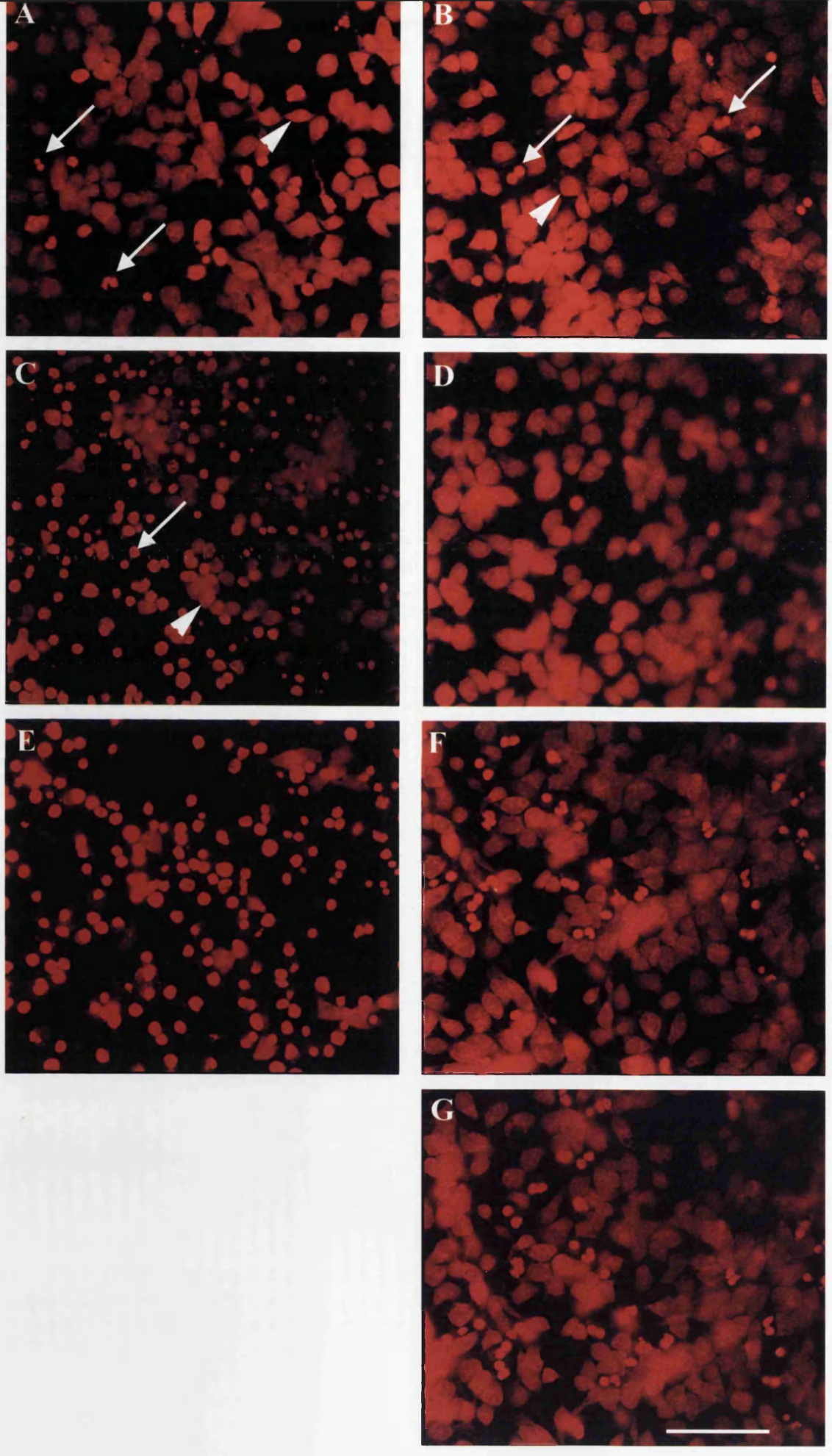
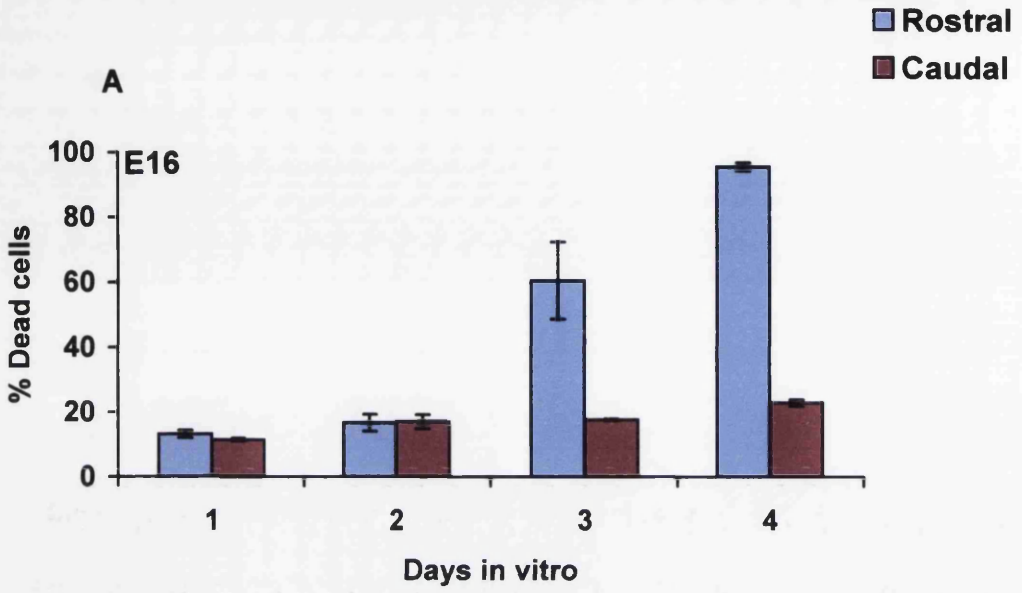

A
B
C
D
E
F
G

Figure 11

A The fraction of dead cells amongst E16 rostral cultures increased significantly ($p<0.05$) after 3 DIV, with almost 100% dead by 4 DIV, compared with less 25% dead in caudal cultures at 4 DIV.

B E15 derived rostral and caudal cultures showed little difference in the proportion of dying cells at the time points examined. The proportion of dead cells was not calculated after 3 DIV due to confluent nature of the cultures. Error bars represent SEM, n=3-6.


Rostral
Caudal
A
E16
100
80
60
40
20
0
% Dead cells
1
2
3
4
Days in vitro

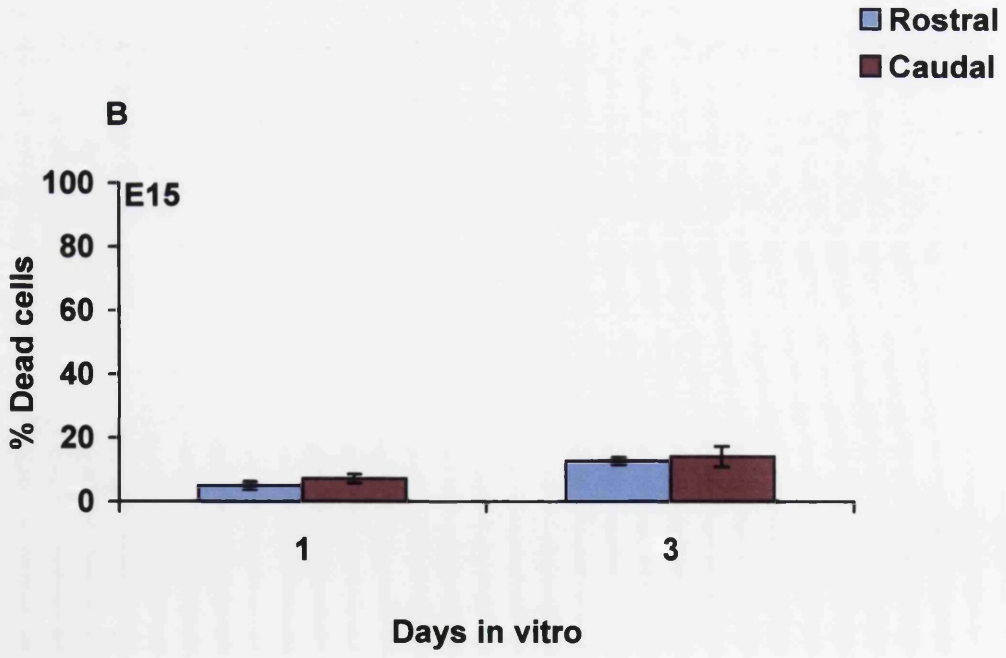

Rostral
Caudal
B
E15
100
80
60
40
20
0
% Dead cells
1
3
Days in vitro

Figure 12

The proportion of dividing cells, determined by controlled BrdU uptake and immunocytochemistry, decreased with increasing age. However at E15 more caudal cells were entering the cell cycle than rostral cells ($P<0.05$). However, there was no difference in the proportion of cells undergoing cell division between rostral and caudal areas of the cortex at the other ages examined. Error bars represent SEM, n=6-9.

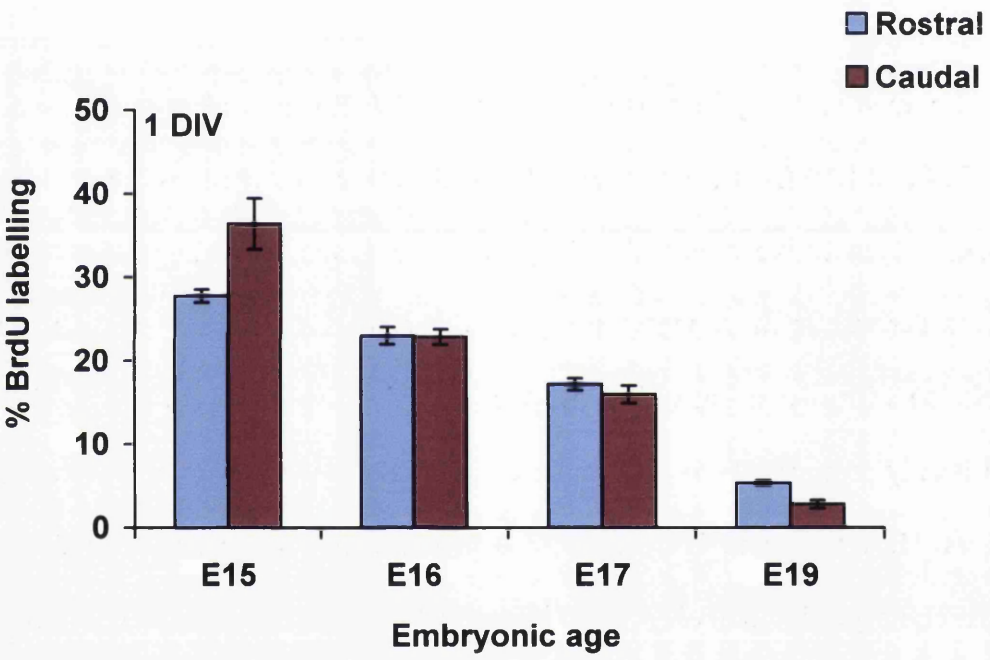

Rostral
Caudal
1 DIV
% BrdU labelling
50
40
30
20
10
0
E15
E16
E17
E19
Embryonic age

Figure 13

BrdU immunoreactivity in cells cultures for 1 DIV. Rostral cells derived from E15, E16, E17 and E19 (A, C, E and G) and caudal cells from these ages are shown in B, D, F, and H respectively. Note that the greatest proportion of dividing cells were seen in cultures derived from younger embryos, with a marked reduction in this specific population with increasing embryonic age, reflecting the trend seen *in vivo*. There was significantly more ($P<0.05$) BrdU uptake in caudal cells at E15 compared with sister cultures. However there was no longer a difference in proportions of dividing cells thereafter, suggesting that differences in survival of cells were not solely attributable to the rate of proliferation. There was variation in the different degrees of labelling of BrdU positive cells, this simply reflected the exposure time to BrdU. More intensely labelled cells were in S-phase for the majority of the time that BrdU was present in the culture medium. Scale bar 30 μm.

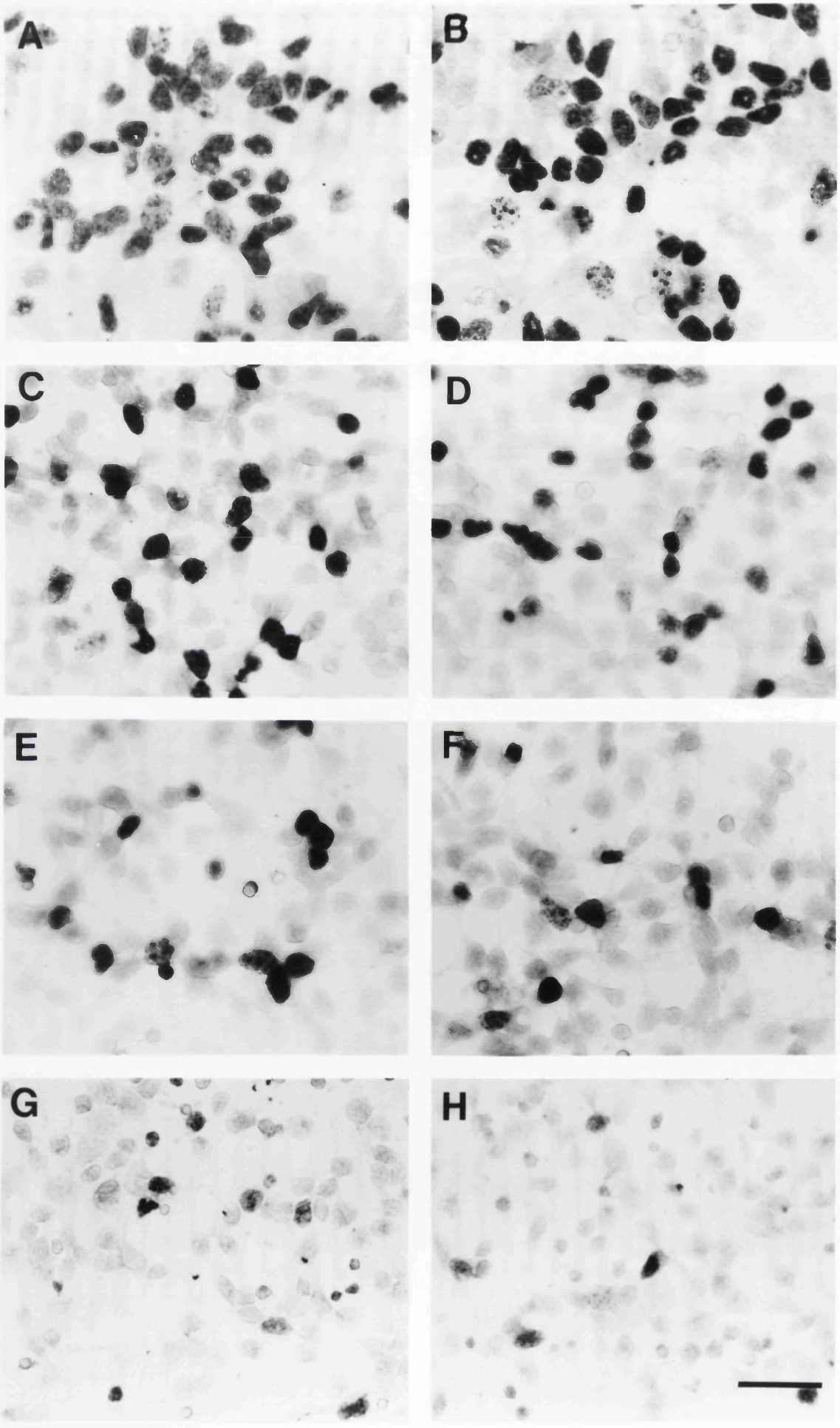
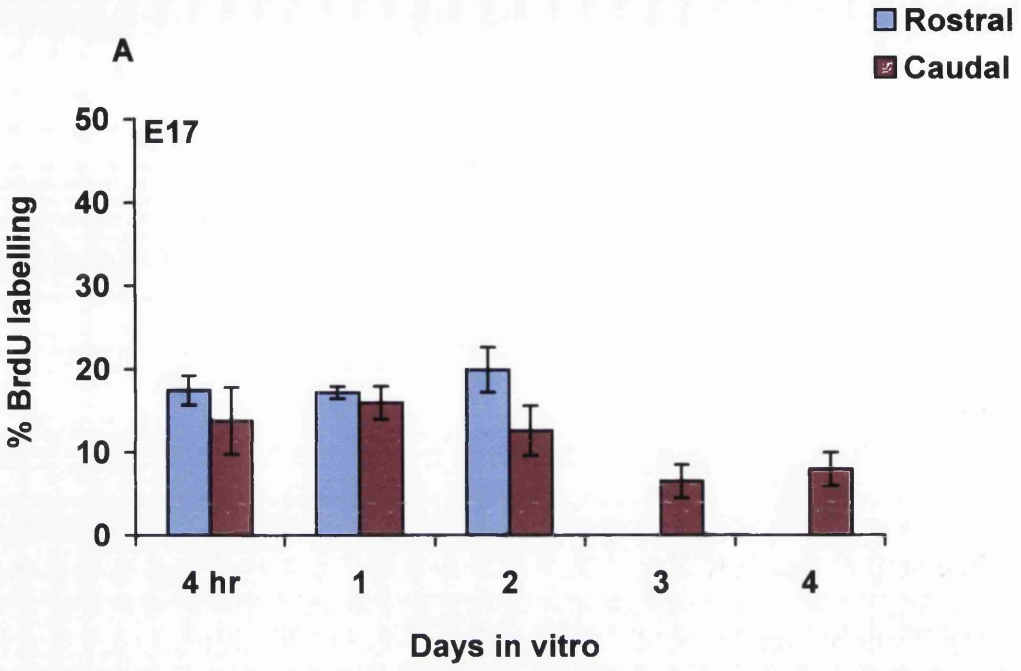

A
B
C
D
E
F
G
H

Figure 14

A The proportion of dividing cells, determined by BrdU uptake, did not change significantly over the first few days in E17 cultures. This demonstrated that the rate of proliferation of caudal cells was not the determining factor for the difference in survival of rostral and caudal cells. The overall proportion of dividing cells amongst the caudal cultures diminished with time.

B BrdU uptake at E15 in rostral and caudal cultures. Initially there was a greater proportion of dividing caudal cells. By 3 DIV the proportions of dividing cells in rostral and caudal cells were equivocal. Error bars represent SEM, n=6-9.


A
Rostral
Caudal
E17
50
40
30
20
10
0
% BrdU labelling
4 hr
1
2
3
4
Days in vitro

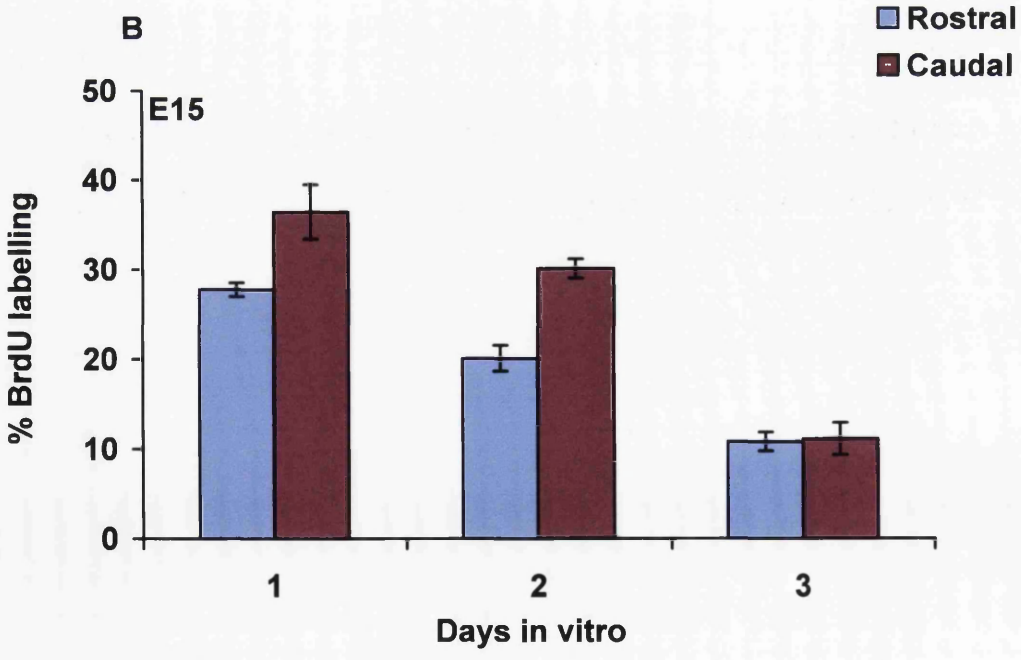

B
Rostral
Caudal
E15
50
40
30
20
10
0
% BrdU labelling
1
2
3
Days in vitro

Figure 15

The proportion of dead cells in E17 derived rostral and caudal cells was compared with the dead cells in mixed rostral and caudal (Rost/Caud) cultures. When rostral cells were mixed with caudal cells, the overall fraction of dying cells was significantly reduced ($p<0.05$). The graph demonstrates that in the presence of caudal cells, over half the rostral cells were rescued from cell death. The proportion of dying cells remained low over the whole culture period examined. Error bars represent SEM, n=6-9.

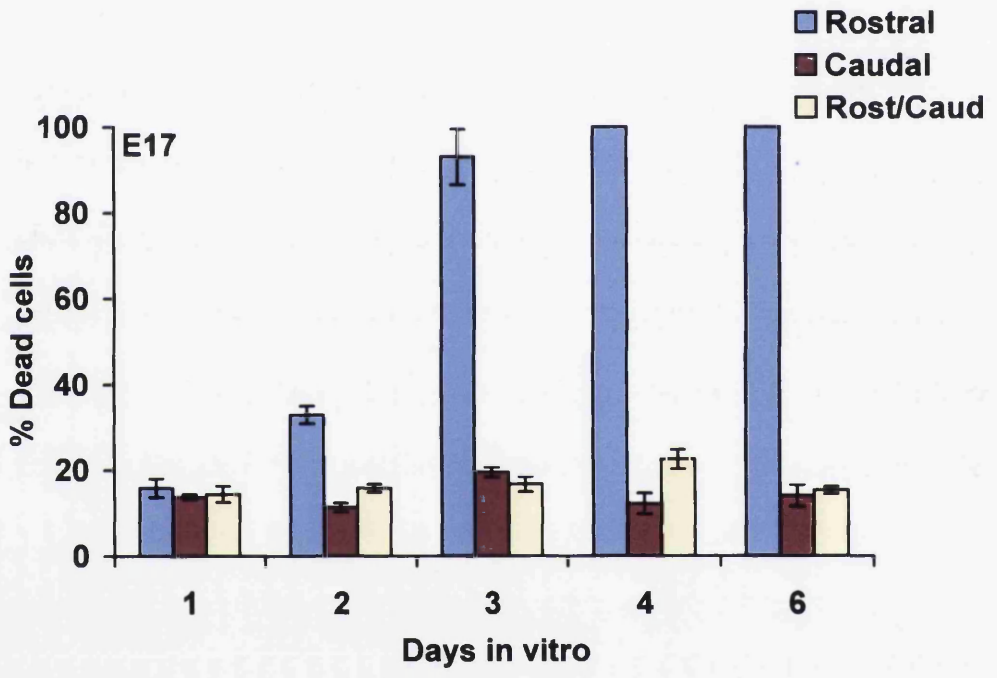

Rostral
Caudal
Rost/Caud
E17
100
80
60
40
20
0
% Dead cells
1
2
3
4
6
Days in vitro

Figure 16

The proportion of dead cells in E17 derived separate co-cultures of rostral and caudal cells, in which rostral cells shared the same environment as caudal cells, in the absence of cell to cell contact, was examined. The proportion of dying cells in such co-cultures remained low (<20%) over 6 DIV. Error bars represent SEM, n=6-9.

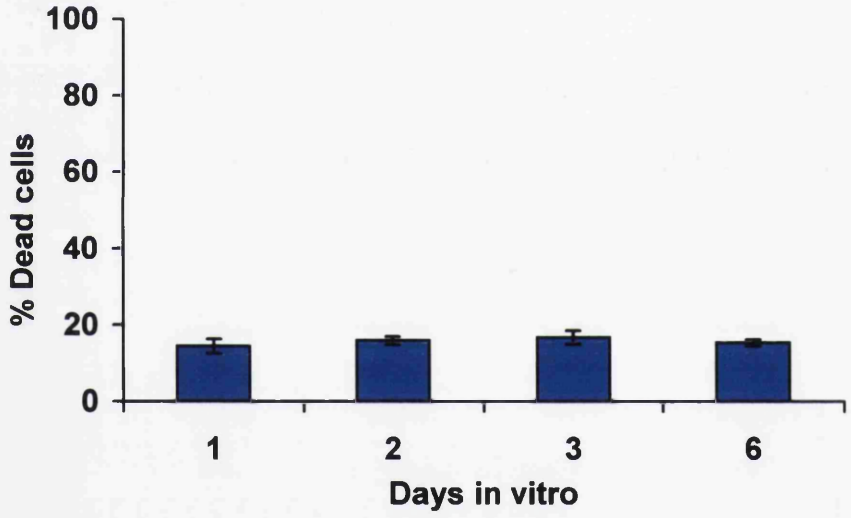

100
80
60
40
20
0
% Dead cells
1
2
3
6
Days in vitro

Figure 17

The proportion of dead cells at 3 DIV, in E17 derived separate co-cultures of rostral and caudal cells, in which rostral cells shared the same environment as caudal cells, in the absence of cell to cell contact (R co C), was examined and compared to the fraction of dead cells in isolated cultures of rostral and caudal cells and mixed cultures (Rost/Caud). The proportion of dying cells in mixed and separate co-cultures was significantly lower than in cultures of isolated rostral cells ($p<0.05$). This demonstrated that in the presence of caudal cells, rostral cells survived longer. Further, in the absence of cell to cell contact, caudal cells were able to promote the survival of rostral cells. Error bars represent SEM, n=9.

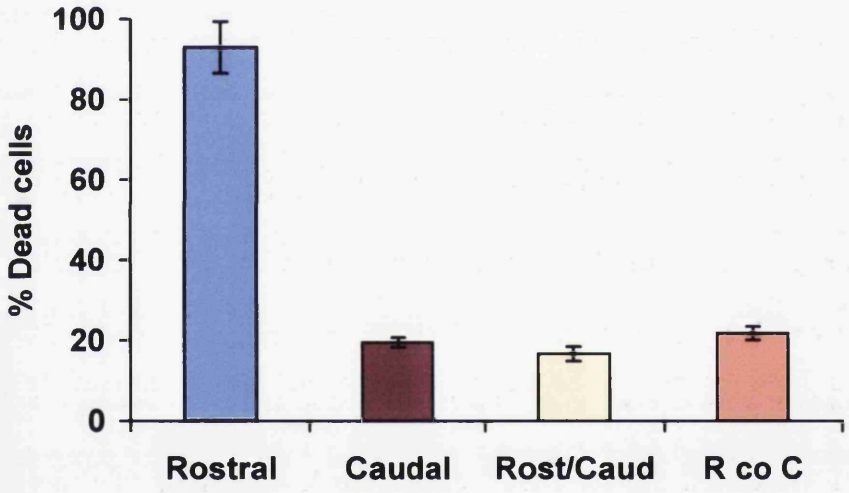
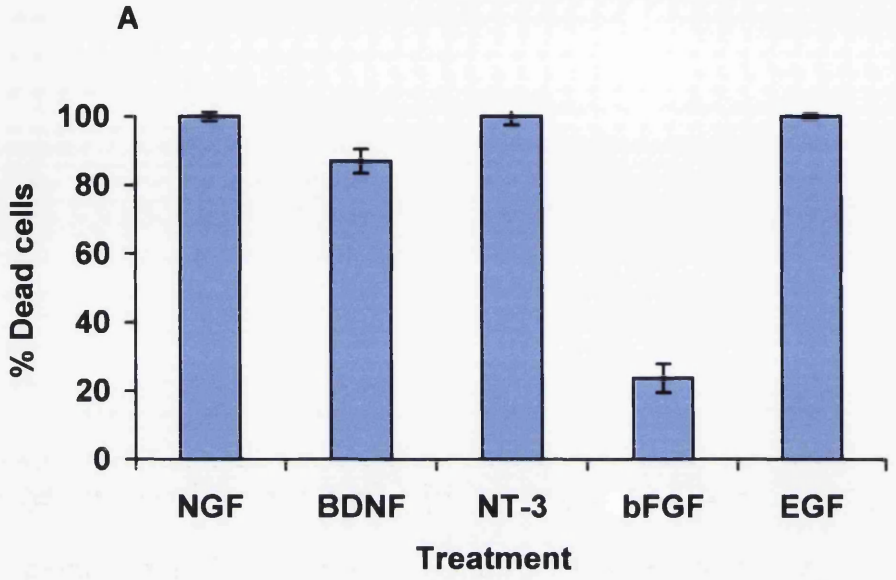

100
80
60
40
20
0
% Dead cells
Rostral
Caudal
Rost/Caud
R co C

Figure 18

A The proportion of dead cells at 3 DIV of E17 derived rostral cells under different treatment conditions was examined. Basic FGF had the most significant cell survival effect compared with NGF, BDNF, NT-3 and EGF. This showed that rostral cells were capable of responding to growth signals, specifically bFGF.

B The proportion of dead cells in E17 derived rostral and caudal cultures compared with the proportion of dead cells in bFGF treated rostral cultures at 1 and 3 DIV.

Error bars represent SEM, n=3.


A
100
80
60
40
20
0
% Dead cells
NGF
BDNF
NT-3
bFGF
EGF
Treatment

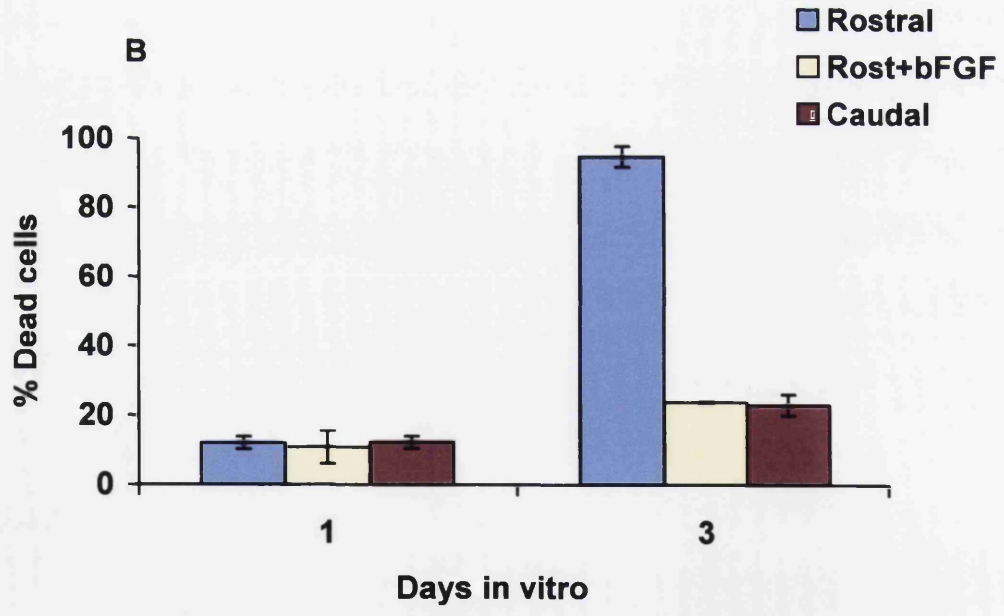

B
Rostral
Rost+bFGF
Caudal
100
80
60
40
20
0
% Dead cells
1
3
Days in vitro

Figure 19

The dose response curve for bFGF treated rostral cultures. Note that increasing concentrations of bFGF promote the percentage survival and the length of survival of rostral cells.

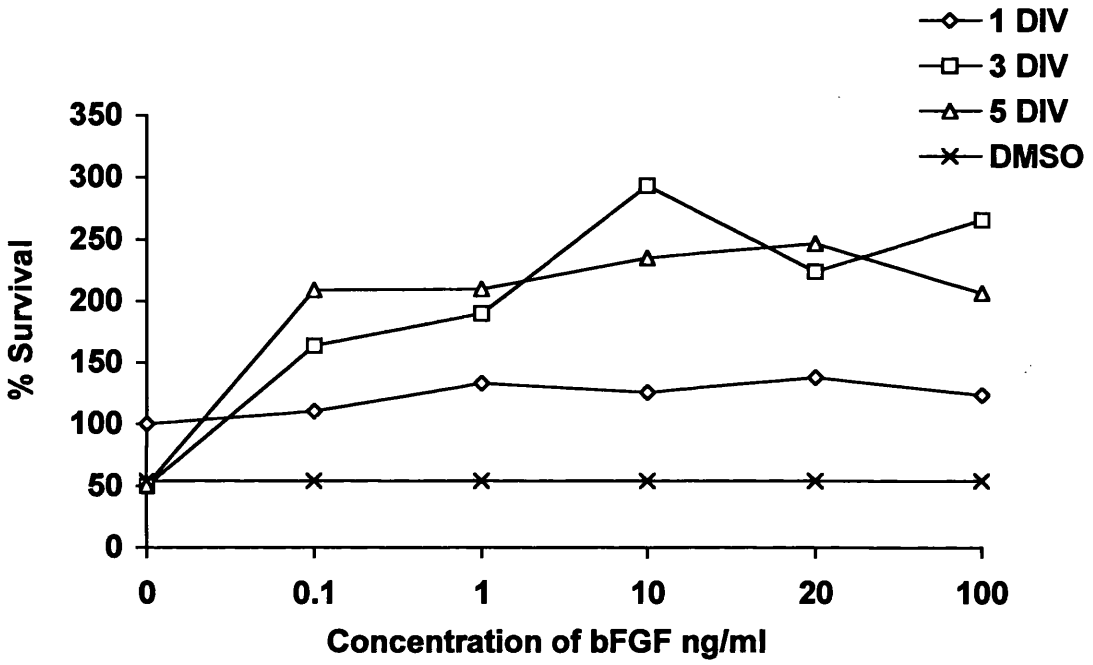

1 DIV
3 DIV
5 DIV
DMSO
350
300
250
200
150
100
50
0
% Survival
0
0.1
1
10
20
100
Concentration of bFGF ng/ml

Figure 20

AraC, a mitotic inhibitor, was used to determine the relationship between cell survival and cell proliferation amongst caudal cells. At non-toxic concentrations, caudal cells continued to survive even in the presence of the inhibitor, suggesting that survival of cells was not entirely dependent on their ability to proliferate.

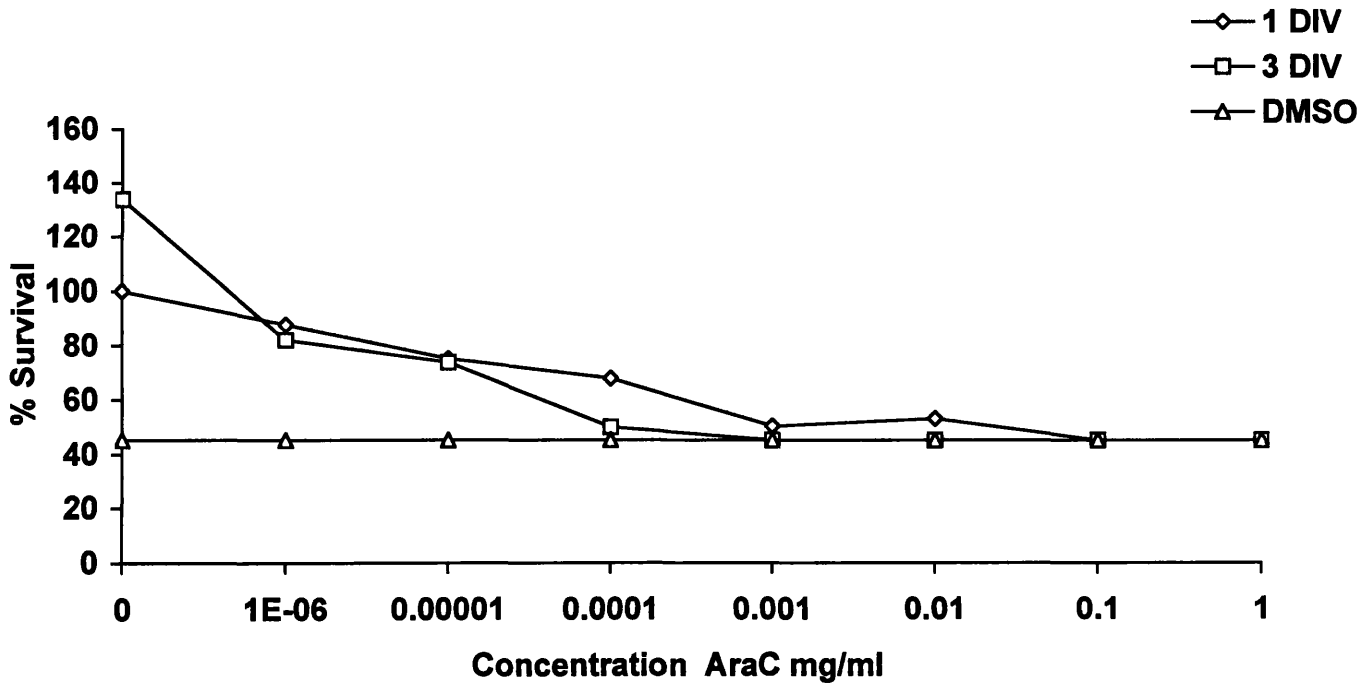

1 DIV
3 DIV
DMSO
160
140
120
100
80
60
40
20
0
% Survival
0
1E-06
0.00001
0.0001
0.001
0.01
0.1
1
Concentration AraC mg/ml

Figure 21

Caudal cells (E17) were cultured in the presence of an inhibitor of bFGF activity (genestein) to determine whether caudal cells continued to survive in the absence of bFGF activity. The histogram shows the proportions of dead cells observed at 1-3 days in culture. In the absence and in the presence of exogenous bFGF, almost all caudal cells underwent cell death by 3 DIV, compared to control in which less than 20% cells died. This confirmed that bFGF was needed for the survival of caudal cells and that this growth factor was produced by caudal cells at E17.

Error bars represent SEM, n=6-9.

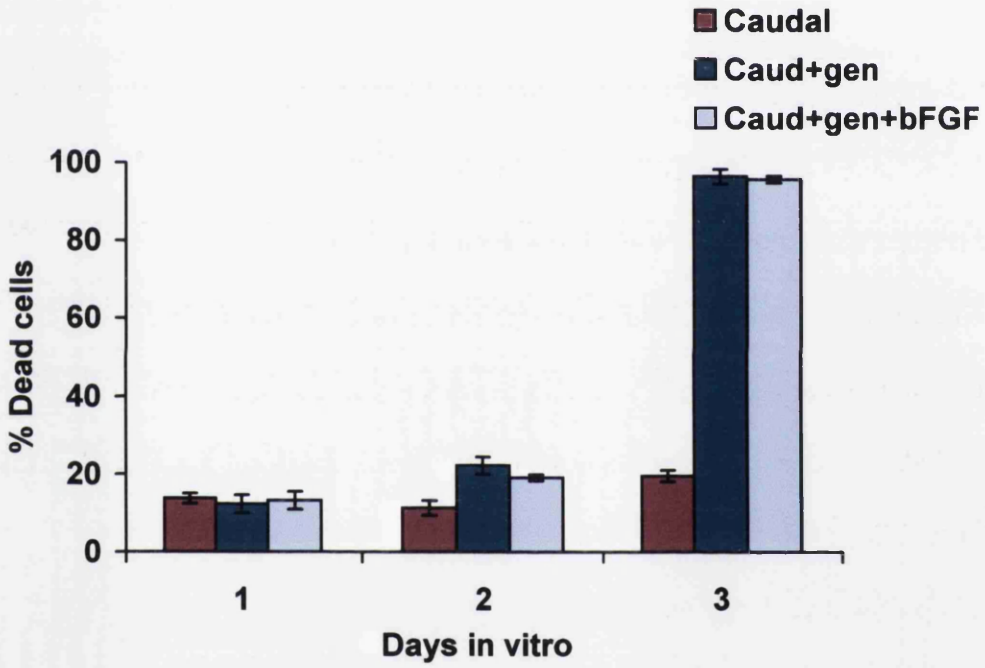

Caudal
Caud+gen
Caud+gen+bFGF
100
80
60
40
20
0
% Dead cells
1
2
3
Days in vitro

Figure 22

The histogram shows the proportions of cells immunoreactive for bFGF using Ab106, and the proportion immunopositive for the FGFR1 in rostral and caudal cells cultured for 1 DIV and derived from E17 rat brains. The proportions of cells positive for both bFGF and the receptor were almost equivocal. This demonstrated that both rostral and caudal cells contained bFGF, and that both cell groups were capable of responding to the growth factor via the FGFR1.

Error bars represent SEM, n=9.

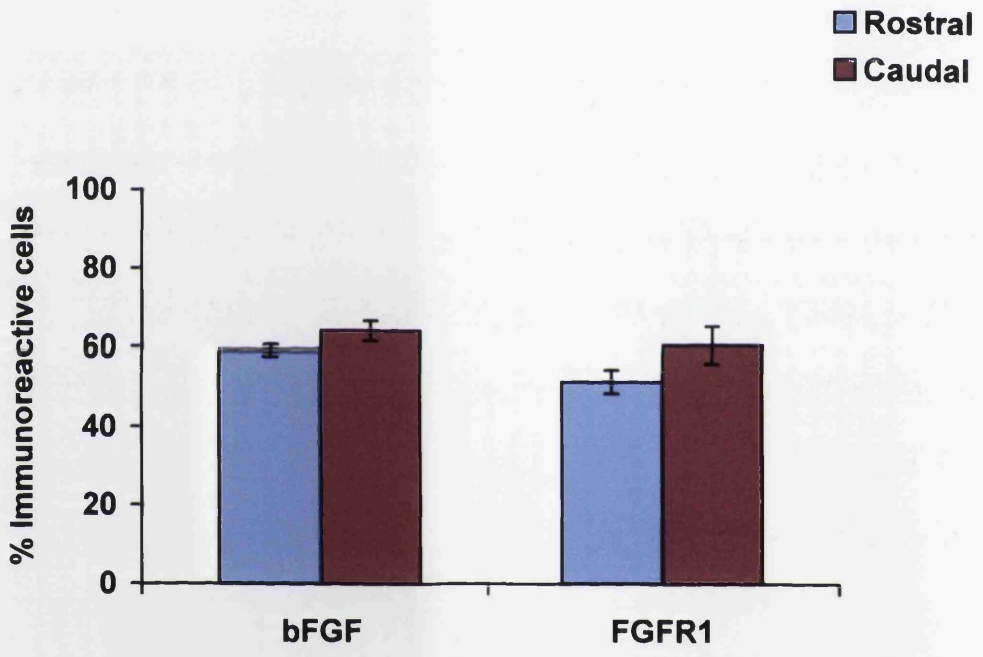

Rostral
Caudal
% Immunoreactive cells
100
80
60
40
20
0
bFGF
FGFR1

Figure 23

Photomicrograph showing anti-bFGF (118) immunoreactivity in E17 cerebral cortical cultures derived from rostral cortex (A), and caudal cortex (C) cultured for 1 DIV. Preabsorbed antibody controls for both rostral and caudal cultures are shown (B and D, respectively). Note that with anti-bFGF (118) the preabsorption controls also showed some degree of immunostaining. However, more importantly figures A and C demonstrate that bFGF immunoreactive cells were present in both rostral and caudal cells derived from E17, with marginally stronger immunostaining in caudal cells. Scale bar 30 μm.

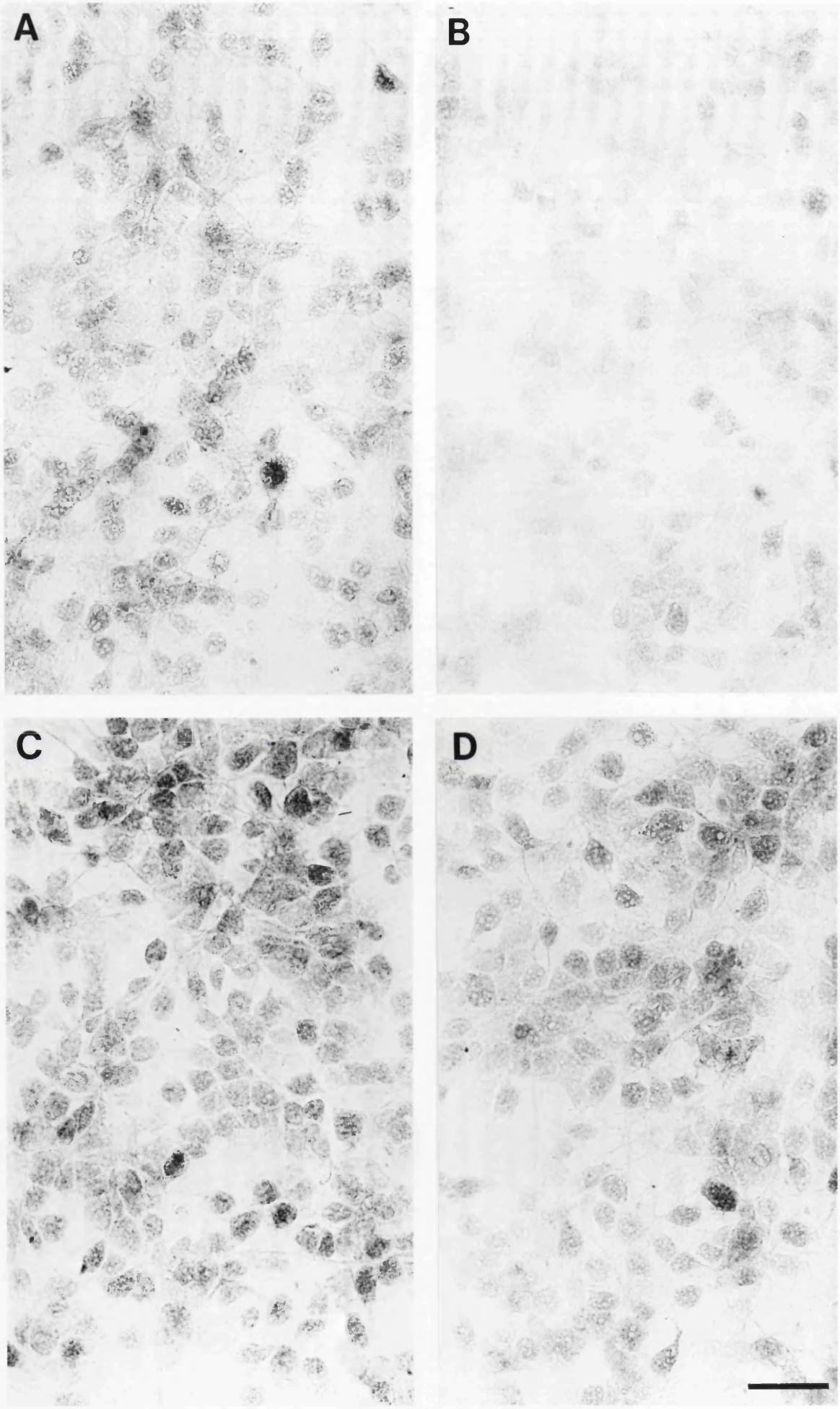
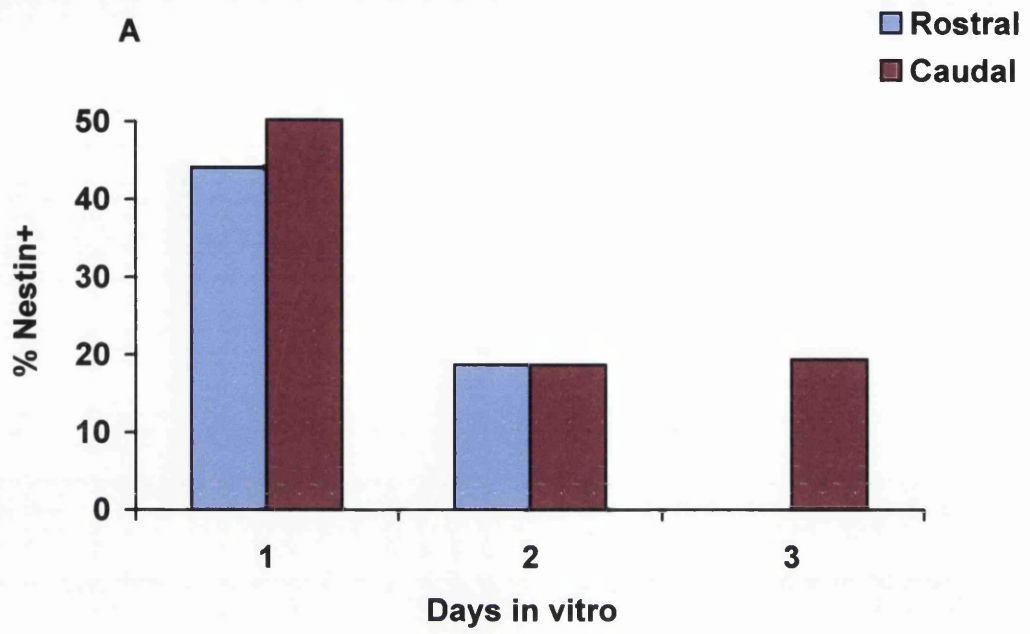

A
B
C
D

Figure 24

A Histograms illustrating the proportions of nestin immunoreactive cells in rostral and caudal cortex obtained from E17 brains over 3 days in culture.

B Histograms comparing the proportions of GABA immunoreactive cells in rostral and caudal cortex obtained from E17 embryos cultured for 3 DIV.

n=3


A
Rostral
Caudal
% Nestin+
50
40
30
20
10
0
1
2
3
Days in vitro

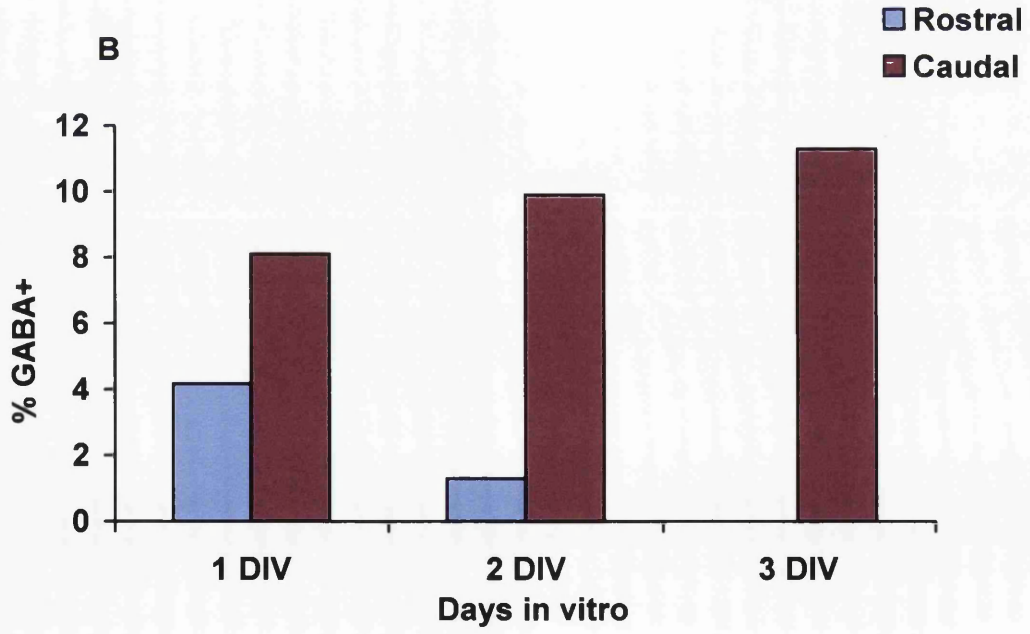

B
Rostral
Caudal
% GABA+
12
10
8
6
4
2
0
1 DIV
2 DIV
3 DIV
Days in vitro

Figure 25

Nestin immunoreactivity (arrows) in cultures prepared from E17 rostral cortex (A and C) at 1 and 3 DIV respectively, and caudal cortex (B and D) at 1 and 3 DIV respectively. There was an approximate 10% differences between the proportion of nestin immunoreactive cells in the cultures of rostral and caudal cells at 1 DIV. After 3 days in culture, the proportion of nestin immunoreactive cells decreased significantly to less than 20%. Although B shows a few rostral nestin immunoreactive cells, the vast majority of cultures had few if any living cells remaining at 3 DIV. Arrow heads show unlabelled cells. Scale bar 30 μm.

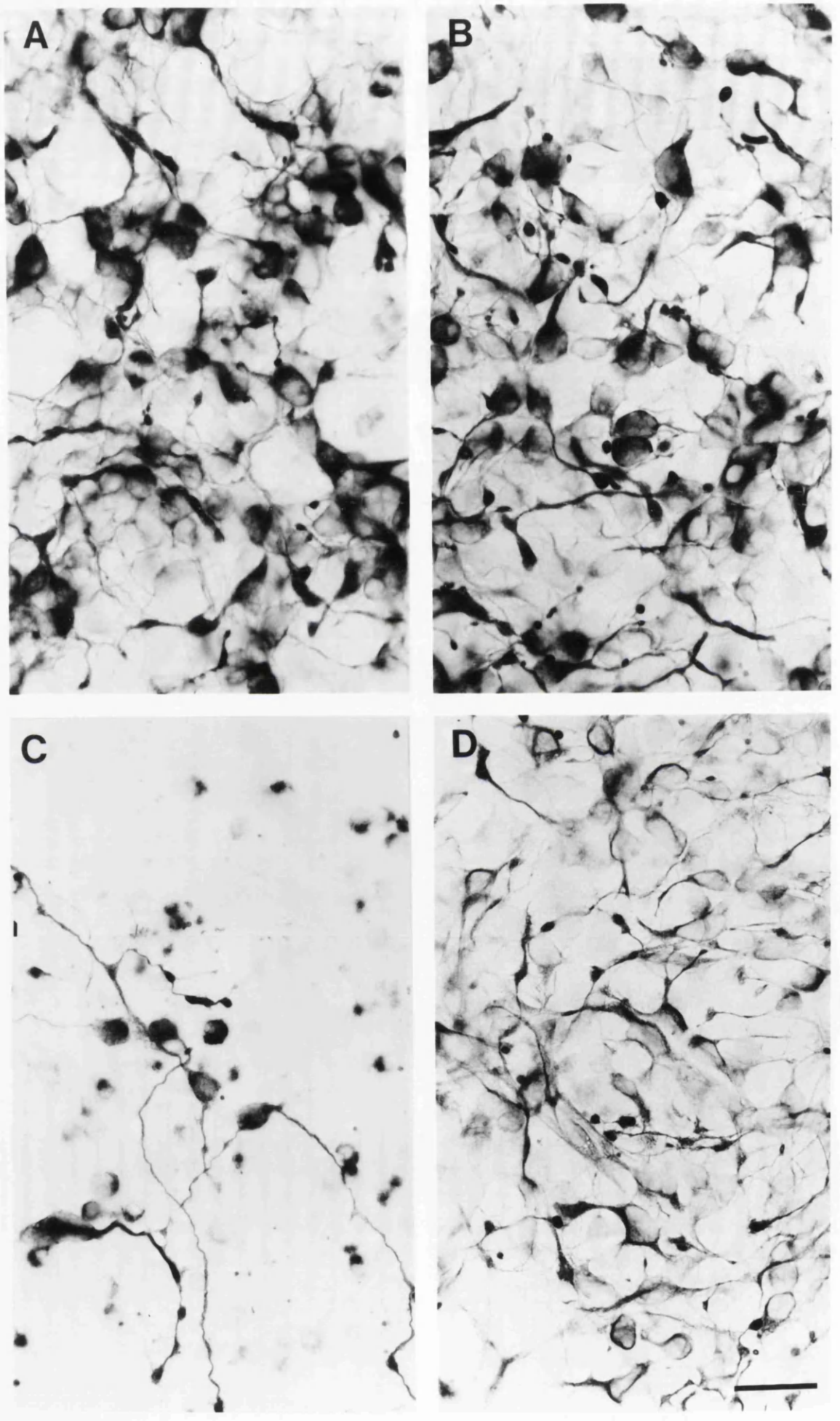
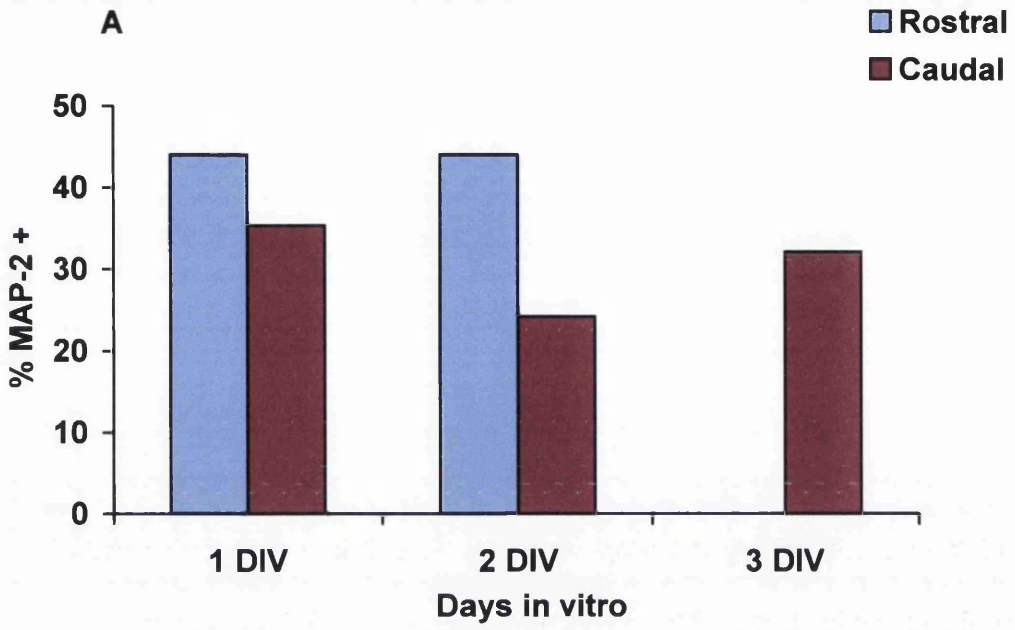

A
B
C
D

Figure 26

A Histograms illustrating the proportions of MAP-2 immunoreactive cells in rostral and caudal cortex obtained from E17 brains over 3 days in culture.

B Histograms comparing the proportions of GFAP immunoreactive cells in rostral and caudal cortex obtained from E17 embryos cultured for 3 DIV.

n=3


A
Rostral
Caudal
50
40
30
20
10
0
% MAP-2 +
1 DIV
2 DIV
3 DIV
Days in vitro

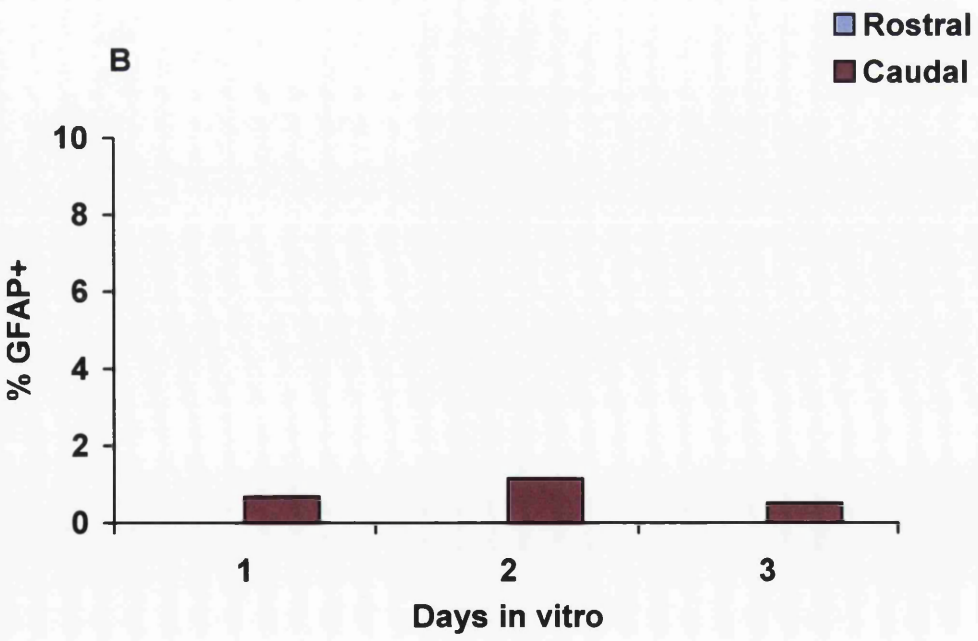

B
Rostral
Caudal
10
8
6
4
2
0
% GFAP+
1
2
3
Days in vitro

Figure 27

MAP-2 immunoreactivity in E17 rostral cortical cells (A and C) at 1 and 3 DIV respectively, and caudal cultures (B and D) at 1 and 3 DIV respectively. Approximately 45% of cells in rostral cultures were MAP-2 immunoreactive, whilst on average 35% were immunoreactive in caudal cultures at 1 DIV. With increasing time in culture, caudal cells continued to differentiate to acquire a neuronal fate, demonstrated by MAP-2 immunoreactivity. Scale bar 30 μm.

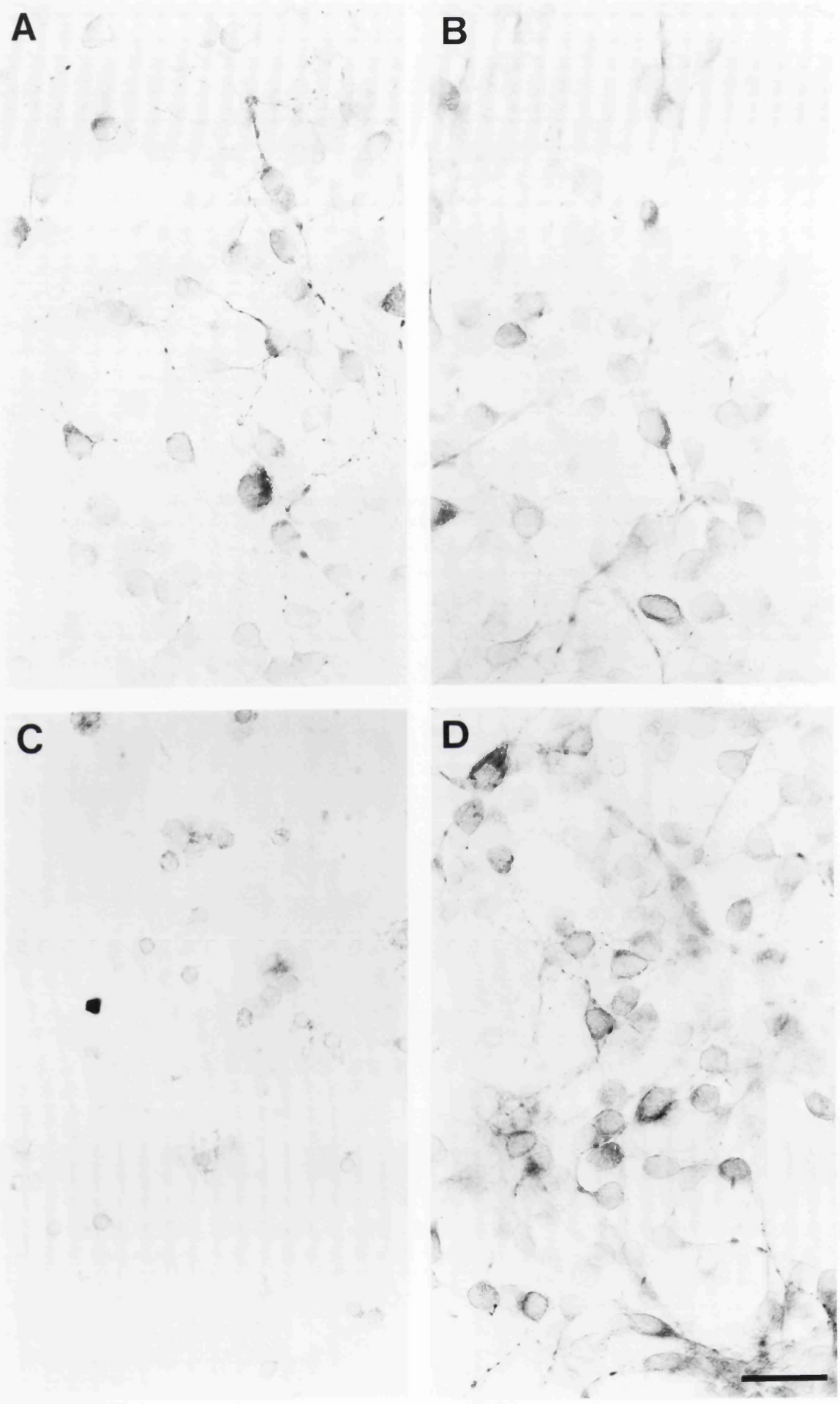

A
B
C
D

Figure 28

GABA immunoreacitivty in E17 rostral cells (A and C) at 1 and 3 DIV, and caudal cells (B and D) at 1 and 3 DIV. Note the low proportion of cells, less than 5% in rostral and less than 10% in caudal cultures at 1 DIV. The proportion of GABA immunopositive cells did not change over 3 days, suggesting that a constant proportion of cells undertook the 'non-pyramidal' lineage. Scale bar μm.

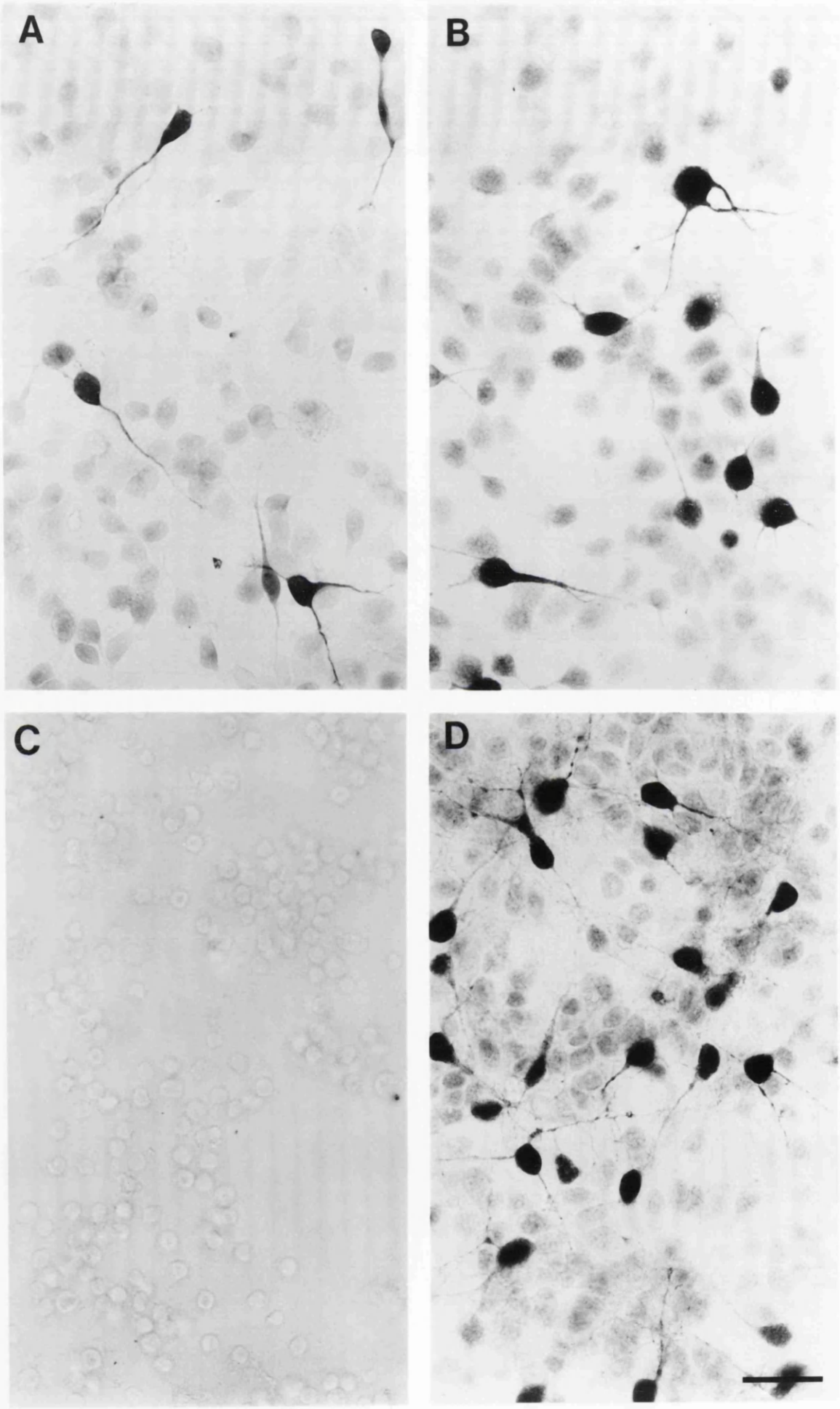

A
B
C
D

Figure 29

A comparison of the proportion of neurons born in rostral and caudal regions was calculated following an injection of BrdU into a pregnant rat, in the different regions of the cortex harvested and cultured for 1 DIV. A marginally greater proportion of neurons were born in the rostral cortex compared to caudal cortex. This suggested that the relative difference in birth rate of neurons was small. Error bars represent standard deviations, n=3.

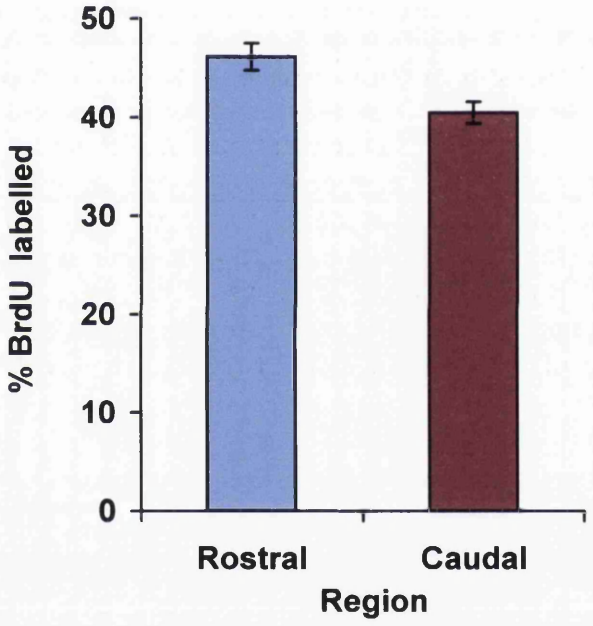
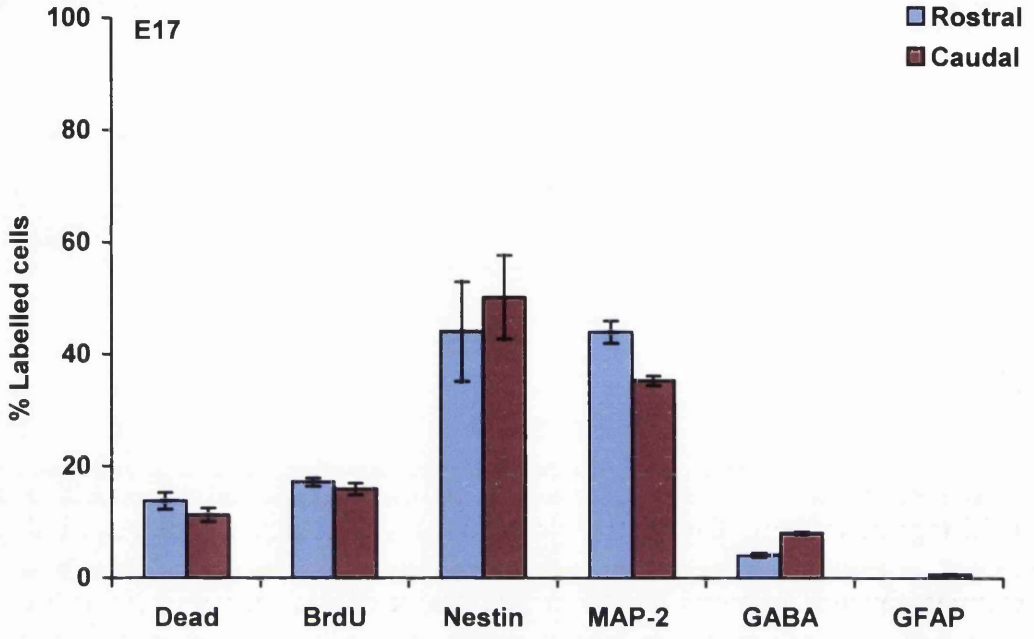

50
40
30
20
10
0
% BrdU labelled
Rostral
Caudal
Region

Figure 30

A Histograms to show the relative proportions of different cell types within cultures obtained from rostral and caudal cortex of E17 embryos. Error bars represent SEM, n=9.

B Histograms to show the relative proportions of different cell types within cultures obtained from rostral and caudal cortex of E19 embryos. n=3


E17
Rostral
Caudal
% Labelled cells
100
80
60
40
20
0
Dead
BrdU
Nestin
MAP-2
GABA
GFAP

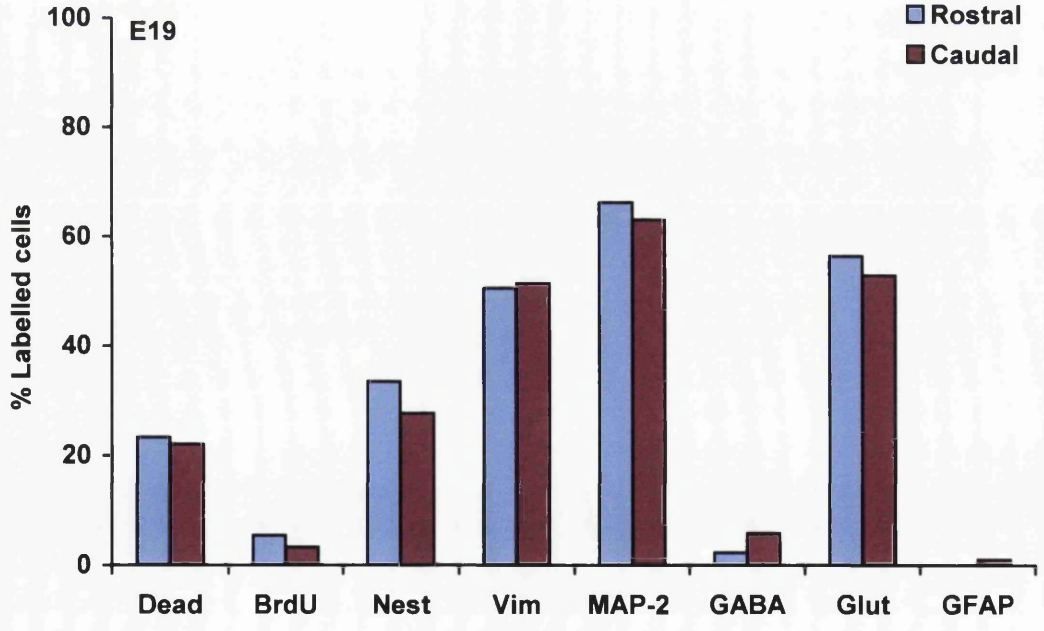

E19
Rostral
Caudal
% Labelled cells
100
80
60
40
20
0
Dead
BrdU
Nest
Vim
MAP-2
GABA
Glut
GFAP

Figure 31

Immunocytochemical labelling for MAP-2 E19 rostral (A) and caudal (B) cells at 1 DIV demonstrating that a considerable degree of differentiation into the neuronal phenotype had occurred. C and D illustrate the vimentin immunolabelling at 1 DIV of rostral and caudal cultures respectively from cells derived from E19 cortices. Note the reduced fraction of vimentin immunoreactive cells reflecting the move towards differentiation rather than proliferation. Scale bar μm.

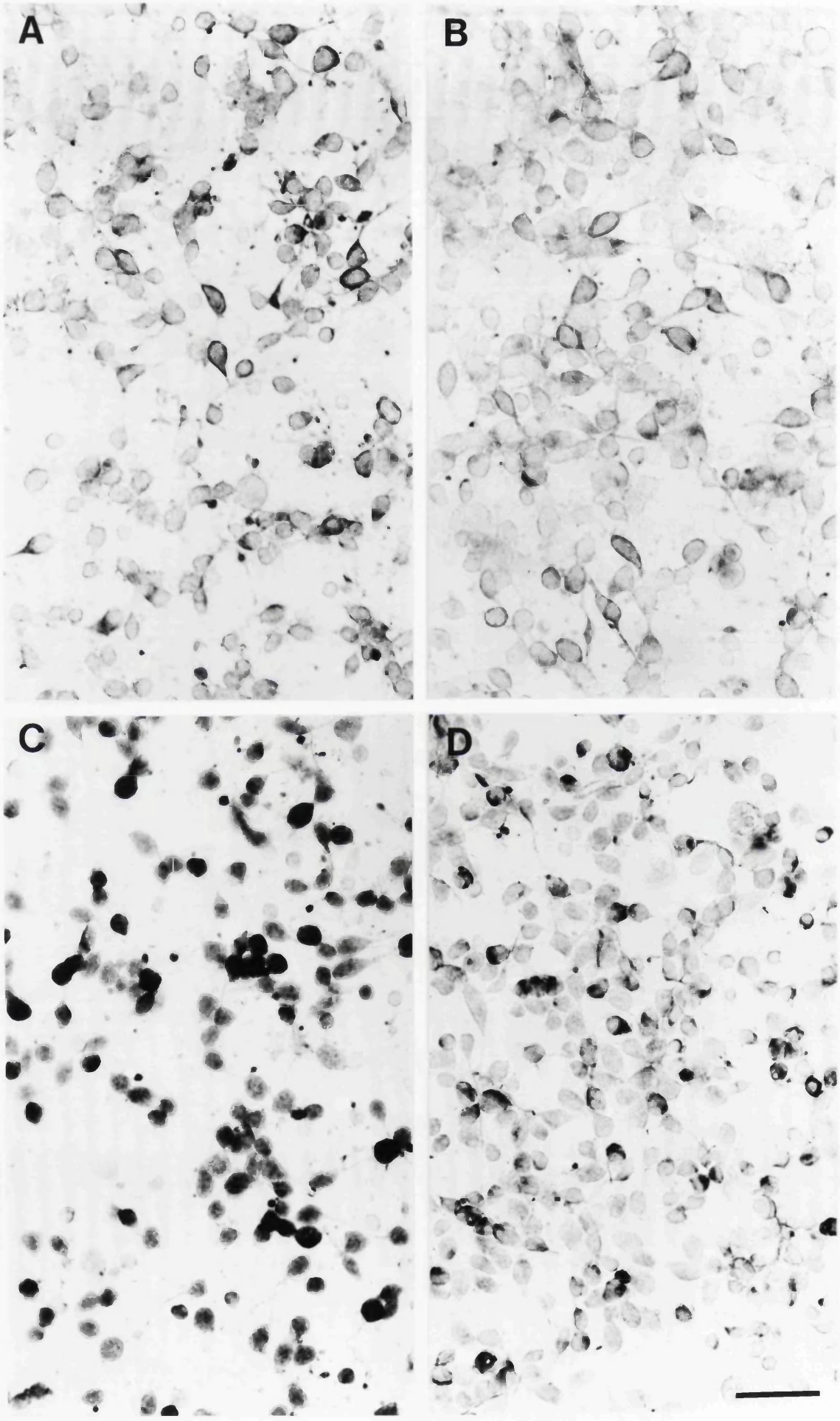

A
B
C
D

Figure 32

Photomicrograph of GABA (A) and glutamate (B) immunoreactivity in E19 caudal cultures 26 hr *in vitro*. Compare the small fraction of GABA immunolabelled cells to the large proportion of glutamate containing cells. The relative fractions reflected the proportions of GABA and glutamate containing cells in the cerebral cortex *in vivo*. Scale bar μm.

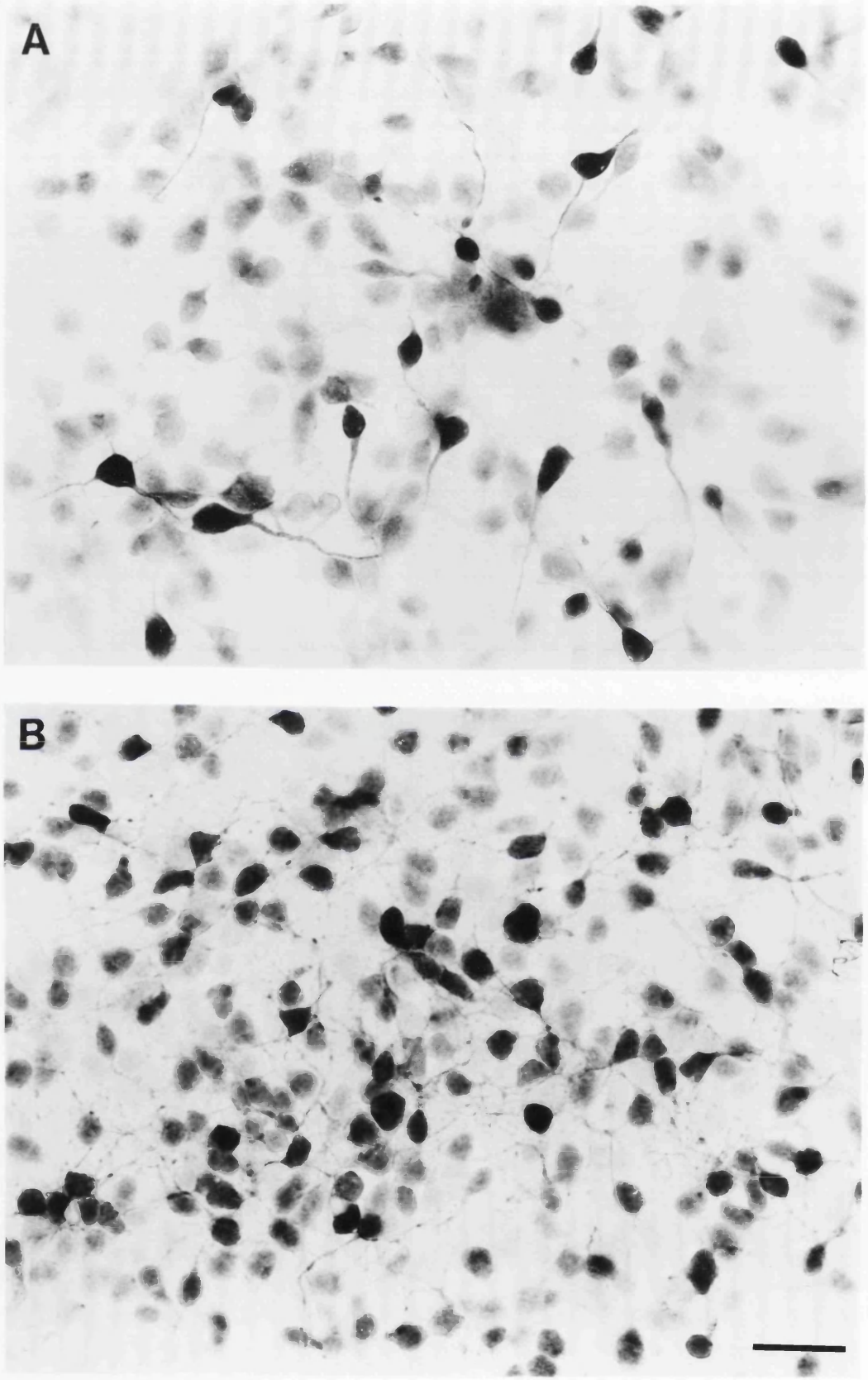
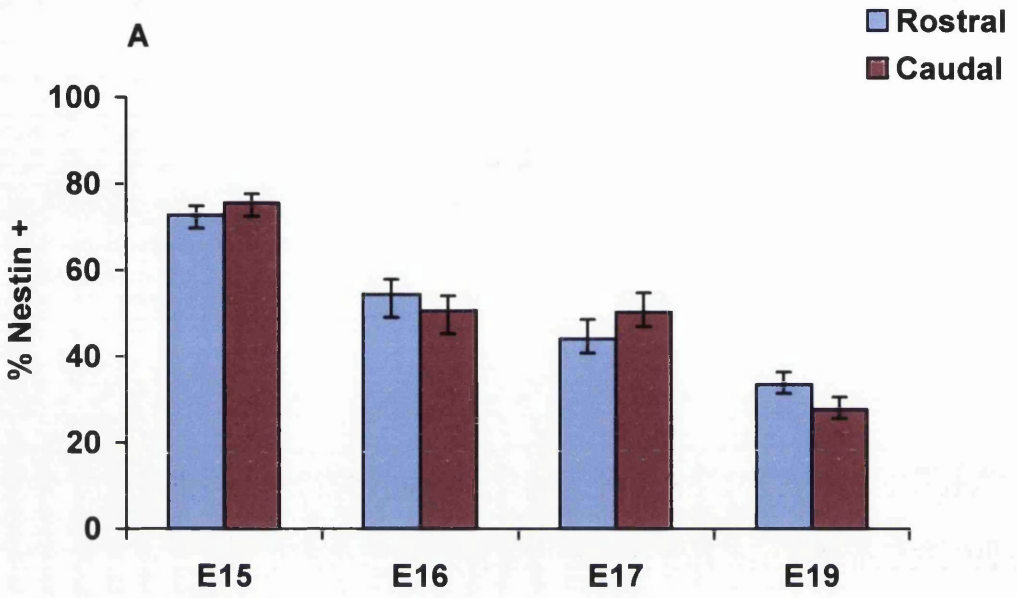

A
B

Figure 33

A Histograms comparing the proportions of nestin immunoreactive cells in rostral and caudal cortex obtained from different ages 1DIV.

B Histograms comparing the proportions of MAP-2 immunoreactive cells in rostral and caudal cortex obtained from different ages cultured for 1 DIV. n=3


A
Rostral
Caudal
100
80
60
40
20
0
% Nestin +
E15
E16
E17
E19

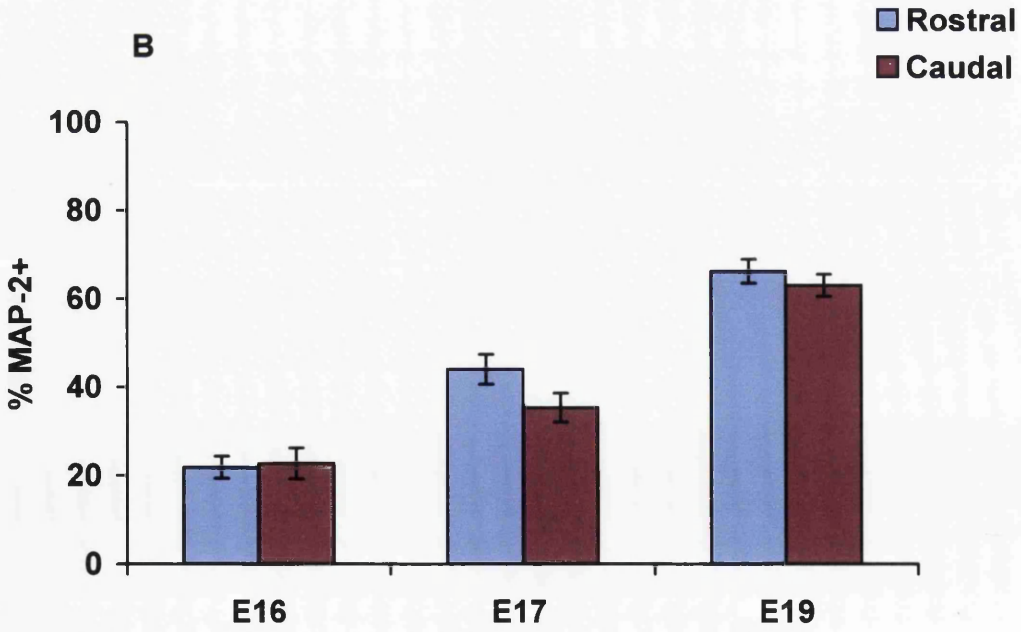
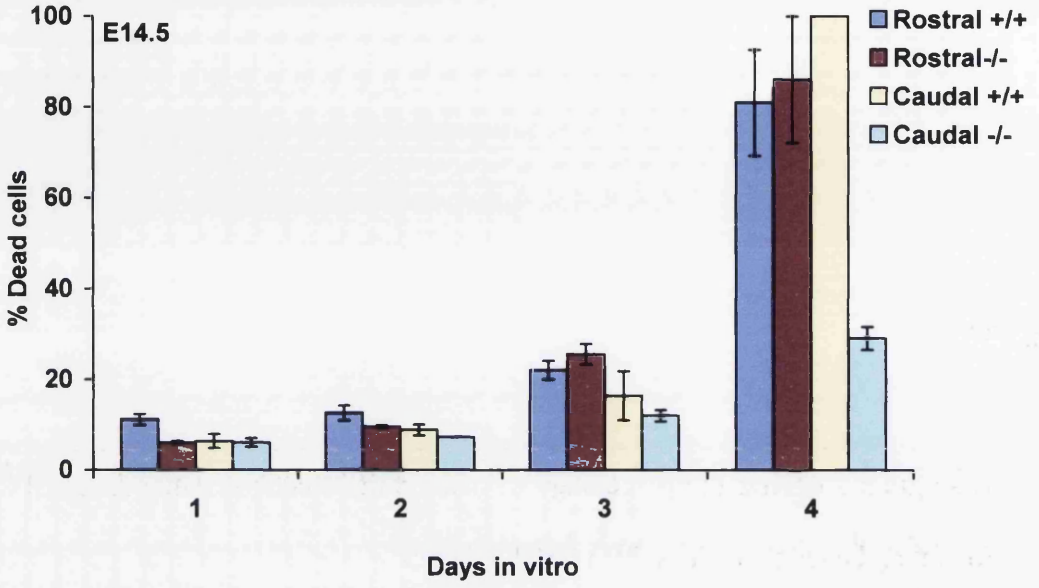

B
Rostral
Caudal
100
80
60
40
20
0
% MAP-2+
E16
E17
E19

Figure 34

A Histograms showing the percentage of cell death in cells isolated from E14.5 rostral and caudal cortices from bFGF -/- mice.

B Histograms showing the percentage of cell death in cells isolated from E15.5 rostral and caudal cortices from bFGF -/- mice.


E14.5
Rostral +/+
Rostral-/-
Caudal +/+
Caudal -/-
% Dead cells
100
80
60
40
20
0
1
2
3
4
Days in vitro

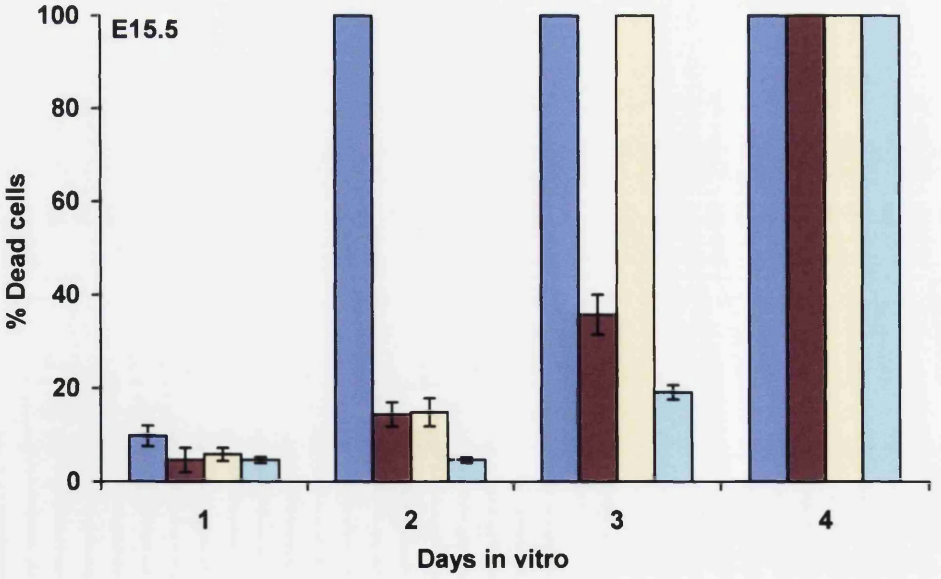

E15.5
% Dead cells
100
80
60
40
20
0
1
2
3
4
Days in vitro

Figure 35

Western blot demonstrating the presence of bFGF protein in rostral and caudal cortex derived from a pool of E17 brains. Antibody used to detect the presence of bFGF was Ab118 which appeared only to detect the 18 kDa isoform. Two specific bands were detected. One band at 18 kDa and one at 10 kDa in rostral and caudal cortex. These bands corresponded to those seen in the lane in which the purified protein was run. The nature of the 10 kDa protein was unclear. Although not quantitated, there appeared to be more bFGF present in the caudal cortex than in rostral cortex, despite approximately the same amount of tissue isolated. Abbreviations, rostral (rost), caudal (caudal).

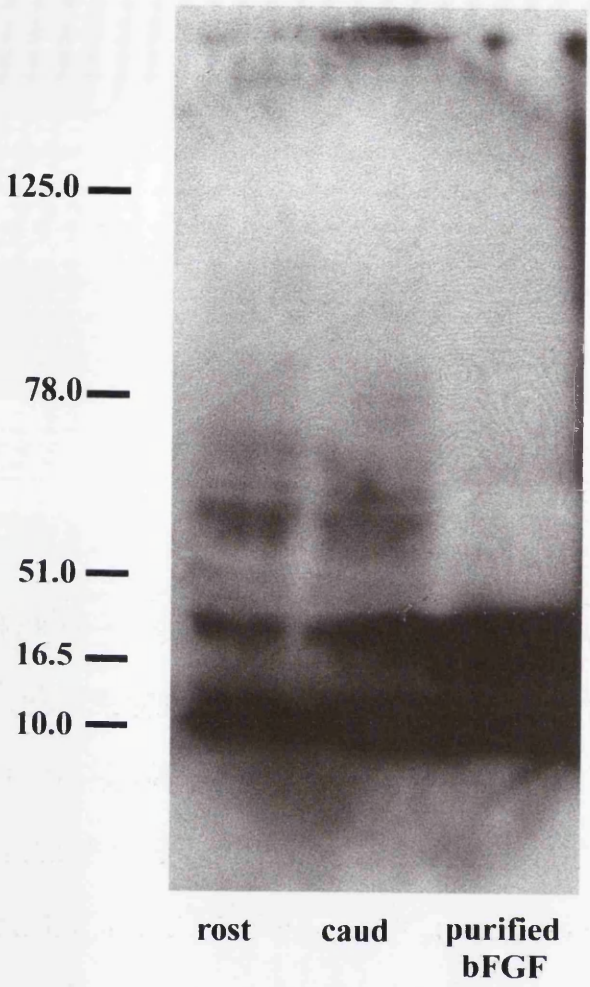

125.0
78.0
51.0
16.5
10.0
rost
caud
purified bFGF

Figure 36

Photomicrograph of E17 sagittal cryostat sections of cortex immunostained for Ab 106 for bFGF. Specifically, Ab106 recognises 18, 21 and 23 kDa forms of bFGF. Basic FGF immunoreactivity was evident in all layers of the cortex in both rostral (A) and caudal (B) regions of the cortex. More intense immunolabelling was observed in the cortical plate (CP, C), subventricular zone (SVZ) and ventricular zone (VZ, D). Arrows show non-immunoreactive cells. The inset in C, demonstrates this more clearly. BFGF immunoreactivity was largely confined intracellularly. The intensity of immunoreactivity was apparently greater in the more caudal than rostral cortex. Abbreviations, corticostriatal sulcus (CSS); marginal zone (MZ); intermediate zone (IZ); lateral ventricle (LV). Scale bar, A, B = 400 μm, C, D = 200 μm.

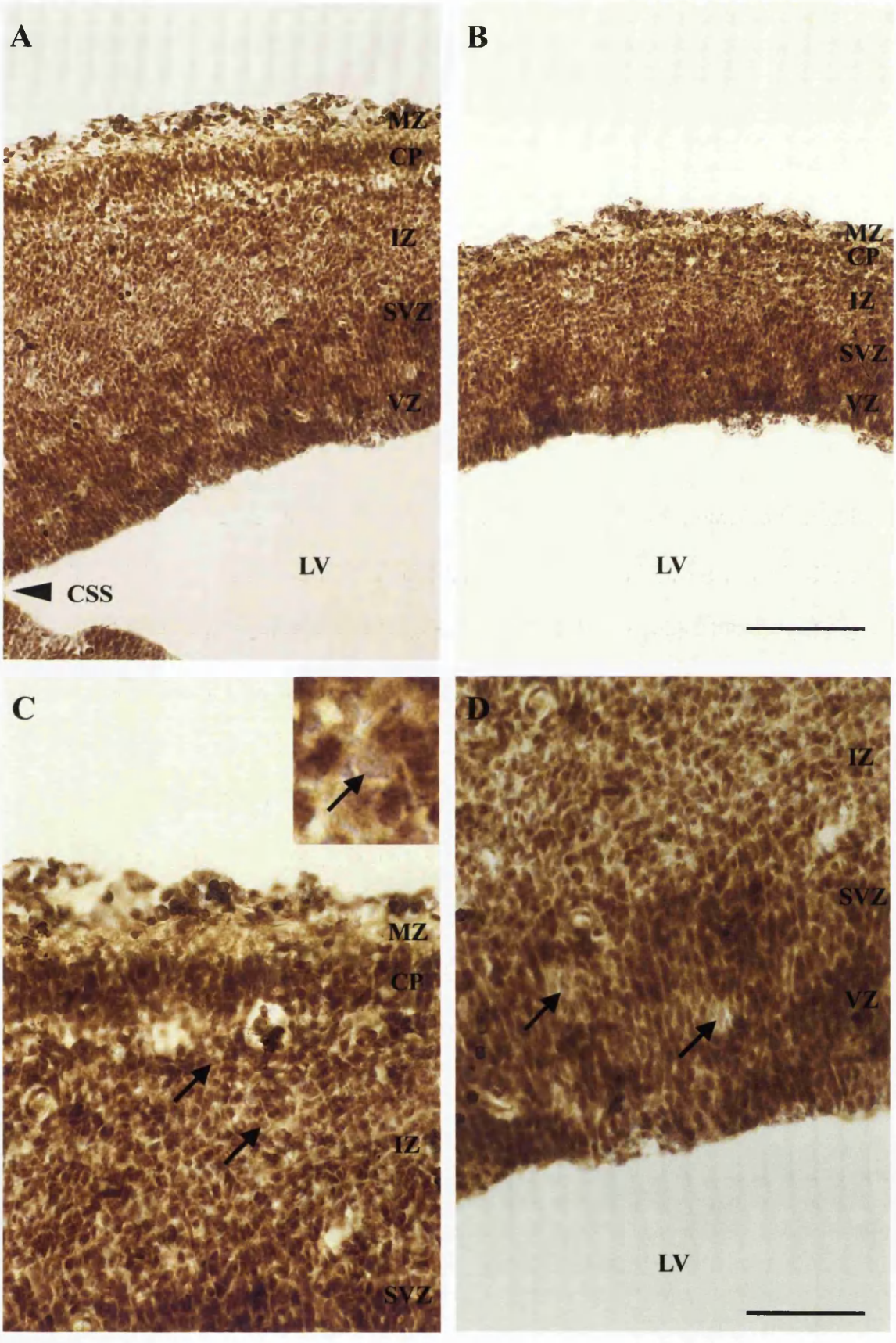

A
MZ
CP
IZ
SVZ
VZ
LV
CSS
B
MZ
CP
IZ
SVZ
VZ
LV
C
MZ
CP
IZ
SVZ
D
IZ
SVZ
VZ
LV

Figure 37

Photomicrographs of paraffin embedded sections of adult cerebral cortex. Ab 106 was preabsorbed with purified bFGF protein prior to application to the slide (A, arrow heads show unlabelled glia). There was clearly no cross reactivity of this antibody with other antigens. In the adult cortex, bFGF was detected specifically in the nuclei of glial cells (small arrows) distributed throughout all layers of the cortex (B, C and D). Unlabelled cells had larger nuclei and were the neuronal cells of the cerebral cortex (long arrows). Blood vessels (BV). Scale bar A = 800 μm, B = 1600 μm, C = 800 μm, D = 400 μm.

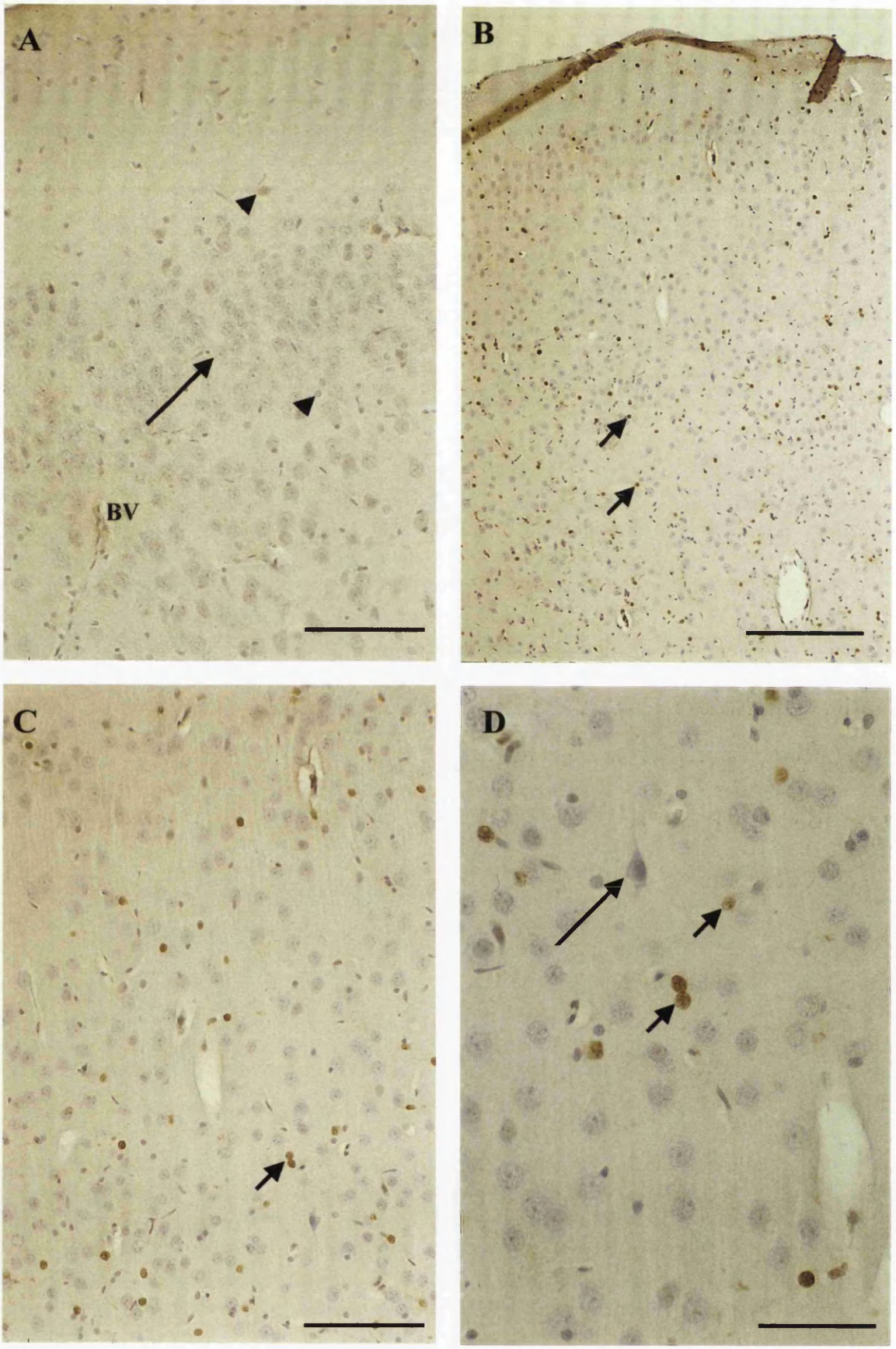

A
BV
B
C
D

Figure 38

Anti bFGF Ab 106 immunoreactivity was observed in some neurones of the cerebral cortex (arrows, A and B). Unlabelled cells are marked by arrow heads. Arrow refers to a labelled neuron. Scale bar A = 800 μm, B = 400 μm.

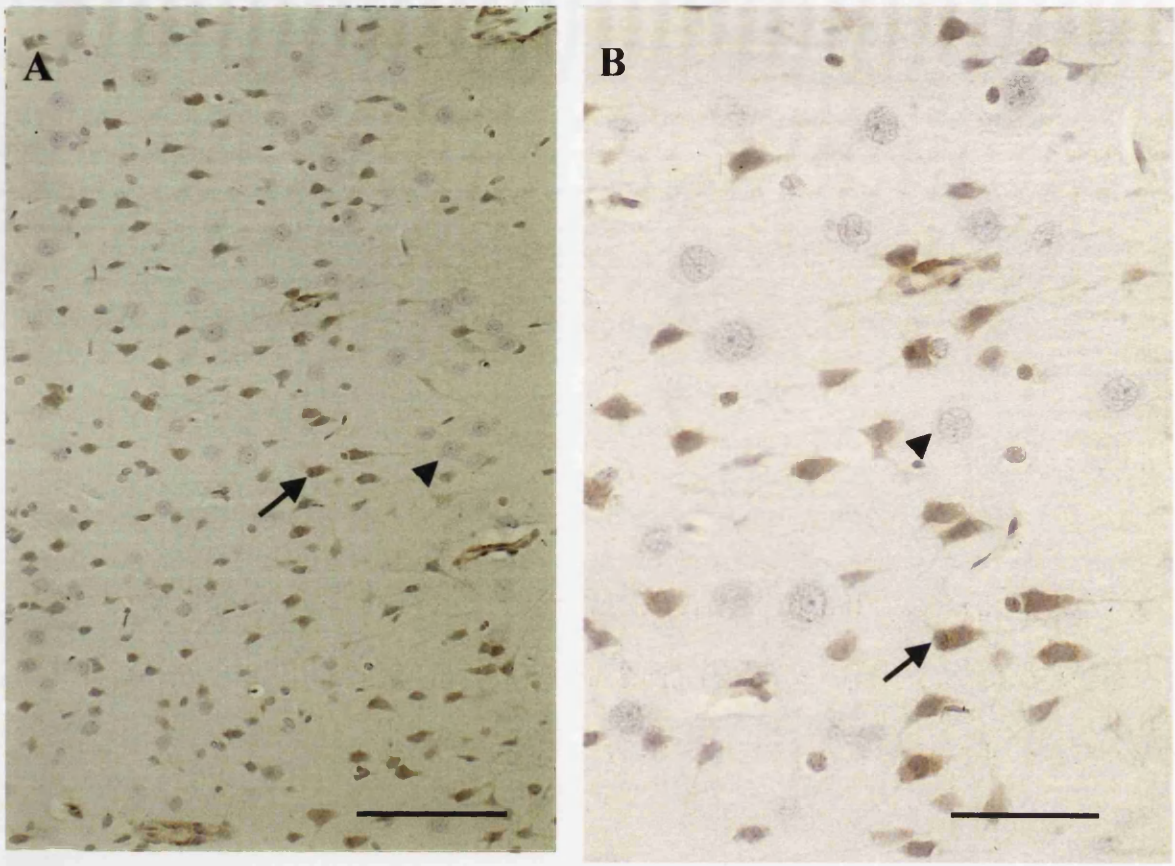

A
B

Figure 39

Immunoreactivity in the rostral (A) and caudal region (B) of the cerebral cortex for FGFR 1. FGFR 1 immunoreactive cells were apparent in the majority of cells in E17 cerebral cortex. Intense labelling was apparent in the cortical plate (CP) and ventricular zone (VZ). Higher magnification revealed immunoreactivity present within cell bodies and their nuclei and cell processes (C and D). There was an apparent rostrocaudal gradient of intensity, with apparently fewer and less intensely labelled cells in the rostral region of the cortex compared to caudal cortex. Abbreviations, marginal zone (MZ), intermediate zone (IZ), subventricular zone (SVZ), lateral ventricle (LV). Scale bar A, B = 800 μm, C, D = 200 V

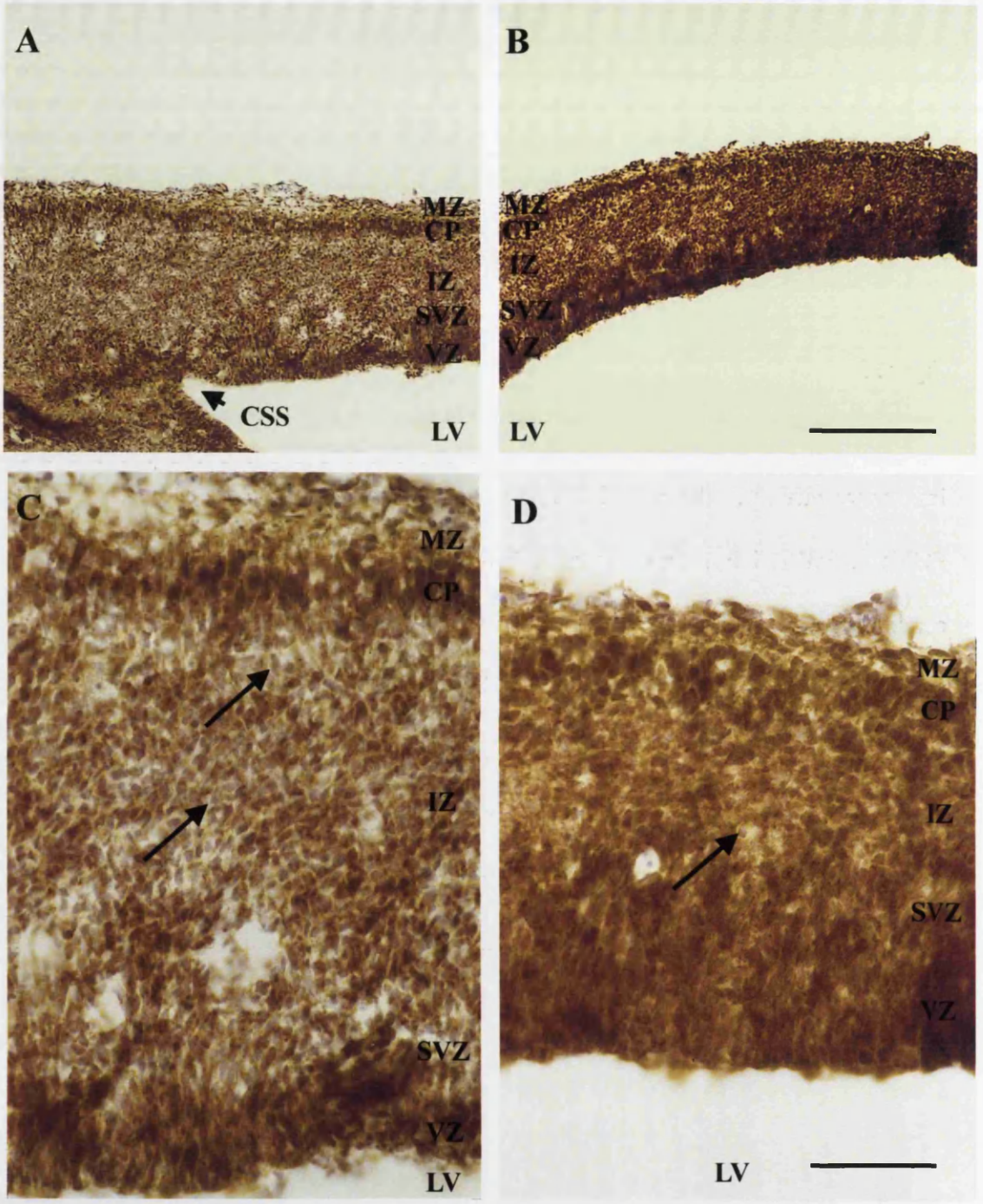

A
MZ
CP
IZ
SVZ
VZ
CSS
LV
B
MZ
CP
IZ
SVZ
VZ
LV
C
MZ
CP
IZ
SVZ
VZ
LV
D
MZ
CP
IZ
SVZ
VZ
LV

Figure 40

In situ hybridisation for bFGF mRNA transcripts. A sagittal section of E17 rat brain demonstrates intense labelling throughout the germinal epithelium (A). There was an apparent reduction in the intensity of signal in the rostro to caudal axis. Panels B and C compare the signal in caudal and rostral regions of the cortex respectively. The majority of cells generating the transcript were present in the ventricular zone (VZ) and the cortical plate (CP). Diffuse labelling was seen throughout the intermediate zone (IZ). Due to the nature of the preparation, the marginal zone (MZ) had dissociated. Some intense labelling was seen in the CP. Panels D and E are higher magnifications of the caudal cortex, respectively. Abbreviations, cerebral cortex (CX), lateral ventricle (LV). Scale bar A = 800 μm, B, C = 400 μm, D, E = 200 μm.

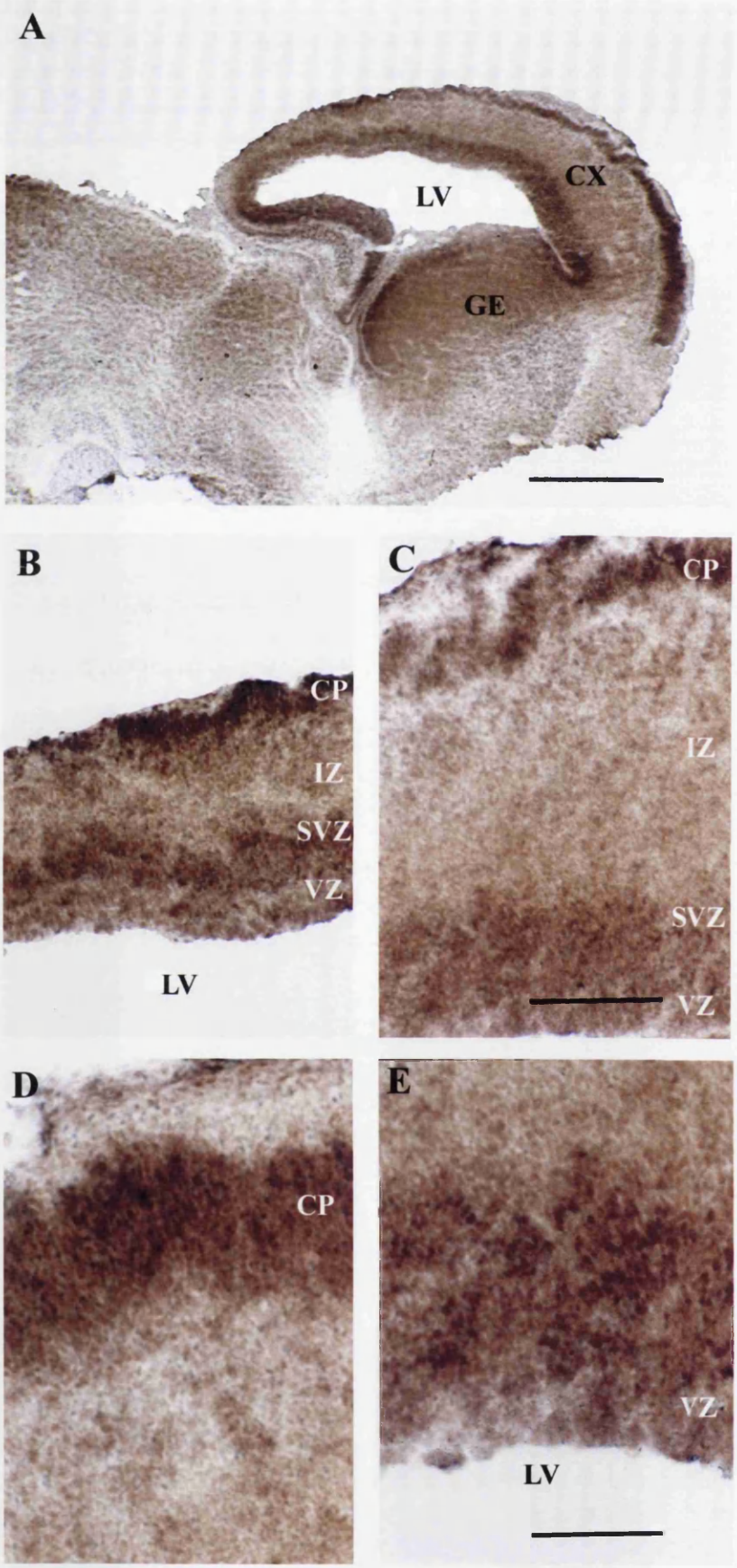

A
CX
LV
GE
B
CP
IZ
SVZ
VZ
LV
C
CP
IZ
SVZ
VZ
D
CP
E
VZ
LV

Figure 41

In situ hybridisation for FGFR 1 in cryostat sections of E17 cerebral cortex. The antisense signal was detected using anti-DIG alkaline phosphatase in sagittal cryostat sections (A). There was no apparent gradient of messenger expression in the rostrocaudal axis. A higher magnification of caudal (B) and rostral (C) cortex confirmed this. Messenger FGFR1 expression was greatest in the cortical plate (CP, D) and ventricular zone (VZ) and subventricular zones (SVZ) (E). Although some cells were labelled in the intermediate zone (IZ). This pattern of expression reflected closely that seen using immunohistochemistry. Abbreviations; lateral ventricle, (LV), cerebral cortex, (CX). Scale bar A = 800μm, B, C = 400 μm, D, E = 200 μm.

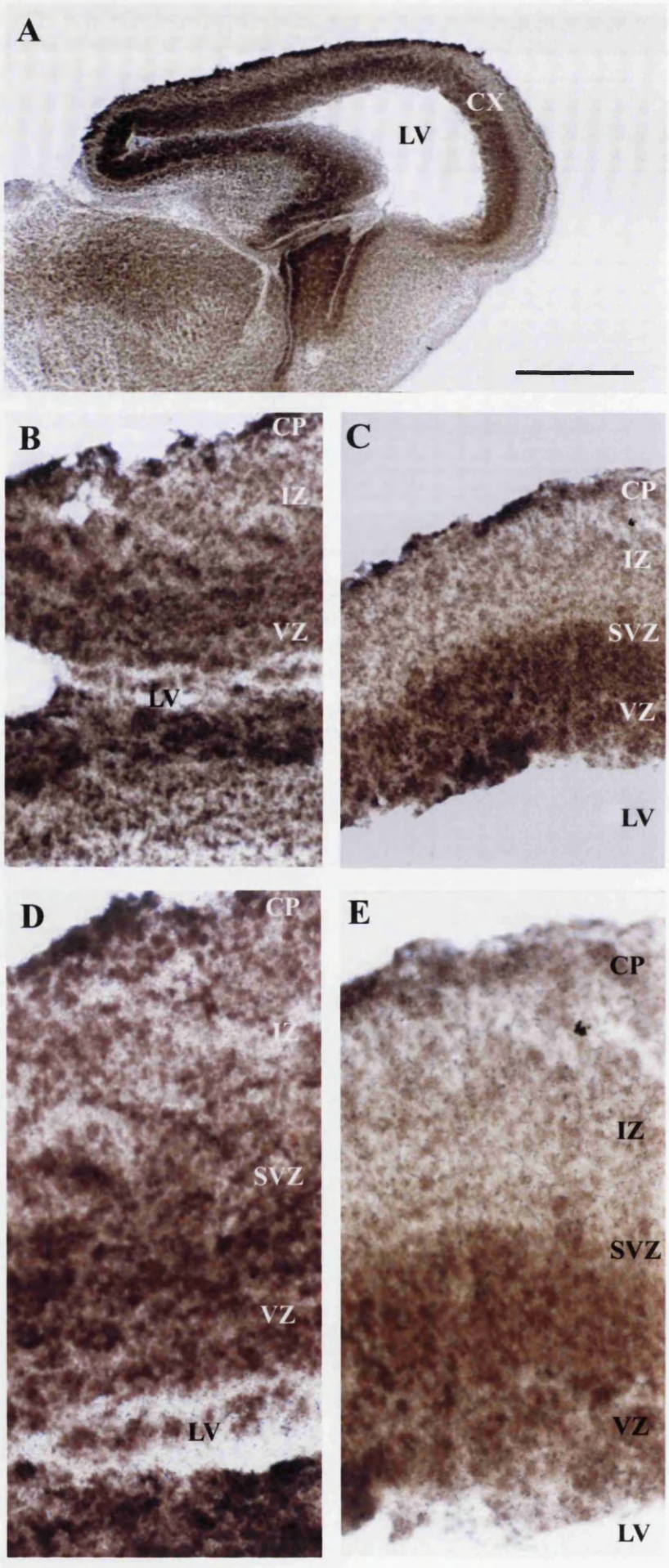

A
CX
LV
B
CP
IZ
VZ
LV
C
CP
IZ
SVZ
VZ
LV
D
CP
IZ
SVZ
VZ
LV
E
CP
IZ
SVZ
VZ
LV

Figure 42

FGFR 1 immunolabelling of E17 rostral cells at 1 and 2 DIV (A and C) and caudal cells (B and D) at 1 and 2 DIV. Antibody omission-controls show absence of immunoreactivity (E). F shows FGFR 1 immunoreactivity at 2 DIV following treatment with bFGF. Scale bar 30 μm.

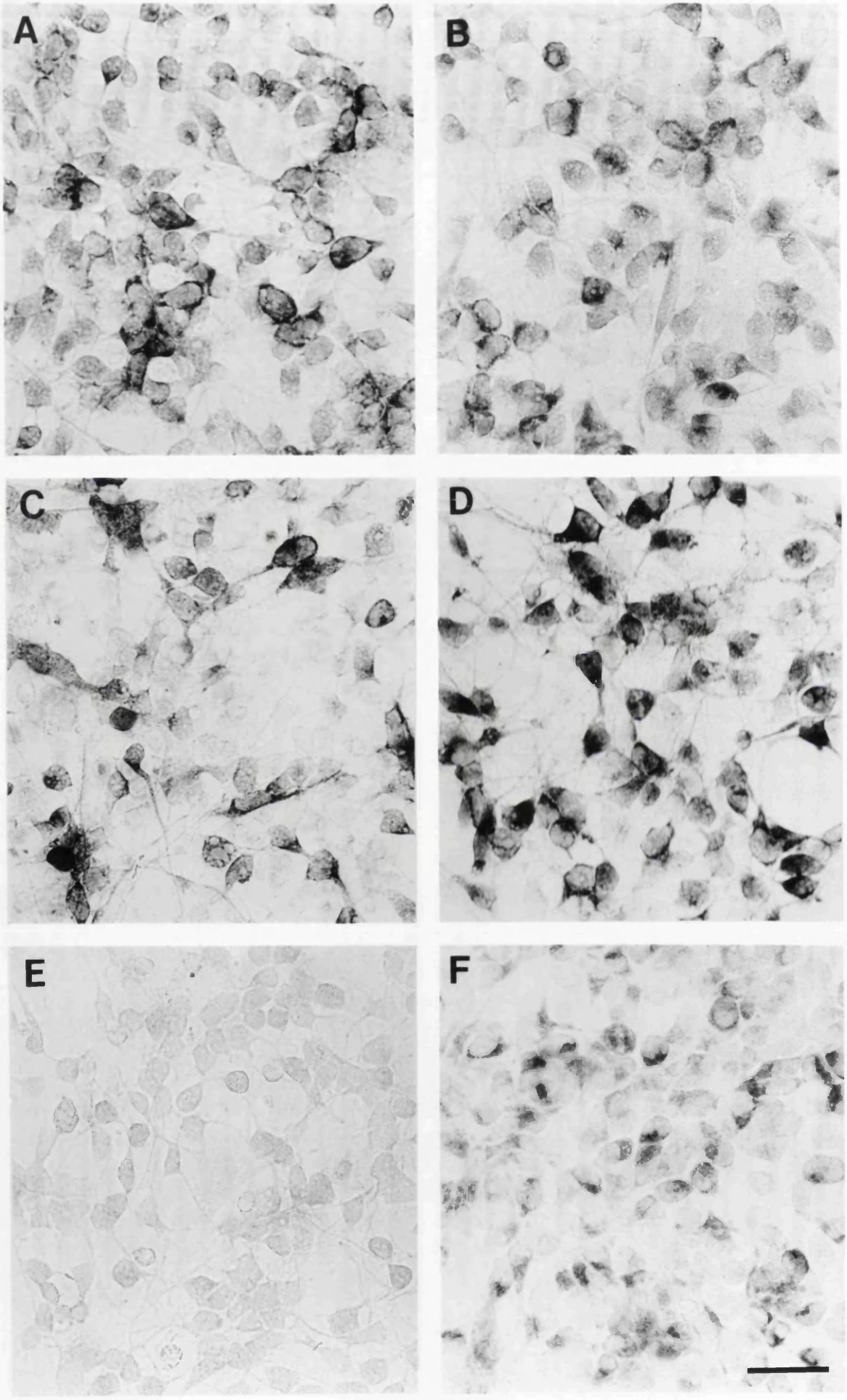

A
B
C
D
E
F

Figure 43

Acridine orange, a vital dye, distinguishes cells actively undergoing cell death and cells in which necrotic changes have taken place (A). Healthy cells had large rounded nuclei and appear green (arrow head, B). Cells undergoing programmed cell death appear green but the nuclei appeared blebbed (small arrow, C). The profiles of cells that appeared orange under the same filter were of cells that have previously undergone the apoptotic pathway but were currently in the secondary necrotic phase of cell death (large arrow, B). Panel D, E and F demonstrate TUNEL labelled cultures. TUNEL labelled cells (green) had blebbed nuclei and were scattered throughout the culture. Counterstaining with propidium iodide (red) showed healthy cells (large rounded nuclei) and cells which had blebbed nuclei but were not labelled with TUNEL (arrow, F). Thus, not all cells undergoing apoptosis were detected by the TUNEL method. Scale bar A, D= 200 μm, B, C, E, F = 20 μm.

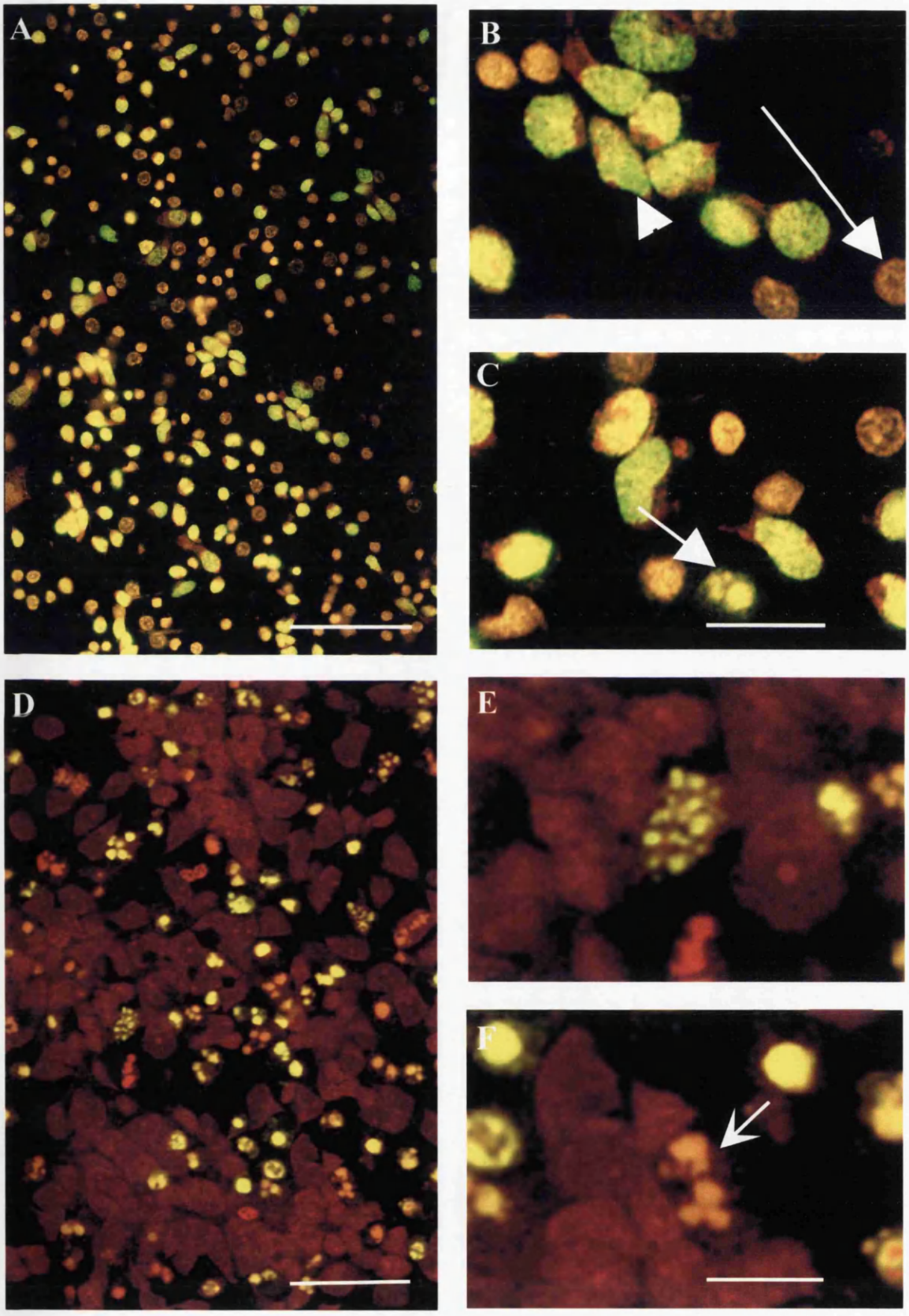

A
B
C
D
E
F

Figure 44

Nestin immunoreactivity demonstrated in E17 rostral cultures in control conditions (A and C) at 1 and 3 DIV respectively, and in the presence of bFGF (B and D) at 1 and 3 DIV respectively. Note that in the presence of bFGF the proportion of cells rescued from cell death was significant ($P<0.05$) after 3 days in culture. This was also reflected in an increase in the proportion of nestin immunoreactive cell population at 3 DIV, however the latter were not quantitated. Scale bar 30 μm.

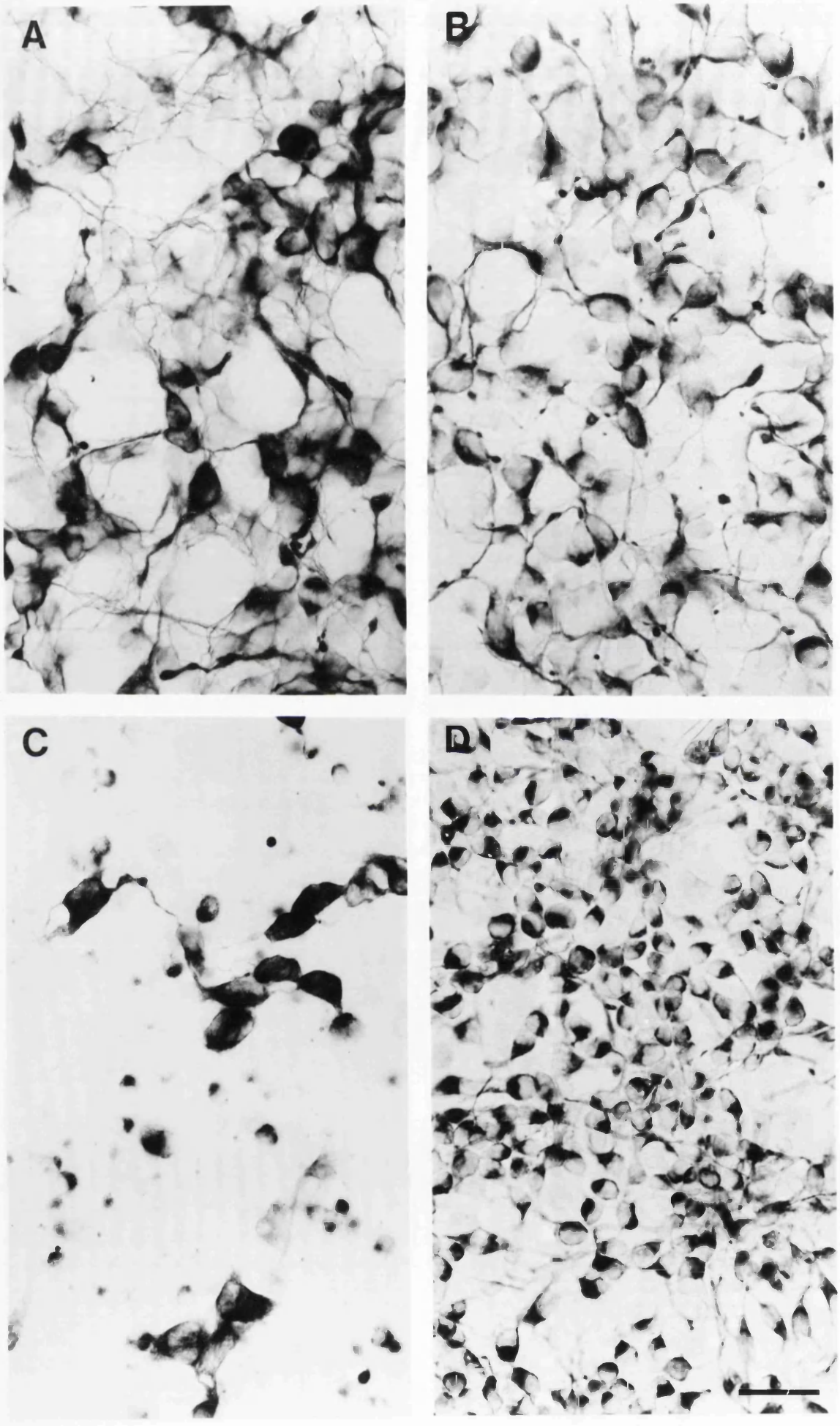

A
B
C
D

Figure 45

Vimentin immunoreactivity at 1 DIV in rostral and caudal cells derived from E15 cortices (A and B) and from E16 cortices (C and D). In all cultures, a high proportion of cells were immunoreactive for vimentin reflecting the high proportion of undifferentiated neuroepithelial cells presented in early embryonic cultures. There did not appear to be a striking difference in the proportion of rostral cells that were immunoreactive for vimentin compared with caudal cultures. Scale bar 30 μm.

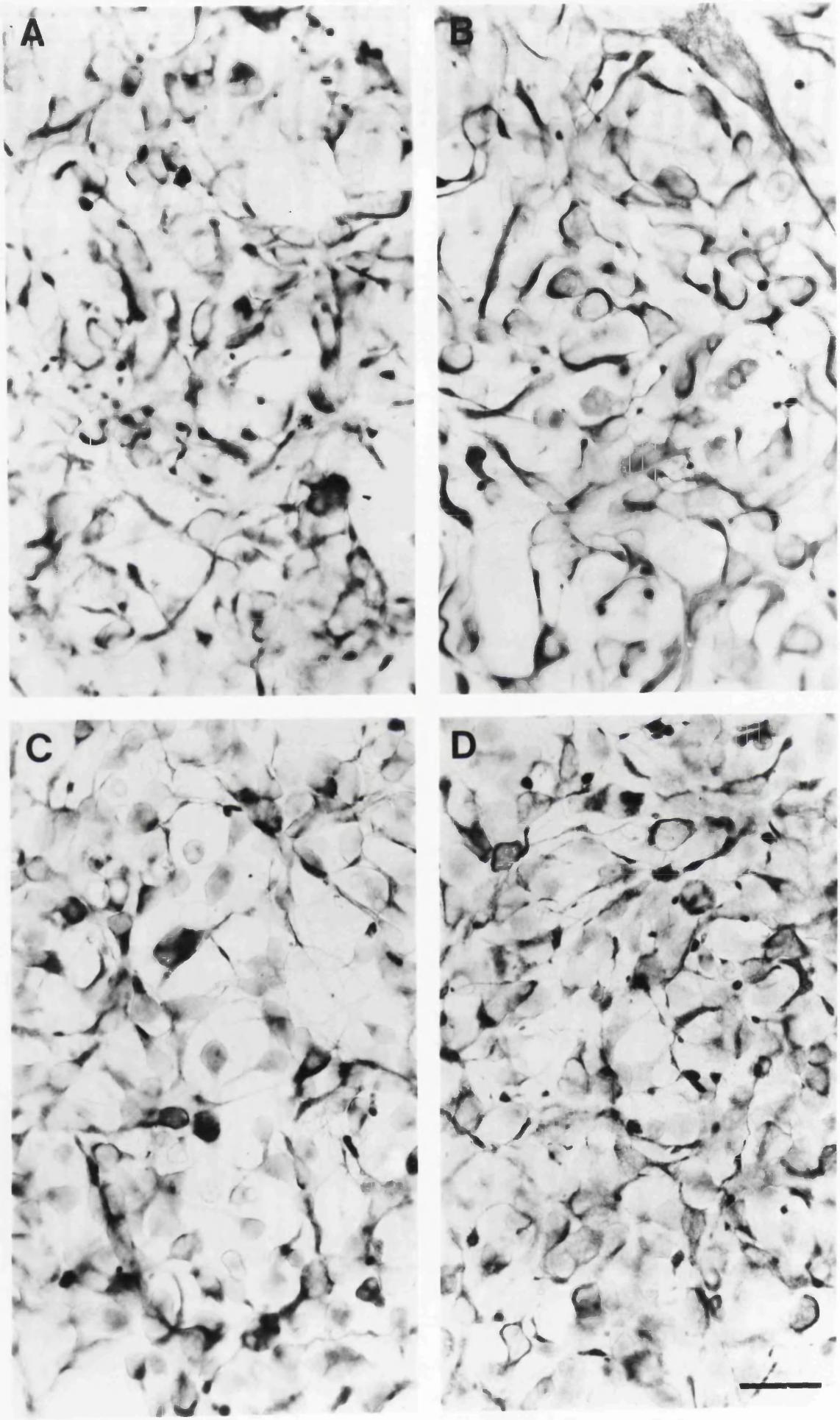

A
B
C
D

Figure 46

MAP-2 immunoreactivity in E16 derived cortical cultures. A, C, and E MAP-2 immunoreactivity in rostral cells cultured for 1, 3 and 4 DIV. B, D and F, MAP-2 immunoreactivity in caudal derived cultures. Note that at 3 DIV fewer rostral cells express the neuronal marker and by 4 DIV almost all the rostral cells have undergone cell death, in contrast to caudal cells in which a significant proportion of cells were immunoreactive for MAP-2. Caudal cells continued to differentiate phenotypically and morphologically. Scale bar 30 μm.

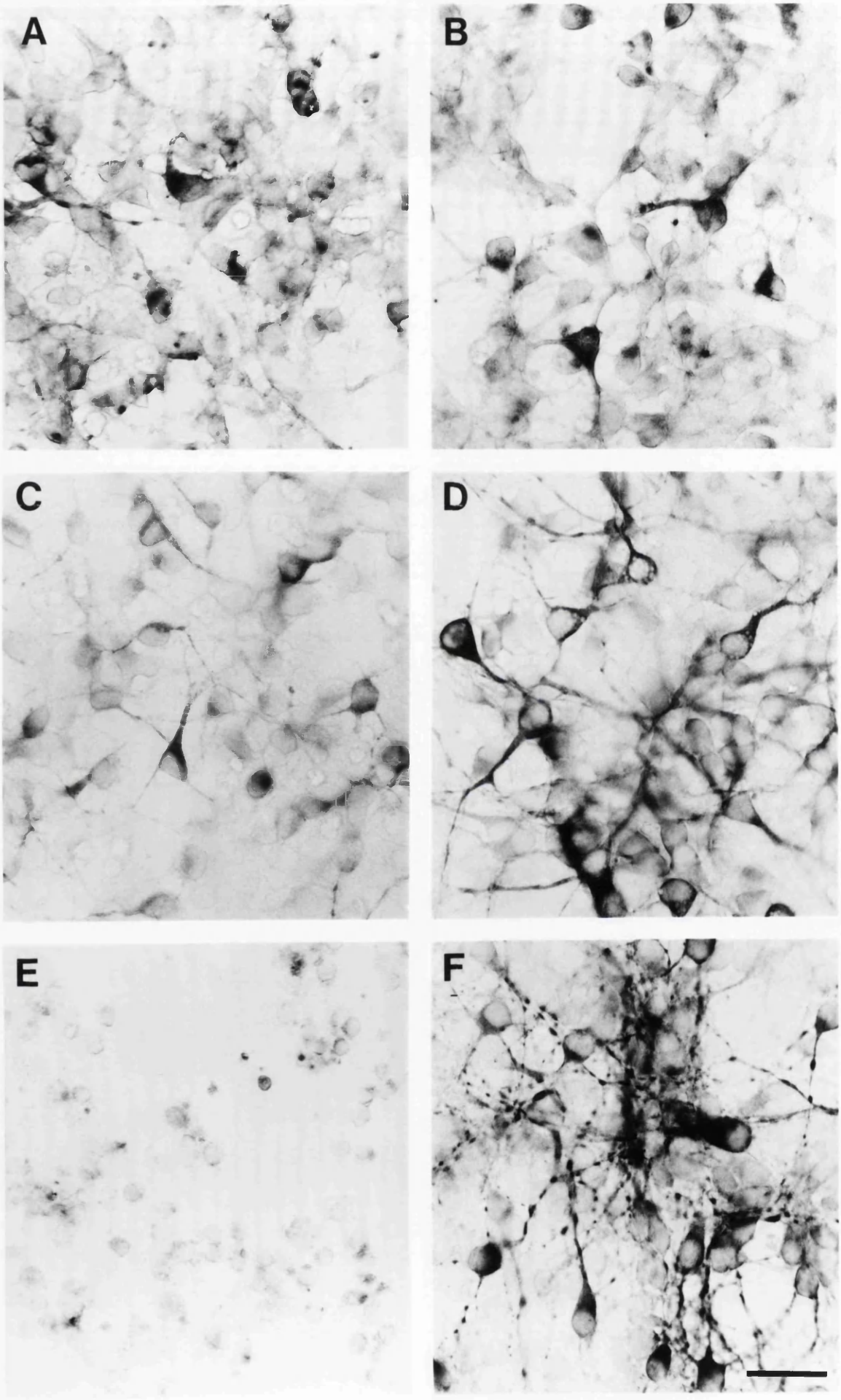

A
B
C
D
E
F

Figure 47

Immunocytochemical labelling for MAP-2 E15 rostral (A) and caudal (B) cells at 1 DIV. A and B demonstrate that *in vitro* even cells taken from cortices as young as E15 some cells undergo differentiation and express neuronal markers following dissociation and culture. However this fraction was considerably low, and no apparent difference was observable between rostral and caudal cells. C and D, immunolabelling for vimentin in E15 derived cultures, rostral and caudal respectively. The vast majority of cells at E15 were immunoreactive for markers of undifferentiated neuroepithelial cells. Scale bar 30 μm.

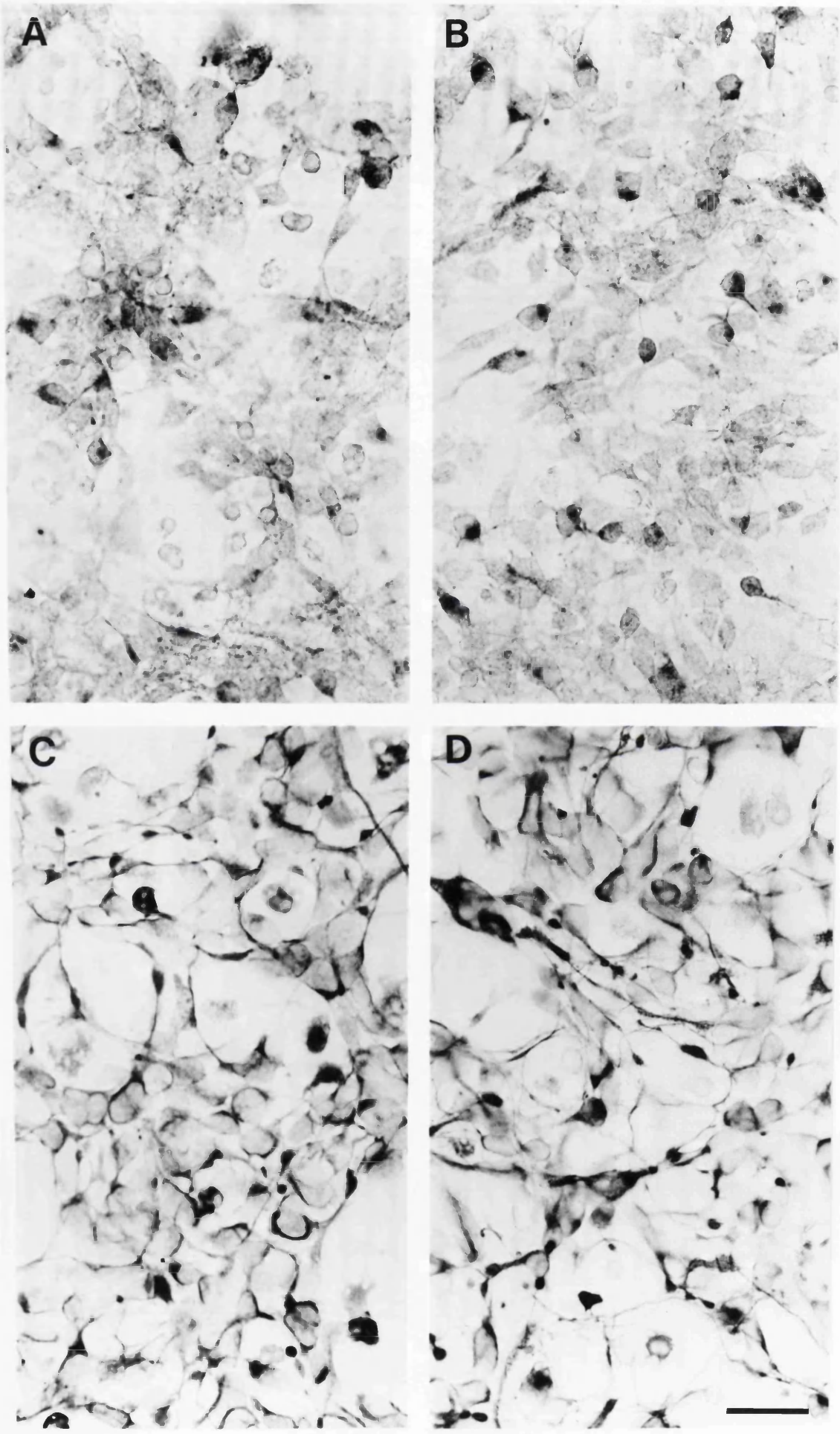

A
B
C
D

Figure 48

GFAP immunoreacitivty in E17 rostral cells under control conditions (A and C) at 1 and 3 DIV, and in the presence of bFGF (B and D) at 1 and 3 DIV. Note the virtual absence of GFAP immunoreactive cells in the rostral cultures under control conditions. Within 24 hr of treatment with bFGF, a greatly increased proportion of GFAP immunoreactive cells became apparent. Scale bar 30 μm.

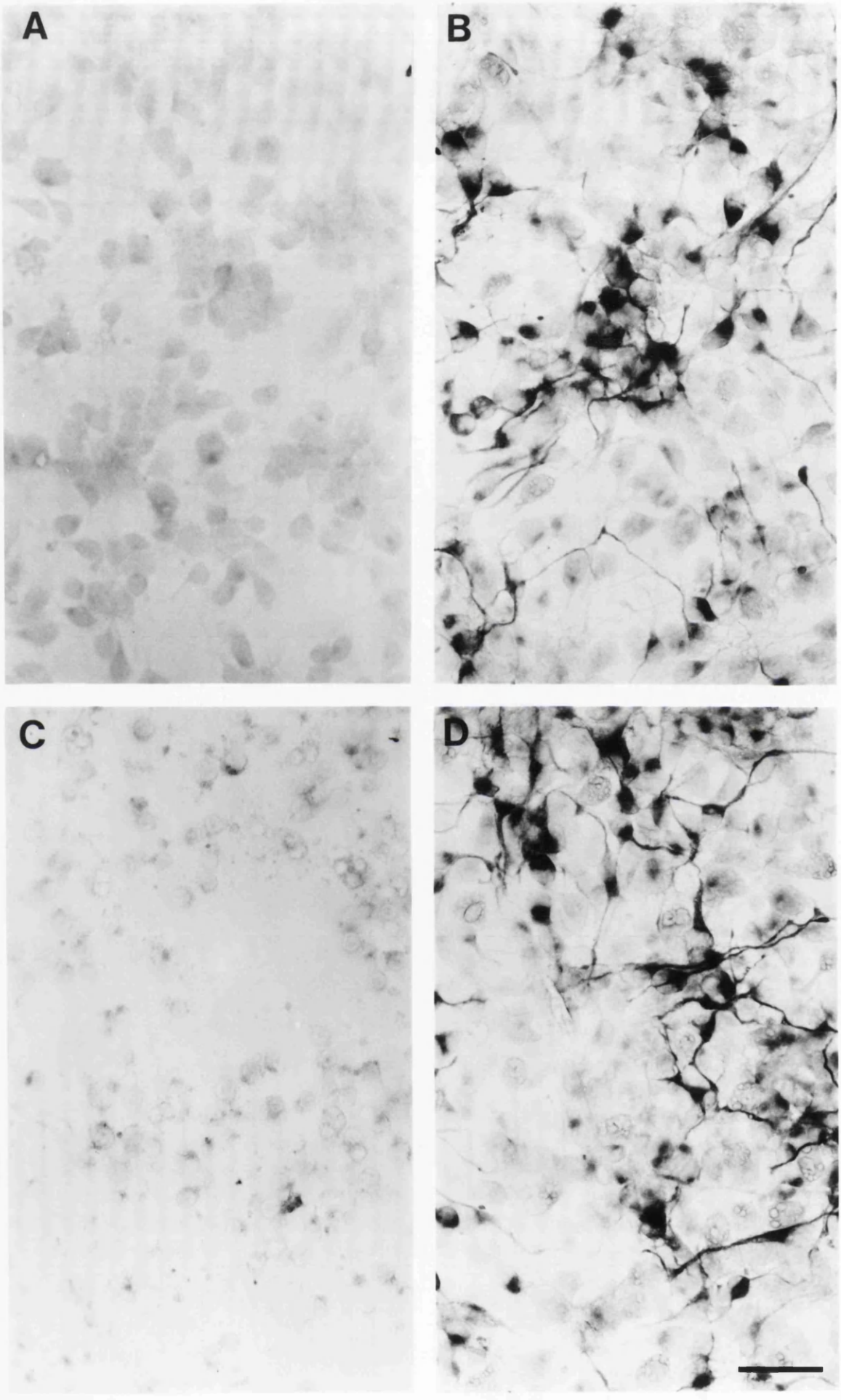

A
B
C
D

Figure 49

Scanning electron microscopic image of E17 cultures at 1 DIV. Note the variety of morphologies present, the extensive network of neurites and the various stages of cell death and degeneration. A degree of cellular debris was observed scattered throughout the field reflecting the end stage necrotic cell death pathway which occured independent of and following programmed cell death in culture, as no macrophages were present to clear cellular debris. Scale bar 20 μm.

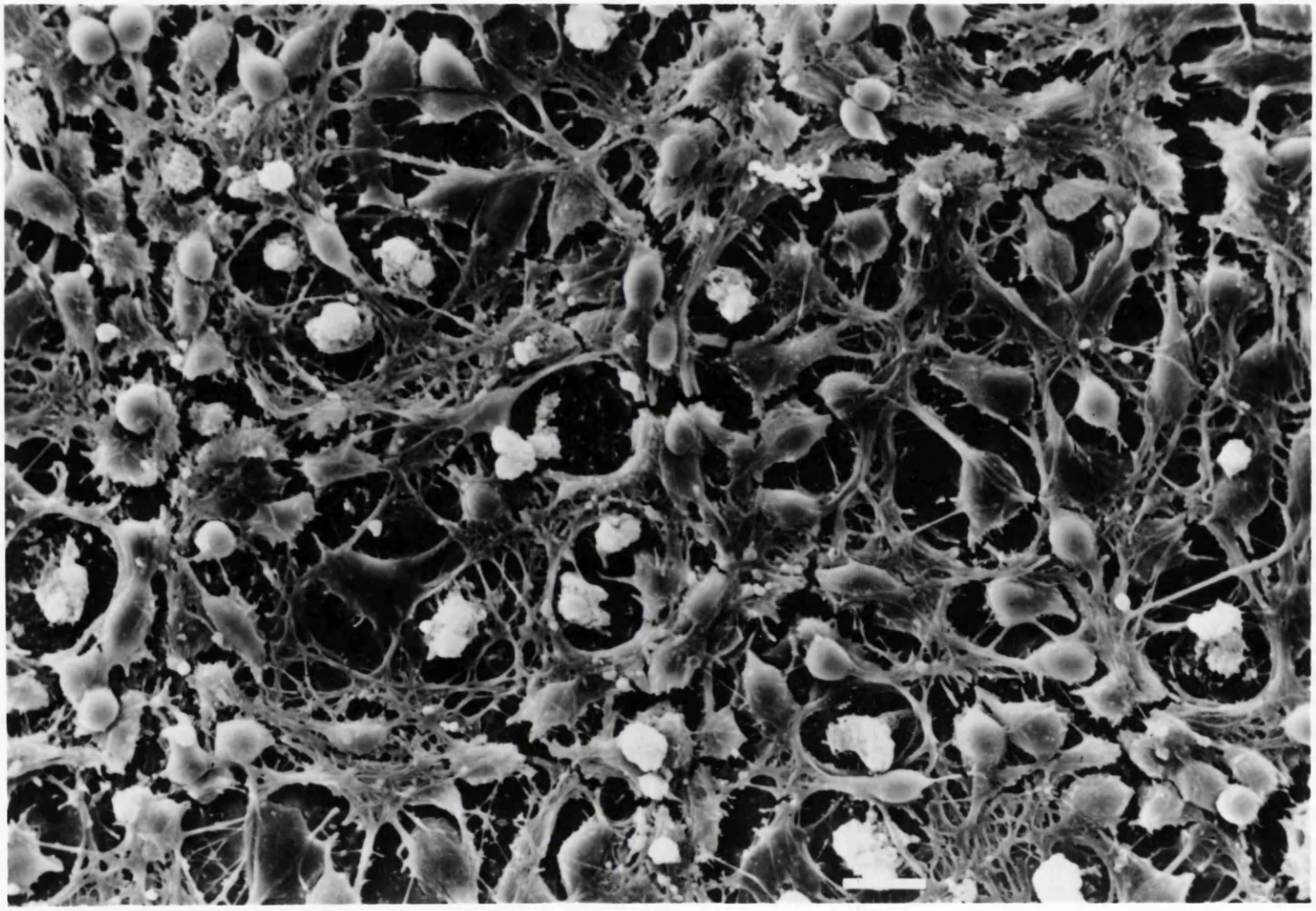


Figure 50

Scanning electron microscopic image of E17 cultures at 1 DIV. A, B and C demonstrate the various morphologies attained by neuroepithelial cells, neuronal cells and cells at various stages between undifferentiated and differentiated states. An extensive network of neurites was apparent very soon after plating. Artifactual breaks in the neuritic connections were possibly due to dehydration during sample preparation. Scale bar = 10 μm.

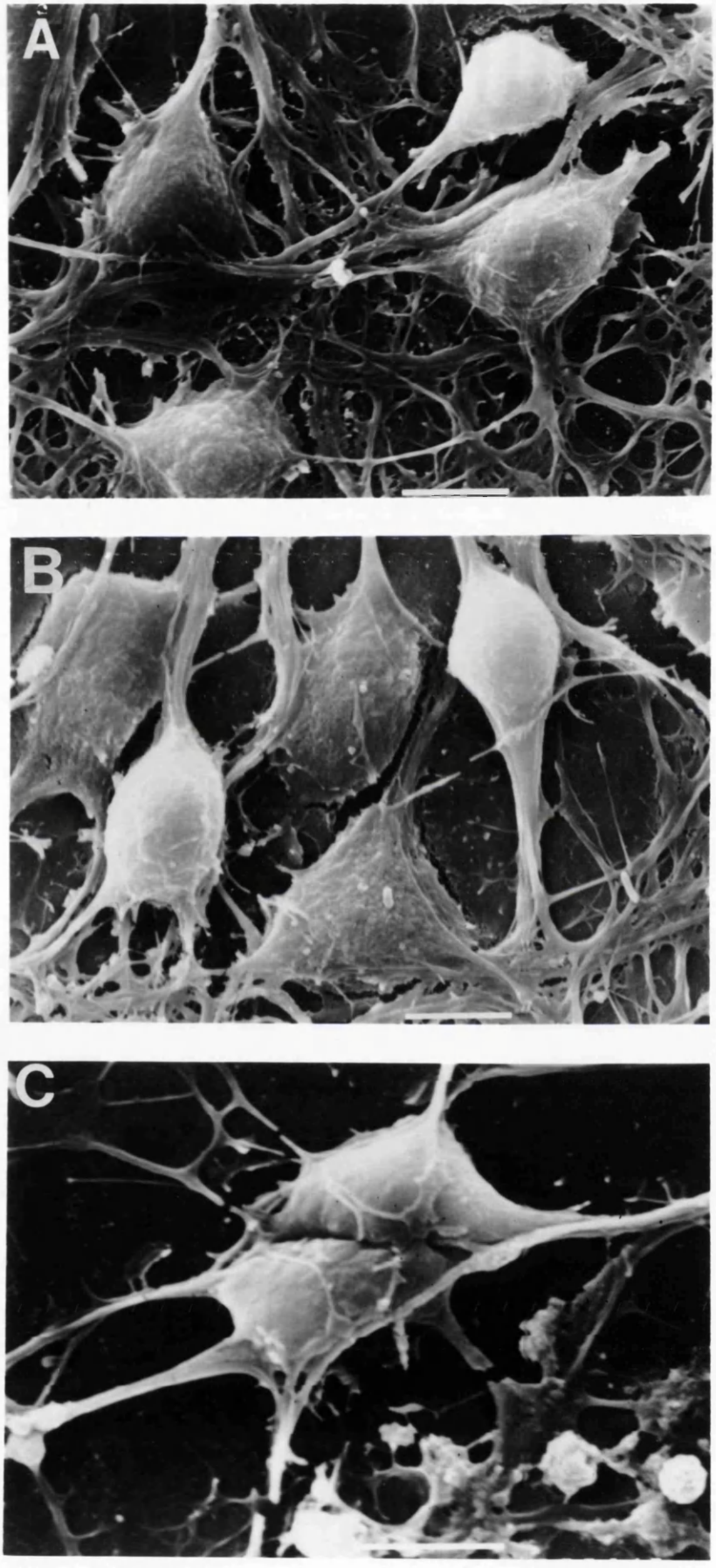

A
B
C

Figure 51

Scanning electron micrograph of cells in cultures derived from E17 cortices at various stages of apoptosis. Cells lost many, but not necessarily all their, neuritic connections, and become rounded (A). The relatively smooth scaly surface of the membrane of the cell became somewhat disorgansised and corrugated in appearance (B, C and D). Magnified views of apoptotic cells revealed blebbling of the surface membrane (D). Scale bar A, B, C = 10 μm, D =2 μm.

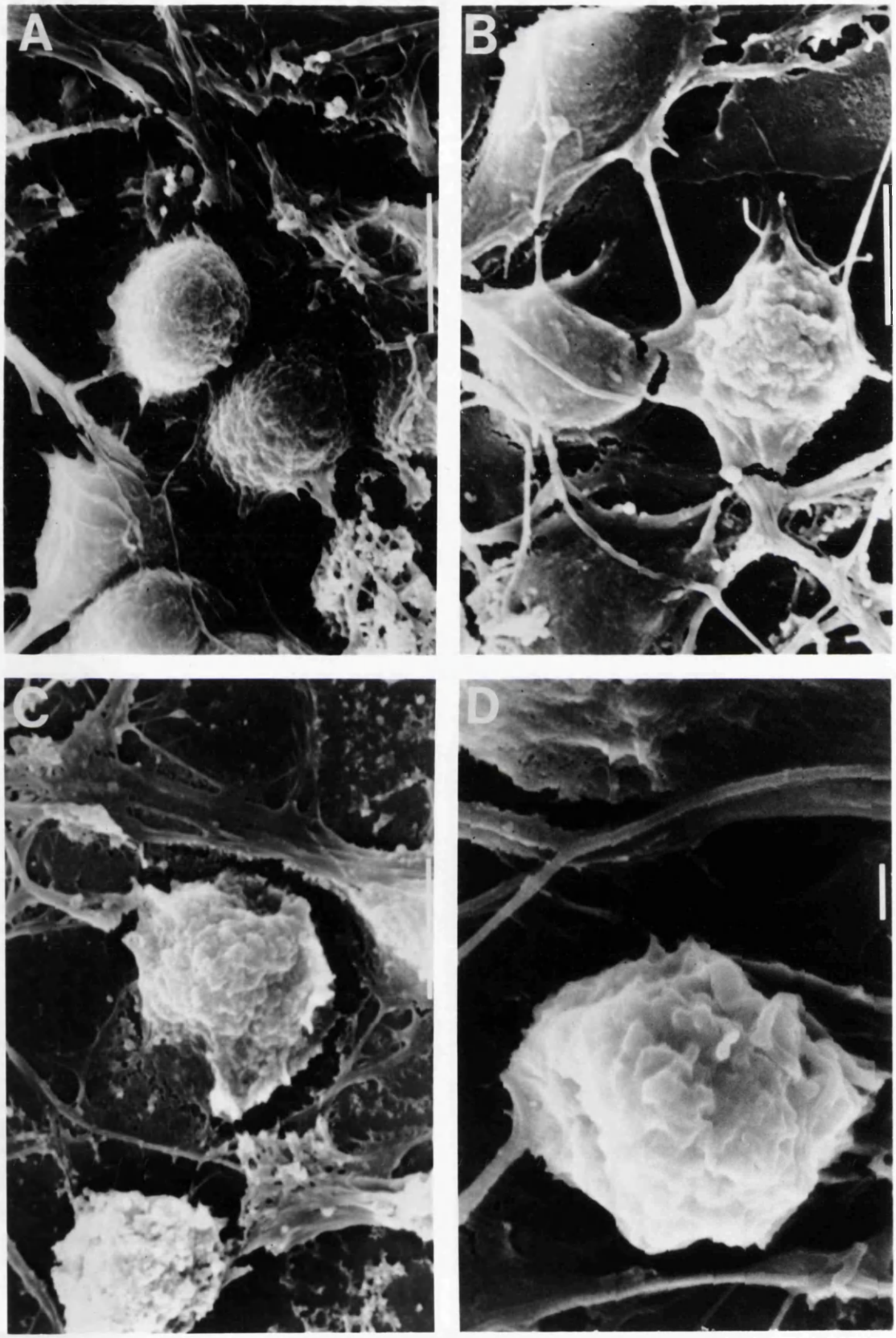

A
B
C
D

Figure 52

Scanning electron micrograph of cells in cultures derived from E17 cortices at various stages of apoptosis (continued from Figure 23). Various intracellular changes which were not apparent under scanning electron microscopy took place with the eventual formation of apoptotic bodies. The membrane integrity remained intact. In the absence of clearance of apoptotic cells in culture, cells underwent a secondary change, secondary necrosis, unique to cultured cells. The membrane integrity was lost and cells become permeable (to vital dyes; A, B, C, D). Whilst A and B exemplify more advanced secondary necrosis, with cytoplasmic membrane rupture, C and D demonstrate a relatively intact membrane with blebbing and indentations. Scale bar A = 10 μm, B = 5 μm, C, D = 1.25 μm.

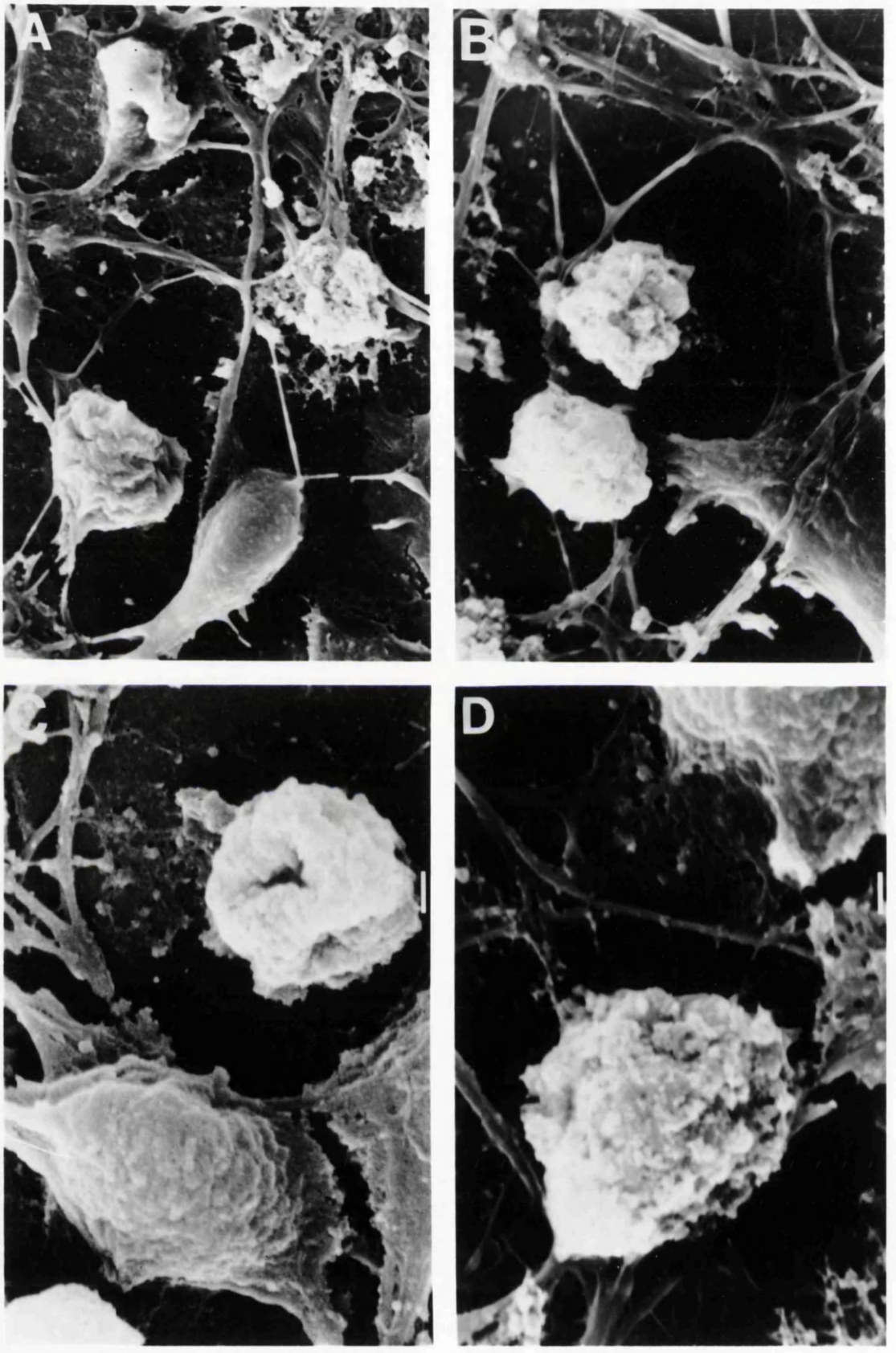

A
B
D

Figure 53

Scanning electron micrograph of cells in cultures derived from E17 cortices at various stages of apoptosis (continued from Figure 23). A, B, C and D illustrate the difference in morphology and size and to some degree electron density between cells undergoing apoptosis and secondary necrosis and healthy living cells. Note that in the majority of instances there was considerable shrinkage and rounding of the apoptotic cells (A and C). With further compromise in membrane integrity, and expulsion of cytoplasmic contents (D) the electron density also altered. It was not surprising that a considerable degree of loss of cells from the substrate occurs as adherent proteins were broken down. Scale bar A, B = 5μm, C = 2.5 μm, D = 10μm.

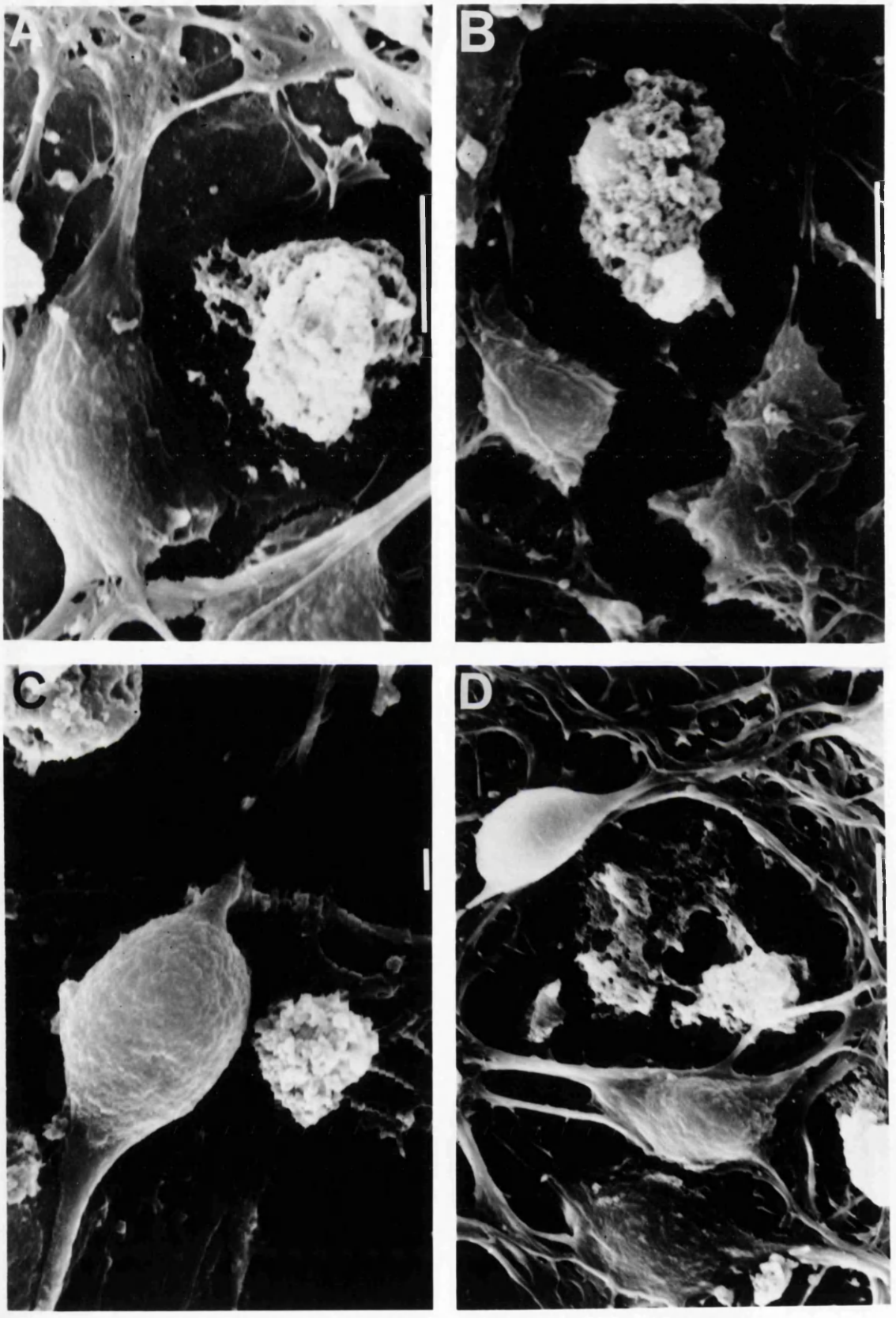

A
B
C
D

Figure 54

Transmission electron micrograph showing a healthy cell in culture derived from E17 embryonic cortex. Note the large rounded, and somewhat lobulated nucleus with uniform distribution of chromatin, extensive ribosomes in the cytoplasm and neurofilaments in the neuronites branching off from the cytoplasm. There was some evidence of connections by other neurites (n). Abbreviations, nucleus (N), mitochondria (m). .Scale bar = 1.5 μm.


N
m
n

Figure 55

Transmission electron micrograph showing a cell undergoing apoptosis in culture. Note the rounded, condensed chromatin. Although the nuclear envelope has been compromised, it was assumed that the preparation of the specimen has caused some artifactual disfigurement of the cell. Note particularly the intact cytoplasmic membrane. There was some swelling of the mitochondria thought to be artifact. Compare with the normal healthy cell..Scale bar = 3 μm.

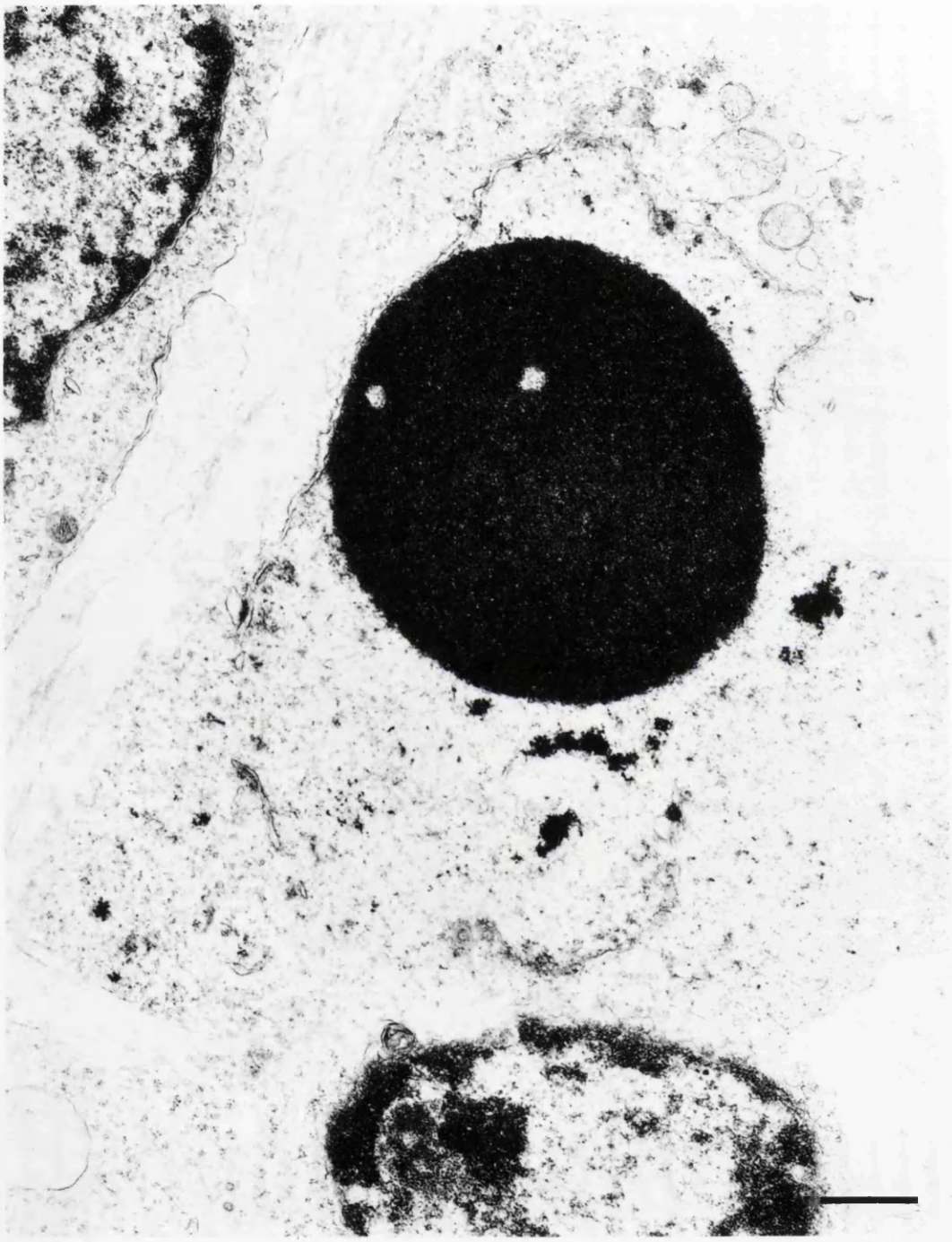


Figure 56

Transmission electron micrograph showing a cell undergoing end stage apoptosis in culture with two apoptotic bodies (ApB). The cytoplasmic membrane remains in tact but dissolution of cytoplasmic organelles was apparent. There was considerable loss of contact with other cells in culture. Scale bar = 3 μm.

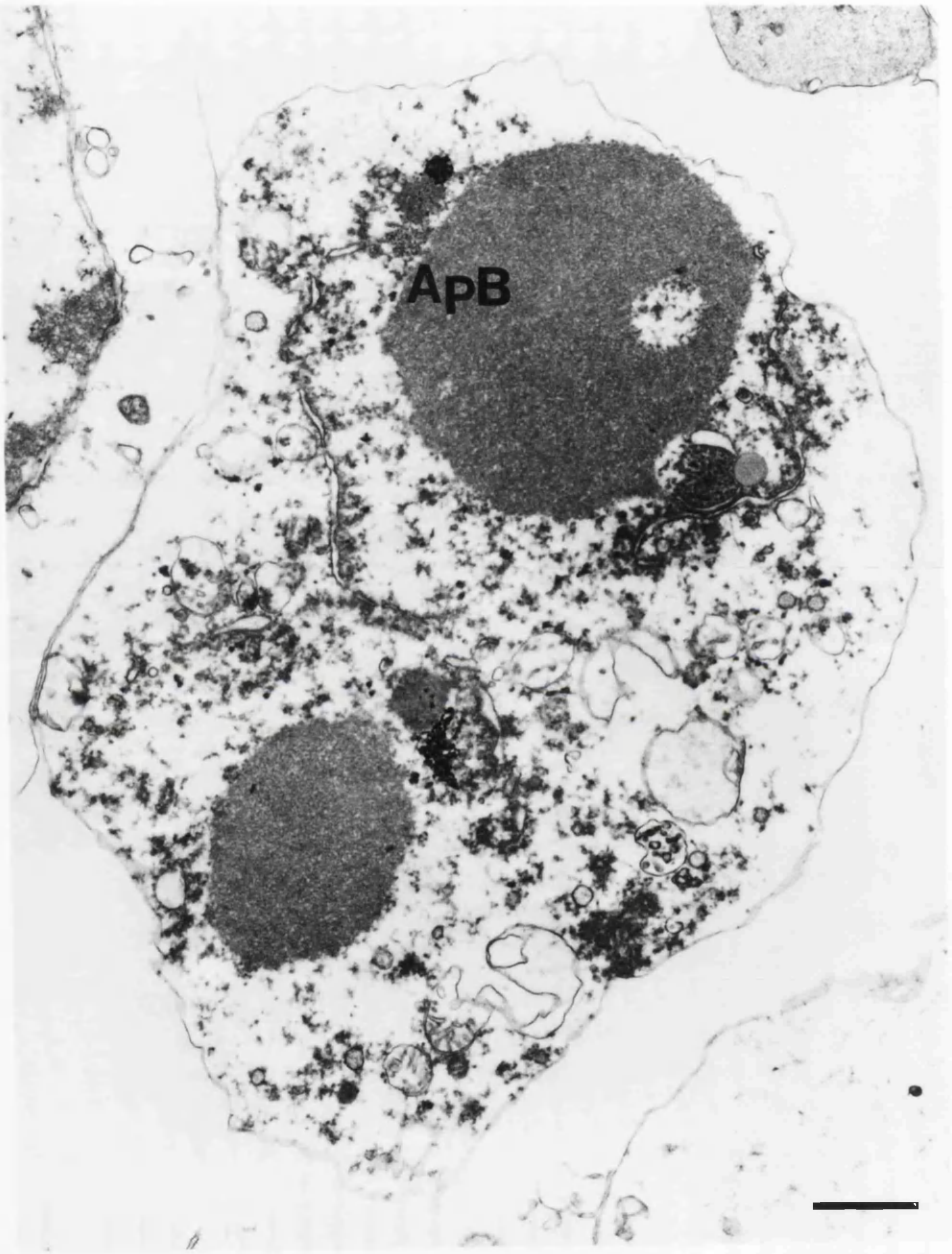

ApB

Figure 57

Transmission electron micrograph showing a cell undergoing early stage apoptosis in culture. The cytoplasmic membrane remains in tact, the organelles remain in tact, there was clear margination of the nuclear contents to the nuclear envelope. The cell continues to contact surrounding cells. A necrotic cell lies adjacent to the apoptotic figure. There was clear vacuolation and disruption of cytoplasmic contents, in contrast to the apoptotic cells. Scale bar 1.5 μm.

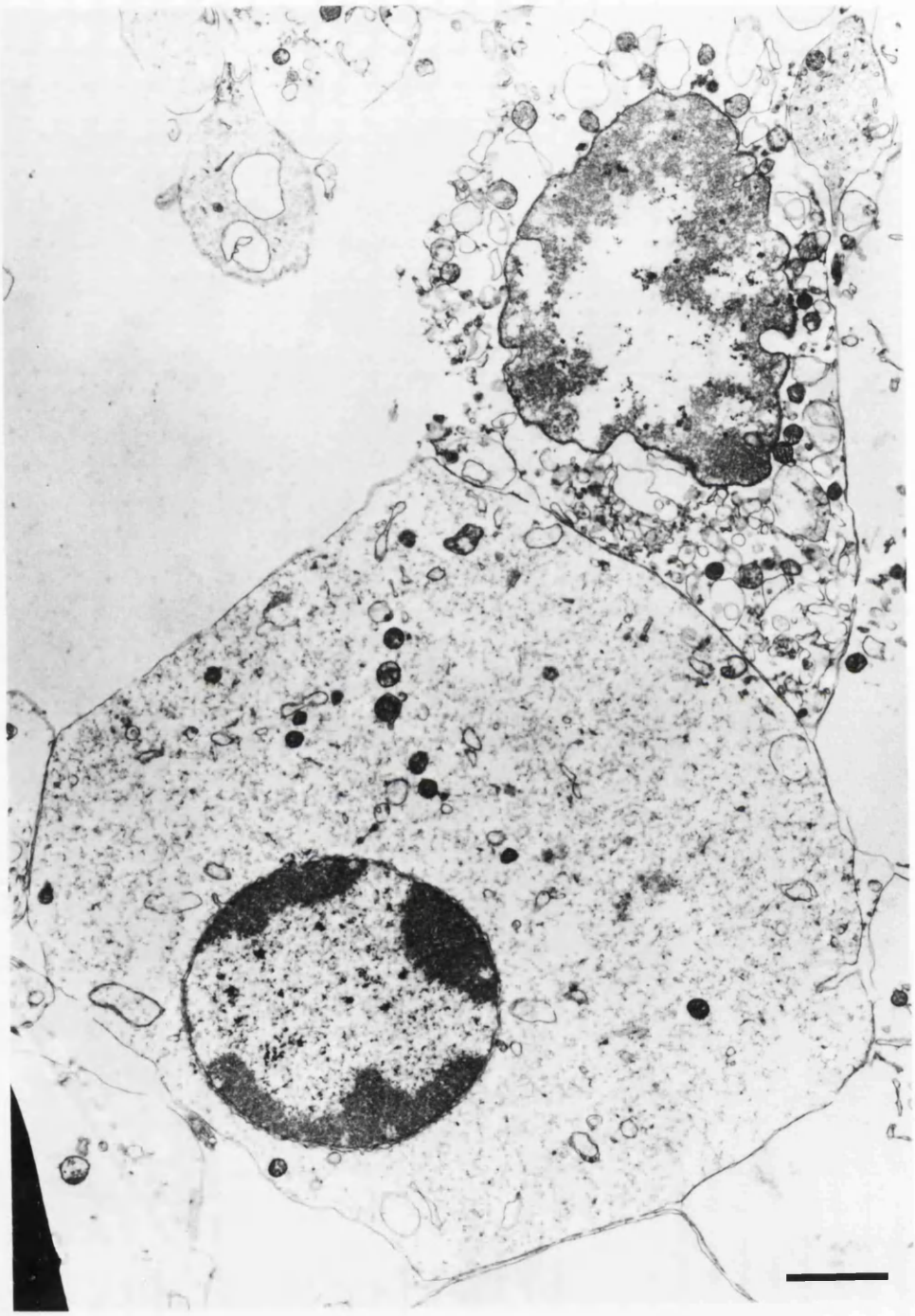


Figure 58

Transmission electron micrograph demonstrating a cell undergoing early to late stage apoptosis in culture (bottom middle). The cytoplasmic membrane was disrupted but crescent shaped margination of the part of the chromatin has occurred, with maintenance of an intact nuclear envelope. A second apoptotic body was also present, without a nuclear envelope. Disruption of the cytoplasmic contents was visible. Scale bar = 1.5 μm.

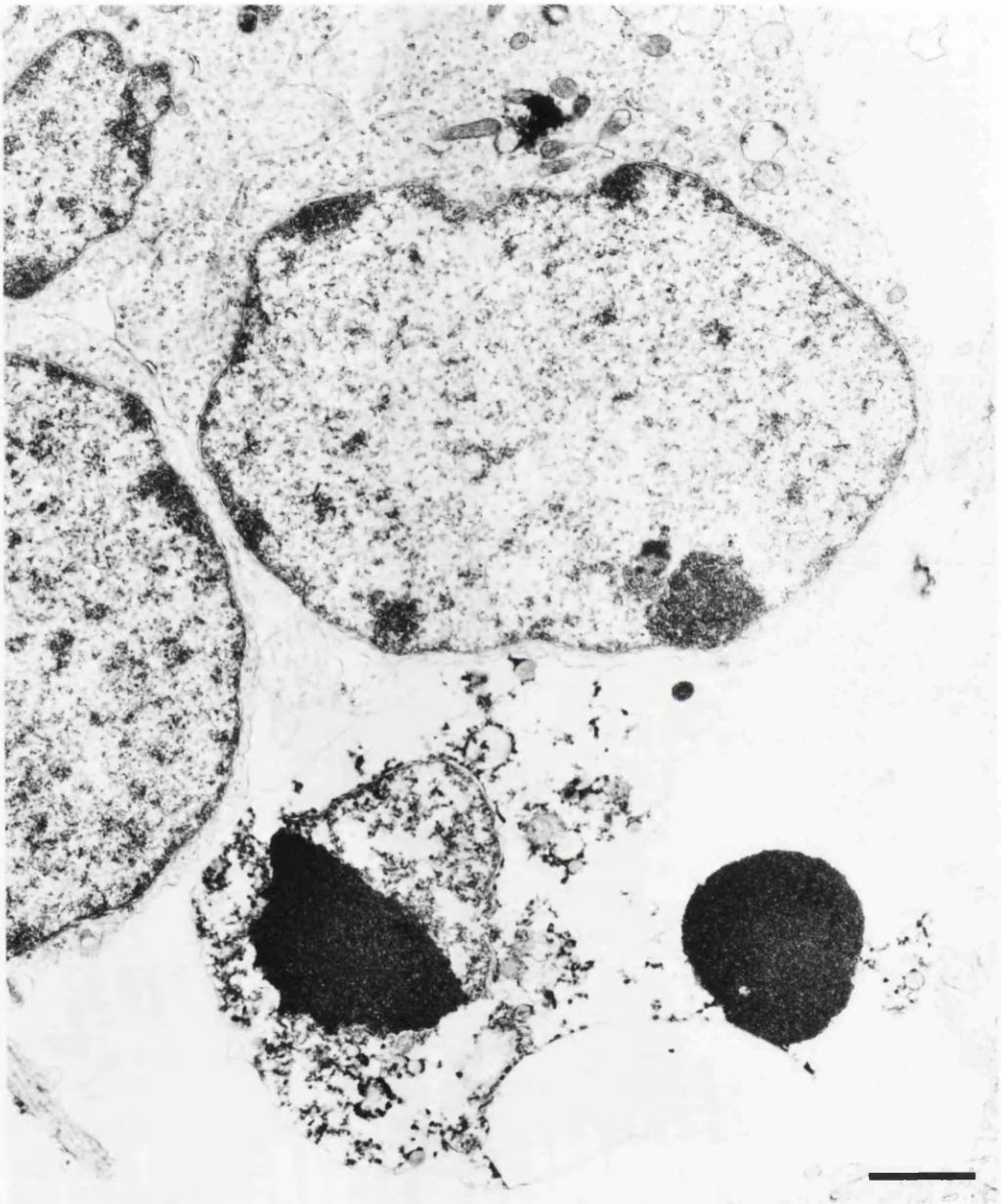
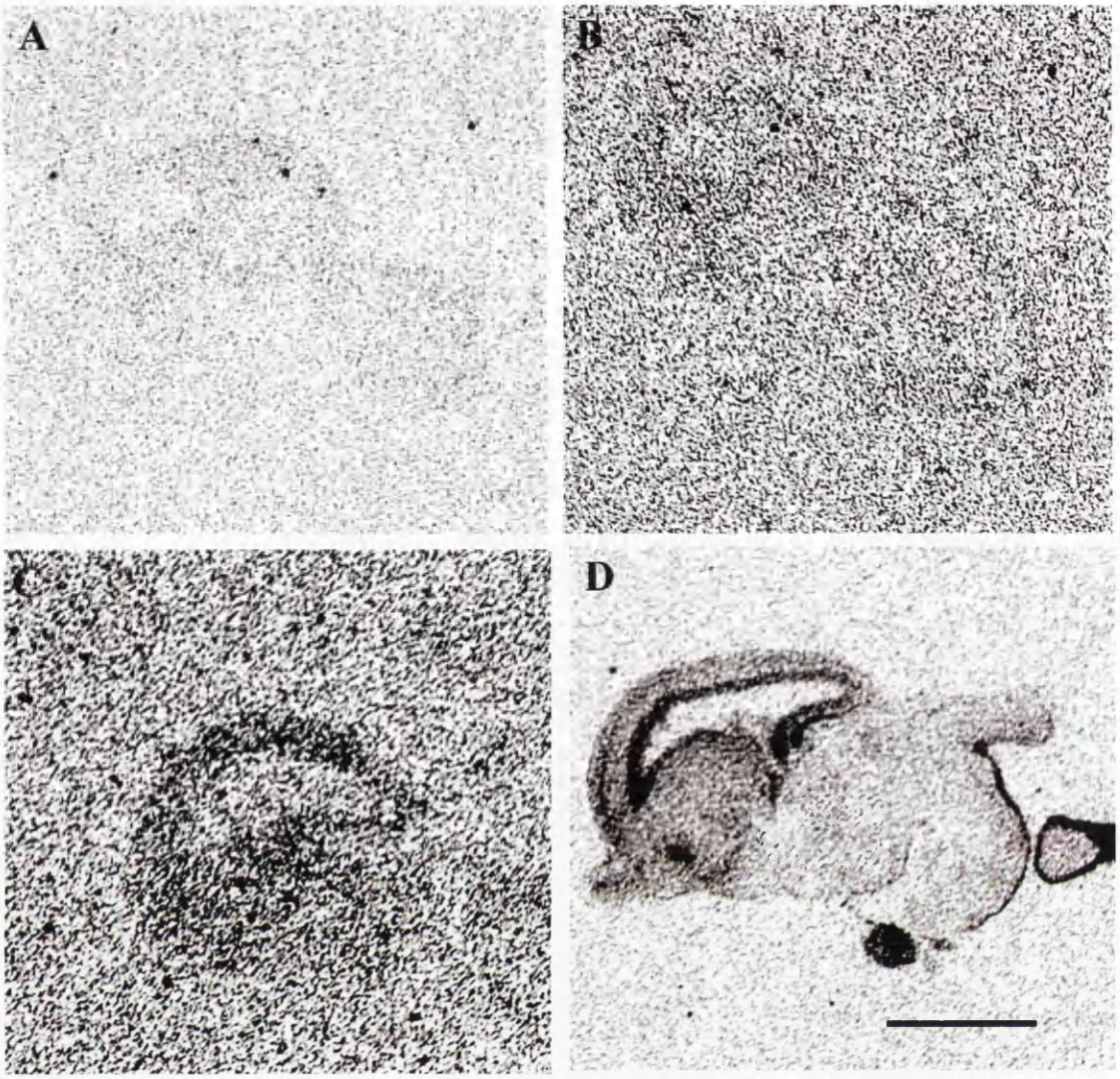


Figure 59

Autoradiograph showing the expression of bFGF mRNA and FGFR1 mRNA (C and D respectively) in E17 sagittal cryosections of brain using radioactively labelled antisense oligonucleotides . Panels A and B are control specimens prepared using sense oligonucleotides. Note the expression of the FGFR1 mRNA closely reflects that seen in immunostaining and was predominantly in VZ and CP. Left is rostral and right is caudal. Scale bar = 1.8 mm.


A
B
C
D