

**THE ROLE OF SEROTONIN AND OTHER
NEUROTRANSMITTER SUBSTANCES IN THE
DEVELOPMENT OF CORTICAL CELL TYPES:
AN *IN VITRO* STUDY**

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November 1997

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ACKNOWLEDGEMENTS

My appreciation and thanks go foremost to John Parnavelas for his guidance and supervision throughout this thesis and in whose laboratory these investigations were conducted. I would next like to thank Dr. Ioannis Pappas for his additional valuable guidance throughout many aspects of this work and Dr. Marina Mione for her helpful comments and advice.

My thanks also go to the following for the technical assistance and advice gratefully received: Dr. Mimika Thomaidou for her help in labelling dying cells, Brett Harris for developing and printing techniques, John Cavanagh for photography techniques, Peter Boardman for his help in maintenance of culture equipment, Dr. Bagi Nadarajah and Dr. Alex Lavdas for their helpful advice and also Dr. Stephen Davies for the use of equipment in his laboratory.

Finally, I would like to say a big thank you to my family: Lascelles, Fay, Jean, Paul and Ruth Dooley for their unending support and priceless encouragement throughout this thesis.

ABSTRACT

The different neuronal and glial cell types that form the mammalian cerebral cortex are generated from a population of epithelial cells lining the embryonic telencephalic ventricles. Cells within this germinal layer proliferate and migrate to form the characteristic six-layered structure of the neocortex. The factors that control the proliferation of cortical progenitor cells and their differentiation into different cell types are still mainly unknown, but recent studies point to the importance of signals from the cells' microenvironment in their commitment to a particular phenotype and laminar fate. Such signals may be provided by growth factors, hormones, neuropeptides, extracellular matrix molecules, or neurotransmitters produced and regulated locally by differentiated cortical neurons or afferent axonal systems.

A host of neurotransmitters have been implicated in regulating the proliferation and differentiation of cortical cell types. Chief among them are the catecholamines, noradrenaline and dopamine, and the indoleamine, 5-hydroxytryptamine (serotonin; 5-HT). The early development of the monoaminergic systems has prompted speculation that they may play a role in a number of developmental processes in the cortex.

In this study I focused on the role of 5-HT during the prenatal period of cortical development by using primary cultures of dissociated neocortex established from embryonic day 14 (E14), E16, and E18 rats. These cultures were grown in defined medium, or additionally exposed to 5-HT and examined for survival effects during 1-11 days *in vitro* (DIV). 5-HT significantly increased survival of these cultures in a concentration dependent manner. E14 cultures showed a 1.3-fold increase at 9 DIV, E16 cultures a 2-4-fold increase between 7-9 DIV and E18 cultures a 1.6-2.2-fold increase from 2-4 DIV. This survival was mimicked by the 5-HT_{2a/2c} receptor agonist α -methyl-5-HT, but not by the 5-HT_{1a} receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin. Survival was predominantly of postmitotic neurons immunopositive for microtubule-associated protein (MAP-2). The other monoamines noradrenaline (NA) and dopamine (DA) also had a survival effect on developing cortical cell types in contrast to the neurotransmitters γ -amino butyric acid (GABA) and acetylcholine (ACh). In conclusion, these results indicate the importance of 5-HT and other neurotransmitter substances in

the survival of cortical cells during development and point to a preferential survival of postmitotic cortical neurons.

PUBLICATIONS ARISING FROM THESIS

Dooley, A.E., Pappas, I.S., and Parnavelas, J.G. (1997). Serotonin promotes the survival of cortical glutamatergic neurons. *Exp. Neurol.* (In Press).

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ABBREVIATIONS

ACh	Acetylcholine
bFGF	Basic fibroblast growth factor
BDNF	Brain derived neurotrophic growth factor
BrdU	Bromodeoxyuridine
Ca ²⁺	Calcium
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DA	Dopamine
DAB	Diaminobenzidene
DIV	Days <i>in vitro</i>
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
E	Embryonic day (e.g. E16)
FCS	Fetal calf serum
GABA	Gamma amino butyric acid
GFAP	Glial fibrillary acidic protein
GLU	Glutamate
HBSS	Hanks balanced salt solution
5-HT	Serotonin
MAP-2	Microtubule associated protein-2
NA	Noradrenaline
NGF	Nerve growth factor
NT	Neurotrophin
6-OHDA	6-hydroxydopamine
PBS	Phosphate buffered saline
<i>p</i> CPA	<i>p</i> -chlorophenylalanine
PLC	Phospholipase C
PNS	Peripheral nervous system
PVE	Pseudostratified ventricular epithelium
SPP	Secondary proliferative population
SVZ	Subventricular zone
TdT	Terminal transferase
TH	Tyrosine hydroxylase
VZ	Ventricular zone

CHAPTER 1

INTRODUCTION

1.1 Preview and Background Information

The mature central nervous system (CNS) of mammals presents us with a precisely organized and structured ensemble of areas and connections which serve to orchestrate our response to various sensory stimuli. As the tools available to scientists became further advanced, so we have been able to follow and describe in detail the events which lead to the presentation of this adult structure.

There are two main classes of cells encountered in the CNS: neurons and glia. Each type of cell itself presents us with many variations dependent upon the area of the CNS to which it belongs. Cell types of the cerebral cortex for example, the largest structure of the CNS, display an array of morphologically, chemically and physiologically distinct neuronal cell types (Szentagothai, 1973; McCormick et al., 1985; Parnavelas et al., 1989; DeFelipe, 1993).

Cortical neurons are subdivided into two principal types: pyramidal and nonpyramidal. Pyramidal cells are the projection neurons known to exert excitatory action in the cortex and in subcortical target areas by releasing L-glutamate (GLU) or L-aspartate (Fagg and Foster, 1983; Dori et al., 1992). The nonpyramidal cells, the cortical interneurons, utilize the inhibitory neurotransmitter γ -aminobutyric acid (GABA) (Houser et al., 1984; Peters and Jones, 1984). Separate developmental patterns have been established for these neuronal cell types (Mione et al., 1997).

Principal in the elucidation of the early development of the cerebral cortex has been the advent of methods to study cell lineage. The most popular type of study, which allowed one to trace a particular cell and its descendants using non-dilutable genetic

markers, has enabled us to determine at least narrow time periods during which cell potentials are restricted (Price and Thurlow, 1988; Luskin et al., 1993; Mione et al., 1994). While such lineage experiments have provided important data as to when cell fate is specified, exactly what factors and signals are involved in cortical cell generation and differentiation is not entirely known. In the peripheral nervous system (PNS), instructional substances such as the neurotrophins, have been found to regulate the survival and differentiation of particular neurons (Snider, 1994). Within the CNS, such factors have also proved instructional (Rohrer, 1990; McConnell, 1991; Pappas and Parnavelas, 1997). There is yet ample evidence as to the actions of growth factors in the regulation of cell proliferation, survival and differentiation in various areas of the CNS including the cerebral cortex. A further source of environmental signals has been found to be provided by neurotransmitter substances (Mattson, 1988; Parnavelas et al., 1988; Lauder, 1993). In particular, evidence suggests that the neurotransmitter serotonin (5-HT) plays a significant role in cortical development (Lauder, 1990).

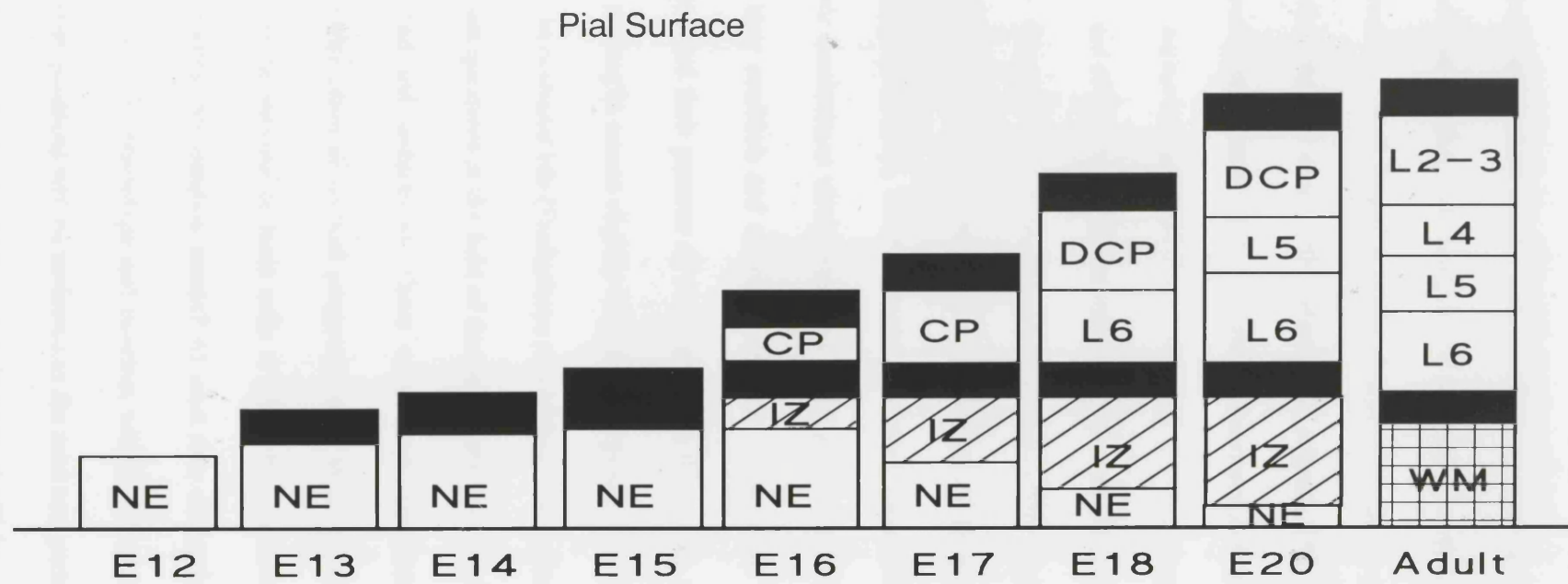
Unraveling the role of environmental factors in the formation of the cerebral cortex has proved a complex task. This project has focused on the actions of one such environmental factor, namely 5-HT, and asked the question as to what actions, if any, does this substance mediate during early cortical development.

1.2 Overview of Cortical Development

One of the major features of the adult mammalian cerebral cortex, which distinguishes it from many other neuronal systems, is its organization into functionally specialized areas coupled with a further distinctive lamination pattern (Lorente de Nó, 1949). During the development of this structure, the neo- (or six layered) cortex is generated from the neuroepithelium of the dorsal telencephalon, each layer being principally distinguished by the morphology, size, shape and density of neurons it contains (Uylings et al, 1990; Bayer and Altman, 1991). Early studies designed to analyze the formation of these layers from neurons contained within the embryonic neuroepithelium, used autoradiographic methods following labelling of these cells with [³H] thymidine. These studies revealed that the first layer of postmitotic neurons produced accumulated above the neuroepithelium and formed the primordial plexiform layer commonly known as the preplate (Marin-Padilla, 1971, 1972). Further generations of incoming neurons which split the preplate into a future layer 1 (or superficial marginal zone) and deep subplate would constitute the cortical plate (Luskin and Shatz, 1985; Kostovic and Rakic, 1990). The cortical plate (which comprises layers II through to VI) revealed an unusual “inside-out” sequence of cell generation with the deepest layers (layer VI and V neurons) produced first followed by the superficial layers (layers IV to II) (Rakic, 1974; Raedler and Raedler, 1978; Caviness, 1982; Smart and Smart, 1982) (See Fig. 1).

FIG. 1. DEVELOPMENT OF CORTICAL LAYERS

The neurons of the cerebral cortex arise from the neuroepithelium (NE) adjacent to the ventricular surface, and migrate towards the pial surface. Sequential distribution is as shown, the important regions to note being the intermediate zone (IZ; future white matter), the cortical plate (CP) which differentiates into layers VI through to II, the dorsal cortical plate (DCP; layers IV-II), and the white matter (WM). (Adapted after O'Leary and Koester, 1993).



Thus, the generation of neurons and final formation of layers in the cortex results in groups of cells which share similar patterns of connectivity and age in relation to their position. In addition to this well-documented scheme of cortical development, further studies indicated an overall frontal to caudal (Miller 1988; Bayer and Altman, 1991) and lateral to medial (Raedler et al., 1980; Bayer and Altman, 1991; Ignacio et al., 1995) gradient of neurogenesis, such that layer specific neurons generated in frontal and lateral regions arrived earlier to their destinations than their caudal and medial counterparts.

The period of production of neurons in the rat occurs between embryonic day 12 (E12) and the time of birth around E21 (Uylings et al, 1990; Bayer and Altman, 1991). Neuronal generation proceeds from the proliferation of progenitor cells lining the neuroepithelium. Eventually, these immature neurons leave their site of origin and migrate to their destinations along processes of radial glial cells (Rakic, 1972; Hatten, 1990) where they establish and maintain synaptic connections. Less is known about glial production and their patterns of colonization in the cortex, suffice to say that the generation of macroglia occurs slightly later than the production of neurons taking place predominantly in postnatal life (Frederiksen and McKay, 1988; Skoff and Knapp, 1991).

Important questions in the field of developmental neurobiology still remain to be fully investigated and understood. These range from questions as to what signals regulate the *proliferation* of cortical progenitor cells in the proliferative zone? Which signals influence the *survival* of these cells at either the immature or later established postnatal stage within the cerebral cortex? At what *time* do cortical stem cells become committed to a specific phenotype and position within the cortex? Studies that have investigated these questions will be reviewed in the next two parts of this thesis. Part I- will focus on the role of intrinsic determinants in cortical cell fate, while Part II- reviews

the importance of environmental signals, with particular emphasis on the role of 5-HT in cortical development.

1.3 Cell Proliferation and Death in the Cerebral Cortex

We start this section by focusing on the events of cell proliferation and cell death, the first involving a mechanism designed to produce more cells, and the latter involved in the elimination of cells from the developing cerebral cortex. These two events at first glance seem mutually opposed in their purpose, yet both processes exhibit certain morphological similarities, such as the retraction and loss of neurites, and the rounding up and bipolar appearance of the cell body (Heintz, 1993; Rubin et al., 1993). It is these characteristics as well as recent biochemical data implicating cell cycle proteins in the process of cell death (Freeman et al., 1994) that has strengthened the hypothesis that the two events may be related. It is now postulated that an inappropriate activation of proliferation could cause a cell to abort and instead follow a pathway leading to death. We shall, therefore, consider both processes in the developing cerebral cortex.

1.3.1 Cell proliferation and the ventricular zone in the cerebral cortex

Early in the development of the CNS, the neural tube appears as a single layer of columnar epithelial cells, between the inner (ventricular) surface and the outer (pial) surface. While these cells proliferate, their nuclei remain close to the ventricular surface whereas postmitotic cells migrate away from it towards the pial surface. As the neural tube thickens, mitotic cells remain close to the ventricular wall and thereby define a layer known as the ventricular germinal zone. In order to guide the migration of cells from the ventricular zone (VZ), a specialized type of glial cell (radial glial cells) remains attached to the ventricular and pial surfaces, forming a scaffolding network (Bayer and Altman, 1991).

The *mechanics* of cell division in the cortex, by which an accurate number and ratio of the major cell types is produced in the correct locations, has been investigated extensively in the mouse cortex (Takahashi et al., 1994). The cell cycle, which results in division of a cell, is divided into 4 distinct phases- G1, S, G2 and M. These involve periods of active synthesis and replication of chromosomes (S phase), nuclear and cellular division (M phase) and intervals of preparation in between (G1/G2 phase). Each phase lasts a defined period of time according to the stage of cortical development and each cell also leaves the cell cycle after a defined number of divisions to become postmitotic. Alternatively, the cell can re-enter the cell cycle to continue the process of division. Thus, the regulation of stem cell proliferation was seen to involve the control of at least four distinct decisions- that of activation and maintenance of the proliferative state coupled with a later stop signal and maintenance of cell cycle repression.

Studies in the mouse found that the developing cerebral wall contains two proliferative populations categorized on the basis of the cell classes which it produces and their proliferative behaviour. The first and largest of the two is the pseudostratified ventricular epithelium (PVE), which has long been known to be the source of most, if not all, neocortical neurons as well as some cells of the glial lineage (Sauer, 1935). The second population of proliferative cells is known as the secondary proliferative population (SPP).

Data on the mechanics and variations of the cell cycle of the PVE population, looking at a developmental perspective between the ages of E11-E17, have shown a general increase in cell cycle length from 8.1 hours at E11 to 18.4 hours at E17 (Takahashi et al., 1995a). Few postmitotic neurons were produced during the early periods up to E14. These investigators also noted variation in the length of individual phases of the cell cycle with development. Taken together, Takahashi et al. (1995a)

concluded that the increase in the cell cycle length observed during development is essentially due to a four-fold increase in the G1 phase, the only phase that varies systematically. Thus, any extrinsic signals that may be involved in the regulation of neuronal number is most likely to affect and modulate the G1 phase of the cell cycle. It has also been postulated that differences in the size of the cortex across species could be regulated by factors which prolong the early period of proliferation, the time of expansion of the progenitor pool. Such factors could achieve this result by shortening the cell cycle or by providing a continuous mitogenic signal (Caviness, 1995).

The significantly smaller population of proliferative cells, the SPP, is principally a source of glial cells arising from the PVE, and extends from the interface of the ventricular and subventricular zone towards the base of the cortical plate (Altman and Bayer, 1990; Takahashi et al., 1993). Studies that focused on the SPP found a high rate of proliferation between E14-E16. The population increased nearly six-fold antecedent to the production of glial cells throughout the neocortex. Quantitatively, it was estimated that 87% of SPP cells would re-enter S-phase during this interval, while only 13% would exit the cell cycle (Takahashi et al., 1995b).

Recent studies have begun to investigate the molecular mechanisms which might regulate cell proliferation in vertebrates. Of note are the investigations in mutant mice expressing a deficiency for the gene *BF-1* (Xuan et al., 1995) in the telencephalic neuroepithelium. These mice showed a dramatic reduction in the size of their cerebral hemispheres. It was postulated that this cell intrinsic transcription factor may regulate the machinery required for the progression of the cell cycle or enhance the effects of mitogens on neuroepithelial cells. However, the importance of a number of genes in cell proliferation has become evident in invertebrate studies which have used the host *Drosophila* (Ebens et al., 1993; Prokop and Technau, 1994; Datta, 1995).

The mode of cell production in the developing cerebral cortex also influences the *number* of cells generated during proliferation. Symmetrical division, produces two daughter cells of which both re-enter the cell cycle. In this process the plane of cleavage of cell division is orientated vertically (or perpendicular) to the ventricular surface. Asymmetrical division, however, results in one of the two daughter cells continuing to proliferate while the other cell becomes postmitotic. In this situation, the plane of cleavage is horizontal to the VZ. Observations made by Takahashi et al. (1995a), showed that many proliferative cortical cells are produced within a short time. They thus hypothesized that the *progenitor* pool is expanded by divisions of a symmetrical nature. Direct evidence for symmetrical and asymmetrical divisions within the proliferative zone came from imaging labelled progenitor cells with confocal microscopy (Chenn and McConnell, 1995). How the cell divisions observed produce *distinct cell types* that display a different proliferative nature has been studied at a molecular level using *Drosophila*. It was observed that products of the genes Numb (a membrane associated protein) and Prospero (a nuclear protein), become differentially segregated during asymmetrical neural precursor cell division (Hirata et al., 1995; Knoblich et al., 1995; Spana et al., 1995; Spana and Doe, 1995). Similar studies in the cortex of vertebrates have shown that the protein Notch 1 is localized close to the upper pial surface of mitotic cells (Chenn and McConnell, 1995). Asymmetrical divisions result in the uneven distribution of this protein and as such provide a mechanism by which distinct cell types can be generated in the mammalian species.

Proliferation and the exit of neurons from the cell cycle during development occurs within a limited period of time (E12-E21) in most regions of the mammalian CNS (Bayer and Altman, 1991). An exception is a population of dividing cells within the subventricular zone (SVZ) of the rat which remain and can continue to proliferate in

the adult (Morshed et al., 1994; Gritti et al., 1996). Such studies have introduced the idea of using adult stem cells in transplantation to replace dying neurons during pathological diseases (Gage et al., 1995).

1.3.2 Cell death in the cerebral cortex

Cell death in many systems is mainly classified into two major types based on morphological and biochemical criteria. Programmed cell death (apoptosis) is prevalent during development, and cells undergoing apoptosis exhibit features such as plasma and nuclear membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. Necrosis, which is the type of cell death apparent in neurodegenerative diseases, involves cell swelling and eventual eruption. The distinction between both these forms of cell death is now less clear than traditionally believed due to similar changes occurring in both pathways i.e: elevated levels of calcium (Ca^{2+}) (Choi, 1992; Richter, 1993).

In the developing nervous system *apoptosis* occurs in a variety of circumstances. Major periods featuring apoptosis include:

- 1) Death of cells after the terminal division of the cell cycle, but before the formation of synaptic contacts. An example of such dying cells are found in the cortical subplate; these cells die after acting as targets of early thalamic afferents (McConnell et al., 1994).
- 2) Cell death resulting from competition between neurons for target derived factors. Such types of cell death has been demonstrated in the chick spinal cord using limiting amounts of target derived factors (Oppenheim, 1991; Raff et al., 1993).
- 3) Death following the loss of pre-synaptic inputs from afferents (Oppenheim, 1991; Catsicas et al., 1992).

Additionally, an earlier scene of cell death is observed when limited amounts of a survival factor are made available to proliferating or early postmitotic cells. This is demonstrated in cells of the oligodendrocyte lineage *in vitro* which show survival responses to various combinations of growth factors (Barres et al., 1992).

Of interest in the field of cell death in the cerebral cortex has been the *extent* to which this process occurs. Earlier studies in the cortex (Ferrer et al., 1992), as well as retina and spinal cord (Young, 1984; Homma et al., 1994) reported insignificant levels of cell death. More recent investigations using an enhanced sensitive assay, reported large amounts (up to 70%) of cell death in the proliferative areas and upper postmitotic layers of the cortex (Blaschke et al., 1996), although these high figures may be due to the sensitive nature of the technique used (Voyvodic, 1996). Recent work in our laboratory has shown a high correlation between dying cells and those that were in G1 phase of the cell cycle (Thomaidou et al., 1997). These results indicate that apoptosis is *prominent* amongst the proliferative population of the developing cortex and that it is *linked* to the cell cycle.

1.4 Early Theories on Cortical Cell Fate (Historical Foundations)

The generation of neuronal and glial phenotypes in the mammalian cortex (i.e: how the specification of neurons occurs from an apparently homogeneous population of stem cells in the VZ and at what time does this happen?) is among the earliest questions asked and investigated in this field. Studies in this area concentrated on the production of the major constituent cell types of the cortex- neurons and glia. By visualizing the ultrastructure of cells contained within the VZ (using electron microscopy and autoradiographic criteria), an hypothesis was formed which suggested that the uniform population of stem cells gave rise first to neuronal elements and later switched to glial production (Fujita, 1963, 1964). Subsequent discoveries, however, revealing the apparent co-existence of neuronal and glial elements in the developing VZ of the cortex (Levitt et al., 1981), led to two early theories gaining prominence. The first postulated by His in 1889, proposed *separate* cell lineages for neuronal and glial cell types in the proliferative zone, whereas Schaper in 1897 suggested single *multipotential* cells which gave rise to the two cell types (reviewed by McConnell, 1988).

The advent of techniques which permanently trace the lineage of cells during their development has revealed new data on cortical cell fate. The use of recombinant retroviruses in cell lineage experiments, for example, in which the *gag*, *pol* and *env* genes are excised and replaced by the *E.coli* β -galactosidase gene (detected by a simple histochemical stain) has overcome the problem of previously dilutable markers such as ^3H -thymidine (Price et al., 1987; Cepko, 1988; Sanes, 1989). Remaining sections in Part I review three main areas of specification shown by cortical progenitor cells: that of commitment to phenotype, cortical layer, and region.

1.5 Cortical Cell Fate and Migration

1.5.1 Commitment to cell phenotype in the cerebral cortex

Information regarding the determination of the neurochemical, morphological and physiological fate of a cell has rapidly increased by the use of retroviral mediated gene transfer to study cell lineage. Experiments that utilized this method labelled cortical cells *in vivo* in the period of neurogenesis (E14-E21), and later analyzed the composition and distribution of cell clones at adult stages. Clones of cells produced which were homogeneous in composition indicated the presence of progenitor cells which at the point of infection were restricted in potential. However, the existence of heterogeneous clones suggested the presence of multipotential stem cells in the VZ. These studies reported that clones of cells analyzed are almost always exclusively composed of one cell type i.e: either neurons, astrocytes or oligodendrocytes (Price and Thurlow, 1988; Grove et al., 1993; Luskin et al., 1993). Thus, it appeared that progenitor cells from an early stage of cortical development (E16) were restricted in their developmental potential. Further evidence for the existence of separate precursor cells came from experiments which showed that the neuronal subtypes- pyramidal and non-pyramidal cells also had defined lineages as early as E15 (Parnavelas et al., 1991; Mione et al., 1994).

Although the above data pointed to the early existence of restricted progenitor cells in the VZ, further experiments revealed complications in the interpretation of these results. One study in which the resultant clones were analyzed at postnatal ages of P7, P14 and P21 (rather than previously as in the adult), showed a significant proportion of clones containing a mixed population of pyramidal and non-pyramidal neurons (Lavdas et al., 1996). It was hypothesized that cell death, or changes in cell phenotype due to

local environmental factors, may produce the homogenous clones observed in the adult. Further complications were evident by the definitions of the spatial dimensions that constituted a clone of cells. Clones were usually defined as a *spatial clustering of labelled cells* in a 500 μm cortical strip, separated from another clone of cells by at least 500 μm . Such clones were considered to be separate and unrelated (Lavdas et al., 1996). The basis for these definitions came from earlier observations of the migratory behaviour of cortical progenitor cells. As such, studies focusing on the migratory behaviour of progenitor cells revealed that there was potential for movement over large distances (average of 100 μm over 8 hr) and in the lateral direction (Rakic, 1972; Fishell et al., 1993; O'Rourke et al., 1995). This mode of movement by progenitor cells provided a mechanism by which widespread related clones could be produced. The existence of these dispersed clones was conclusively identified by use of a modified retroviral technique (Walsh and Cepko, 1992, 1993).

What would be the significance of related widespread clones? Experiments labelling clones with multiple, distinct, retroviral vectors, reported that *widespread clones* were systematically spaced at 2-3 mm intervals and often had distinct laminar and morphological properties to their related cousins. At E15 48% of clones analyzed were widespread and heterogeneous in nature for neuronal or glial cell types, while 52% consisted of small or single homogeneous cell clusters (Reid et al., 1995). Thus, widespread clones were proposed to be the result of progeny of migratory multipotential progenitor cells in the VZ (Reid et al., 1995). In order to reconcile previous lineage data it is proposed that multipotential stem cells migrate laterally in the VZ and stop to give rise by asymmetric division to a non-migrating sibling. This sibling could produce a homogeneous cluster of cells, whereas its migrating sister at a considerable distance

produces a different but related homogeneous cluster of another cell type (Reid et al., 1995).

In vitro studies also provides evidence for multipotential cells in the cerebral cortex (Davis and Temple, 1994; Reynolds and Weiss, 1996) it should be noted, however, that while *in vivo* retroviral analysis documents the ultimate fate of clonally related cells, tissue culture manipulations reflect the range of cell potential. In other regions of the developing CNS the early presence of multipotential cells is well-established and as such has emphasized the importance of environmental factors in specification of cell fate (Wetts and Fraser, 1988; Galileo et al., 1990; Turner et al., 1990; Halliday and Cepko, 1992).

Studies aimed at identifying genes which specify commitment to the neuronal or glial cell lineages have focused on *Drosophila* and to a lesser extent the mammalian species. These investigations revealed the existence of *proneural* genes in *Drosophila* such as the *Achaete-Scute* complex (AS-C) and *atonal* which endow progenitor cells with the ability to adopt a neuronal fate (Jan and Jan, 1995). Vertebrate homologues of these genes (termed *Mash 1*, *Math 1*, *Math 2* and *neuroD*) are also reported (Johnson et al., 1990; Akazawa et al., 1995; Lee et al., 1995). As well as proneural genes, neurogenic genes are also evident which confer neuronal differentiation (Jan and Jan, 1995). Two principal neurogenic genes in *Drosophila* (Notch and Delta) have homologues which have been characterized in mammals (Artavanis-Tsakoonas et al., 1995; Bettenhausen et al., 1995; Nye and Kopan, 1995). Furthermore, there is the *glial cells missing* (*gcm*), a *Drosophila* gene which may be involved in the choice between a neuronal or glial cell fate (Jones et al., 1995; Hosoya et al., 1995). These studies, which have identified the first gene known to function as a switch between glial and neuronal

cell fate, reported that loss of *gcm* resulted in failure of glial cell differentiation. Gain of *gcm* however, promoted presumptive neuroblasts to a glial fate.

1.5.2 Commitment of cells to laminar and regional fates in the cerebral cortex

Neurons with similar morphologies, functional properties, and patterns of connectivity divide the cortex into distinctive layers. Thymidine autoradiography studies established that neurons in a given layer are born at about the same time (reviewed by McConnell, 1988). Recent investigations (McConnell and Kaznowski, 1991) has shown that the laminar identity of a cell is determined during the S-phase of a cells *final* mitotic division in the VZ. Such studies, using cell transplantation, have tested the importance of the environment and cell lineage in this aspect of specification. They observed that environmental signals, which may be cell-cell interactions, were able to change the final position of a cell, however, only during the S-phase of the cell cycle.

Transplantation experiments have additionally provided data on the commitment of stem cells to distinct cytoarchitectonic regions of the cerebral cortex. Such studies, in which regionally derived neurons were challenged to form connections appropriate for their new location have proposed a late phenotypic specification of postmitotic neurons to region (Stanfield and O'Leary, 1985; Schlagger and O'Leary, 1991). These studies suggested that the fetal neocortex is functionally equipotential throughout its tangential extent, with environmental factors in the form of incoming afferents conferring regional specificity (O'Leary et al., 1994). However, other studies proposed a model of early specification of area specific features (Barbe and Levitt, 1991; Ferri and Levitt, 1993; Cohen-Tannoudji et al., 1994). Thus, although the cortex can exhibit a high degree of

plasticity in axonal connections and cytoarchitecture, yet there remain regional differences across the VZ from an early period during development.

In conclusion to this section on cell commitment in the cerebral cortex, it is clear that various aspects of cell fate (i.e: cell phenotype, layer position and region), are regulated by neurodevelopmental mechanisms involving both intrinsic and extrinsic stimuli. In Part II, we take a closer look at the range of these environmental stimuli and the situations in which they are effective in the developing cerebral cortex.

2. Overview of Environmental Factors

Environmental factors play an integral role in the process of cell survival, proliferation, differentiation, growth, and plasticity during the development of the CNS. The nature of these signals can be broadly divided into four categories of 1) growth factors, 2) neurotransmitters and neuropeptides, 3) extracellular matrix molecules and 4) cell-cell interactions (reviewed by Loughlin and Fallon, 1993). This study will focus on the above two categories of epigenetic stimuli, namely, growth factors and neurotransmitters.

Growth factors are small, soluble, polypeptides synthesized and secreted by cell types. These factors include the major groups of neurotrophins, fibroblast growth factors, epidermal growth factors, and insulin-like growth factors, as well as other molecules such as platelet-derived growth factor (PDGF) (Loughlin and Fallon, 1993). Within the neurotrophin family, 4 main members have been identified: nerve growth factor (NGF: Thoenen et al., 1987), brain-derived neurotrophic factor (BDNF: Leibrock et al., 1989), neurotrophin (NT)-3 (Ernfors et al., 1990), and NT-4/5 (often termed NT-4: Berkmeier et al., 1991). All of these neurotrophins bind to the low-affinity receptor p75, as well as a specific receptor tyrosine kinase of the Trk family (Trk A for NGF, Trk B for BDNF and NT-4, and Trk C for NT-3). The involvement of neurotrophins in a spectrum of biological processes is widespread (reviewed by Bonhoeffer, 1996; Henderson, 1996). The FGF family is composed of at least seven heparin-binding proteins that share a 40%-60% sequence homology. Their main members include basic FGF (bFGF or FGF2), and acidic FGF (aFGF or FGF1) (Morrison et al., 1986; Walicke, 1988). Additional factors termed FGF3-7 are also identified (Burgess and Maciag, 1989; Coulier et al., 1991).

Neurotransmitters, although conventionally assigned roles as intercellular mediators of communication within the adult nervous system, are now regarded to play a wider *neurotrophic* role in development (Lauder, 1988; Mattson, 1988a; Lipton and Kater, 1989; Schwartz, 1992). Such ideas have followed the early localization and transient nature of expression shown by neurotransmitters substances and their receptors during neurogenesis, as well as the significant pharmacological plasticity which can be induced by their reduction or depletion. Within the CNS, such neurotransmitters include the amino acids *glutamate* (GLU) acting through ionotropic and metabotropic receptors (Watkins et al., 1990) and γ -*aminobutyric acid* (GABA), the physiological actions of which are mediated through GABA_A and GABA_B receptors (Barnard, 1988a,b). Additionally, there are the monoamines, *serotonin* (5-HT), *noradrenaline* (NA), and *dopamine* (DA) (reviewed further in latter sections), and finally *acetylcholine* (ACh) which interacts with muscarinic (Bonner et al., 1987) and nicotinic receptors (Deneris et al., 1991). Furthermore, a proportion of GABA-containing nonpyramidal neurons have been shown to contain biologically active peptides (Papadopoulos et al., 1987; Parnavelas et al., 1989). Peptides demonstrated to be localized in cortical neurons include: somatostatin, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), and corticotrophin-releasing factor (CRF) (Parnavelas et al., 1989).

The idea that each of these environmental factors has a single trophic role in the regulation of a cell type or neuronal system is proving rather a simple classification. Rather, it is apparent that each system and cell type responds appropriately to a particular factor depending on the age, the spectrum of genes expressed, and availability or levels of factor present.

2.1 Growth Factors in the Developing CNS

2.1.1 Growth factors and cell survival

The role of growth factors in the regulation of cell survival was first demonstrated for NGF in the PNS (Levi-Montalcini, 1987). These studies showed that treatment of neonatal mice or rats with anti-NGF antiserum resulted in virtual degeneration of sympathetic neurons. Further studies revealed that the effects of NGF on sympathetic neuronal survival were closely related to connectivity with its target i.e: whereas axotomy of these neurons resulted in their degeneration, treatment with NGF prevented axotomy-induced cell death (Hendry, 1975a, b; Levi-Montalcini, 1987). Thus, the final cohort of a developing population of cells may be regulated as they compete for limited amounts of target-derived or afferent provided trophic factor (Oppenheim, 1991). Within the CNS, for example, this has been shown by developing retinal ganglion neurons which project to the optic tectum. These neurons die upon transection of the optic nerve (Perry and Cowey, 1979; Linden and Perry, 1983), but BDNF, which is released by tectal cells was found to promote the survival of retinal ganglion neurons (Barde, 1989; Rodriguez-Teber et al., 1989).

In vitro experiments have provided appropriate conditions to expose the survival effects of growth factors on different populations of neurons. Particularly, bFGF is noted to affect neurons of the cerebral cortex and hippocampus (Morrison et al., 1986; Walicke 1986), while BDNF is involved in activity-dependent survival of developing cortical neurons (Ghosh et al., 1994). Of additional interest is the action of NTs in promoting the survival of progenitor cells. Several groups have distinguished the survival action of NT-3 on sympathetic and sensory PNS cells to affect *proliferating* progenitor cells during neurogenesis as opposed to *postmitotic* neurons (DiCicco-Bloom

et al., 1993, ElShamy and Ernfors, 1996; ElShamy et al., 1996). Also studies with oligodendrocytes and their precursors indicated that long-term survival may be regulated by *multiple* factors (Barres et al., 1992, 1993). Similar investigations have shown that distinct populations of cells respond to more than one growth factor i.e: BDNF and bFGF can both promote the survival of cultured dentate neurons (Lowenstein and Arsenault, 1996), while BDNF, NT-3 and NT-4/5 together increase striatal survival (Ventimilia et al., 1995).

The prominence of NTs in regulating the survival of the developing nervous system is also reflected in the adult where they prevent degeneration and facilitate the recovery of injured neurons (reviewed by Hefti et al., 1989; Gage et al., 1990). These factors have been proposed as potential therapeutic agents for treatment of the structural deterioration that occurs in neurodegenerative diseases, aging and trauma. Attention in the CNS has focused on the survival of cholinergic neurons of the forebrain (Knusel et al., 1991), as well as dopaminergic mesencephalic neurons (Hyman et al., 1994).

2.1.2 Growth factors and cell proliferation

In the CNS, the growth factor bFGF dominates proliferative regulation of both neurons and glia. Present in the telencephalon from E9.5, bFGF is associated with cell membranes and extracellular matrix. The mitogenic actions of bFGF are reported to elicit a 2-fold increase in the number of E13 cortical *neuronal precursors* from the rat CNS (Ghosh and Greenberg, 1995), mouse neuroepithelium (Kilpatrick and Bartlett, 1993), rat retinal precursors (Lillien and Cepko, 1992), hippocampus (Vicario-Abejón, 1995), and mesencephalic dopamine precursors (Bouvier and Mytilineou, 1995), as well as regulate the proliferation of purified cultures of *oligodendrocytes* (Bogler et al., 1990; McKinnon et al., 1990).

bFGF can interact with other factors in the stimulation of neuronal synthesis. This is shown by the addition of NGF to murine embryonic striatal neuroectoderm cells which significantly augmented bFGF induced proliferation (Cattaneo and McKay, 1990), while in the neuroepithelium bFGF acted in conjunction with insulin-like growth factors to stimulate DNA synthesis (Drago et al., 1991). It is interesting to note, however, that early experiments using PNS sympathetic neuronal precursors assigned a differentiative action to bFGF and NGF (Greene and Tischler, 1976; Anderson and Axel, 1986) and as such underlines the diverse effects of these factors upon different cell types in various systems.

Many studies in this area have asked the question as to which *type* of progenitor cell responds to the mitogenic actions of bFGF? Multipotential cells from the adult SVZ have been implicated (Gritti et al., 1996) as well as neuronal precursor cells from the embryonic striatum (Vescovi et al., 1993), olfactory epithelium (DeHamer et al., 1994) and the cerebral cortex (Vaccarino et al., 1995). In the cortex it is shown that bFGF promotes the proliferation of a restricted neuronal progenitor cell dedicated to glutamatergic production (Vaccarino et al., 1995). Further information has also been obtained as to how the *termination* of progenitor cell division is maintained. Oligodendrocyte precursor cells undergo a fixed number of cell divisions *in vitro* indicating the existence of an intrinsic “clock” mechanism which limits precursor proliferation (Temple and Raff, 1986). It has been hypothesized that cell division counting mechanisms are activated by the presence of cell surface receptor mitogens such as bFGF or NT-3, while signals such as hormones that activate intracellular receptors may terminate cell proliferation (reviewed by Barres and Raff, 1994).

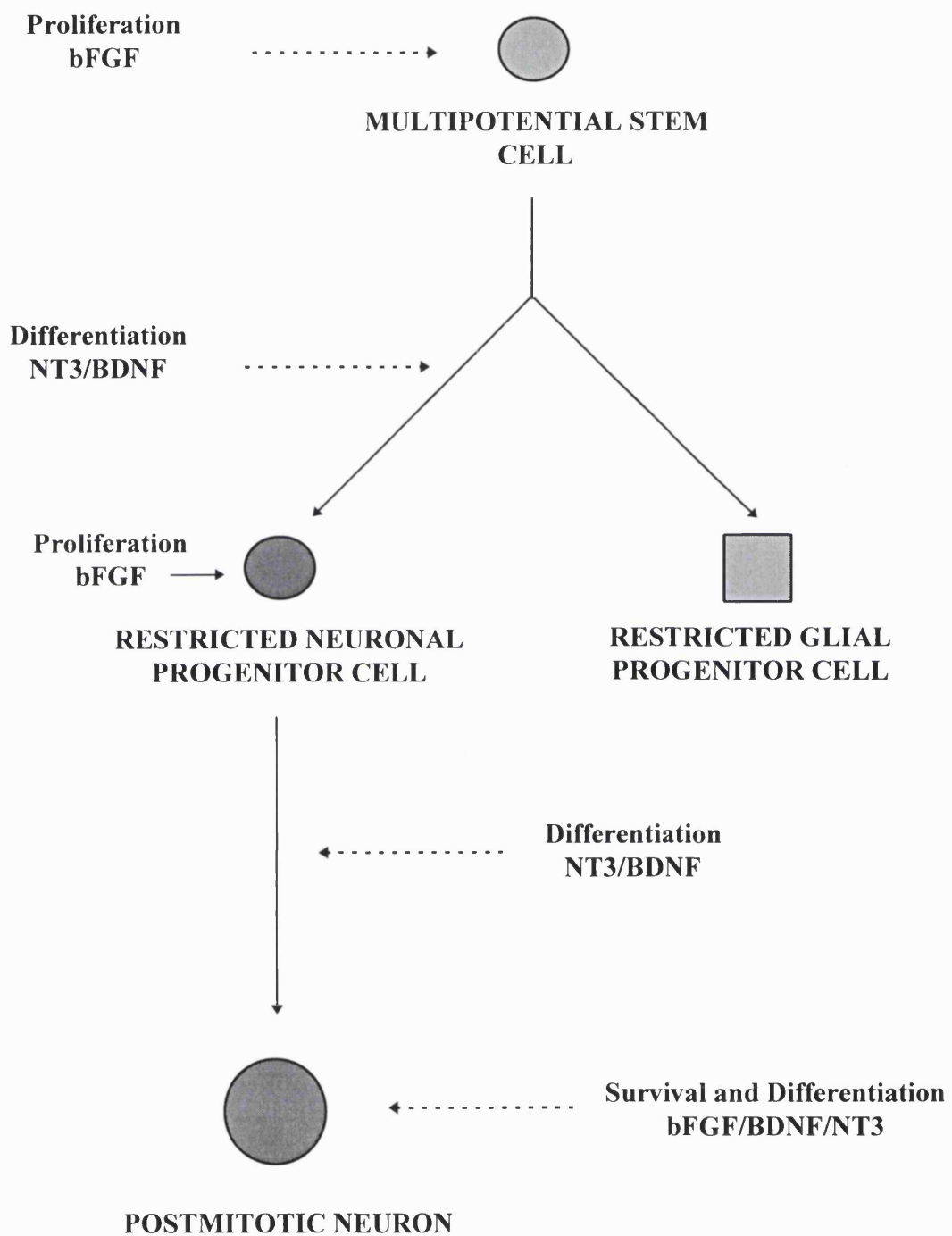
2.1.3 Growth factors and cell differentiation

Within the PNS, it is established that environmental factors are principal regulators of neurochemical differentiation i.e: *in vivo*, cytokines promote the acquisition of the cholinergic phenotype in sympathetic noradrenergic neurons (Rao and Landis 1993; Schotzinger et al., 1994). Such investigations have led to the characterization of factors which may regulate CNS cell fate. The effects of NTs and bFGF have been studied extensively in two regions- the cerebral cortex and hippocampus. It was shown that while bFGF stimulated the division of cortical neuroectodermal cells, NT-3 further induced these cells to differentiate and express the neuronal marker MAP-2 (Ghosh and Greenberg, 1995). Also, both BDNF and NT-3 increased the number of cells expressing a Ca^{2+} -binding protein in the hippocampus (Ip et al., 1993; Vicario-Abejón, 1995; Marty et al., 1996a). It is unknown as to which progenitor cell type (i.e, multipotential or restricted) these factors are acting upon to influence cell fate, however, another interesting result has been the *dual* function of bFGF as an activator of proliferation and a promoter of the latter phases of differentiation in hippocampal cells (Vicario-Abejón, 1995). Furthermore, in the cerebral cortex NTs have been attributed additional roles in the promotion of morphological specification and chemical differentiation. BDNF and NT-3, for example, regulate dendritic growth (McAllister et al., 1997), while BDNF increases peptide expression (Nawa et al., 1993). Growth factor actions are noted in other regions of the CNS i.e, the striatum (Mizuno et al., 1994), septum (Grothe et al., 1989; Alderson et al., 1990), and mesencephalon (Hyman et al., 1994). Interestingly, interactions between both growth factors and neurotransmitters have been reported to regulate the phenotypic traits of CNS neurons (Cohen-Cory et al., 1991; Zhou et al., 1994; Du and Iacovitti, 1995; Marty et al., 1996b).

In summary, within the cerebral cortex, specific growth factors have been identified which regulate the process of cell proliferation, survival and differentiation (see Fig. 2). Information regarding the action of these factors is confined to the neuronal lineage and includes the early stimulation of multipotential stem cells to proliferate and survive. Later, these factors act on restricted precursor and postmitotic neurons to influence both survival and differentiation. Our knowledge of factors that specify cell fate is largely confined to the determination of their chemical and morphological phenotypes.

**FIG. 2. SUMMARY OF ACTIONS OF NEUROTROPHINS IN THE
DEVELOPMENT OF CORTICAL CELL TYPES *IN VITRO***

Basic fibroblast growth factor (bFGF), promotes the proliferation of multipotential progenitor cells contained within the VZ to give rise to more proliferative progeny. Other neurotrophins, such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) enhance the differentiation of these cells to a progressively restricted fate resulting in presumptive neuronal and glial cells. *In vitro* analyses suggest that trophic factors continue to specify neurochemical, morphological and, perhaps, regional aspects of differentiation, as well as the maintenance of survival of these neurons (Adapted after Temple and Qian, 1995).

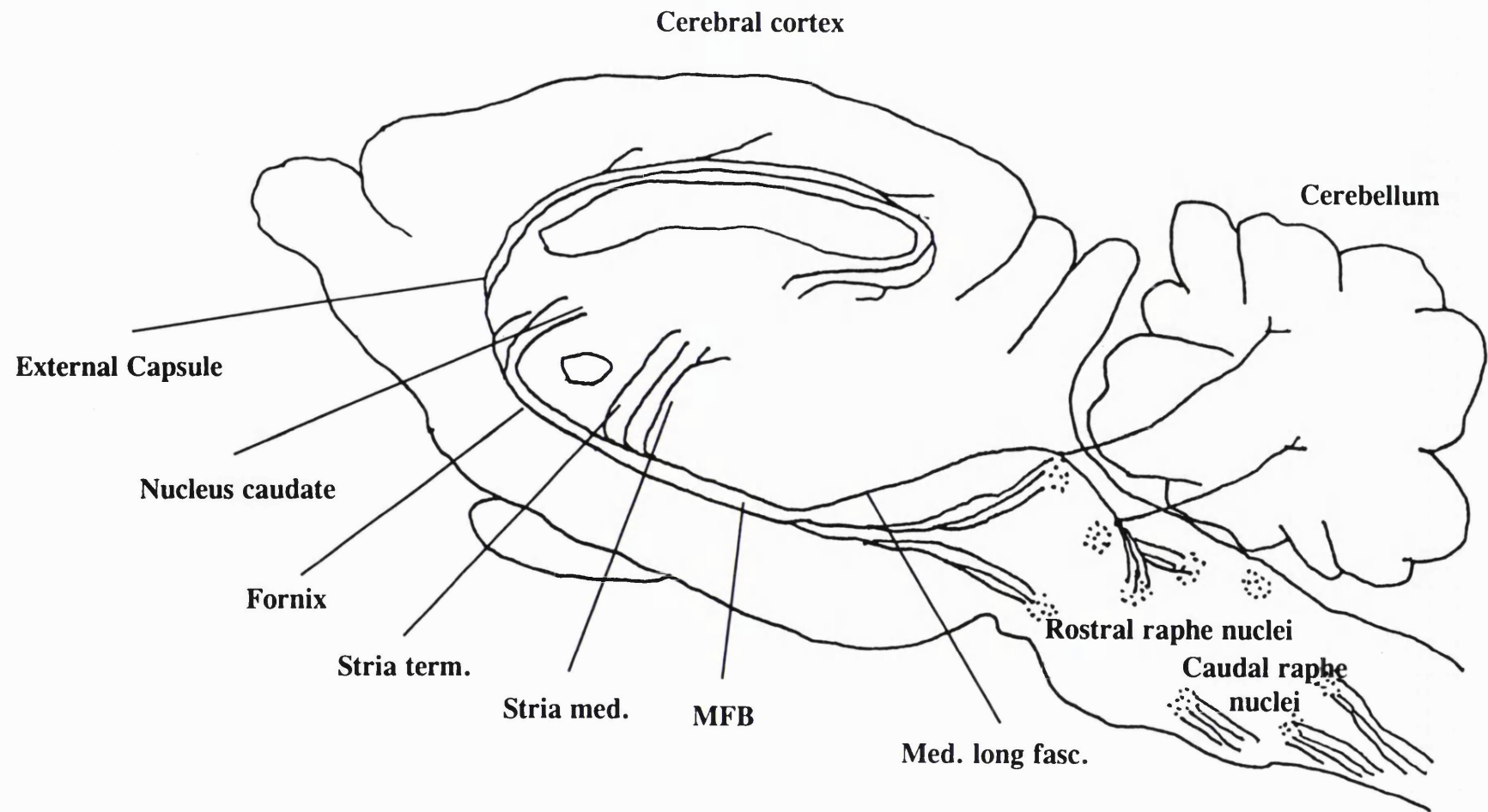


2.2 Neurotransmitters in the Cerebral Cortex: Appearance and Distribution

The early synthesis and appearance of neurotransmitter substances in target regions *before* the onset of synaptogenesis is an indication of their neurotrophic roles in the development of the CNS. These substances are in a position to regulate early developmental events such as survival, proliferation, migration and differentiation in neuronal systems. The serotonergic system has been characterized using immunocytochemistry in the prenatal rat CNS. Serotonin-containing cell bodies are localized in the brainstem raphe nuclei and send projections using ascending and descending afferent fiber pathways to target regions (Steinbusch and Nieuwenhuys, 1983). Expression of the serotonergic phenotype begins in cells of the rostral raphe nuclei at E12 (See Fig. 3). This complex of neurons termed the B7-9 group (which includes the dorsal and median raphe nuclei of earlier conventions) increases in size until E15 when generation is complete. It is from these cells that formation of ascending 5-HT fiber projections rapidly ensues, their axons reaching the frontal neocortical pole by E17 (Lidov and Mollivier, 1982a,b; Wallace and Lauder, 1983). Upon reaching the ventral edge of the cortical plate 5-HT axons bifurcate into two sheets, one traversing the subplate layer and the other traversing the marginal zone in a rostrocaudal direction to cover the entire cortex by the end of the first postnatal week. 5-HT axons at this time arborize extensively in all areas and distinctive regional and laminar patterns have been described. Hence dense plexuses of 5-HT axons are generally observed in layers I and VI of the cortical plate, with additional numerous ramifications in the somatosensory region and layers IV and VI (reviewed by Parnavelas et al., 1988; Dori et al., 1996).

**FIG. 3 SCHEMATIC DIAGRAM ILLUSTRATING THE MAIN
SEROTONERGIC PATHWAYS INNERVATING THE ADULT RAT
CORTEX**

Abbreviations: Stria term., Stria terminalis; Stria med., Stria medullaris; MFB, Medial fascicular bundle; Med. long. fasc., Medial longitudinal fasciculus (Adapted after Cooper et al., 1991)

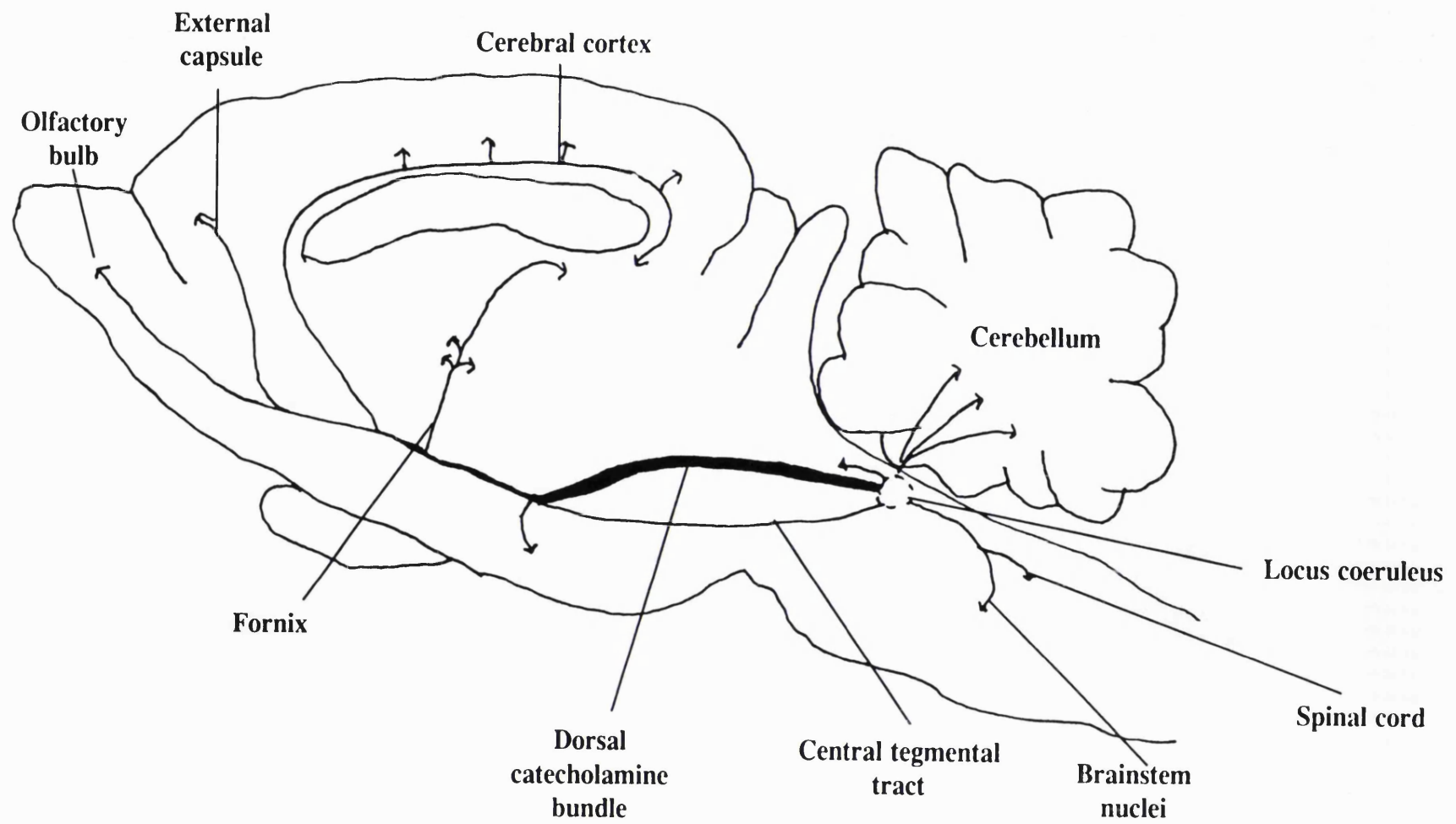


In addition, a transient expression of serotonergic immunoreactivity is reported during the postnatal development of somatosensory areas (D'Amato et al., 1987; Rhoades et al., 1990). By P12 an adult-like distribution is achieved. Other groups of 5-HT neurons of the medulla oblongata, namely the caudal raphe neurons (or B1-3 group), are shown to send descending projections to the spinal cord. However their generation and distribution of projections is not further reviewed here.

The central noradrenergic innervation arises from a small group of neurons termed the locus coeruleus (LC) which is located in the pontine tegmentum (See Fig. 4). This nucleus provides a diffuse innervation of diverse brain structures by both ascending and descending pathways. Differentiation of noradrenergic neurons begins during E10-E13 in the rat, with synthesis of NA apparent by E13-E14 (Olson and Seiger, 1972; Lauder and Bloom, 1974). Projections of NA fibers to the cerebral cortex and innervation has been shown by immunochemistry to occur by E17, with two fibre bundles reaching the rostral cortex and traversing the marginal and intermediate zones in a rostrocaudal direction (Schlumpf et al., 1980). A rapid maturation pattern by NA fibres ensues with the whole hemisphere covered by birth. By the end of the first postnatal week the distribution pattern and neocortical density has reached relative maturity (Levitt and Moore, 1979; reviewed by Parnavelas et al., 1988).

**FIG. 4. SCHEMATIC DIAGRAM ILLUSTRATING THE MAIN
NORADRENERGIC PATHWAYS INNERVATING THE ADULT RAT
CORTEX**

(Adapted after Cooper et al., 1991)

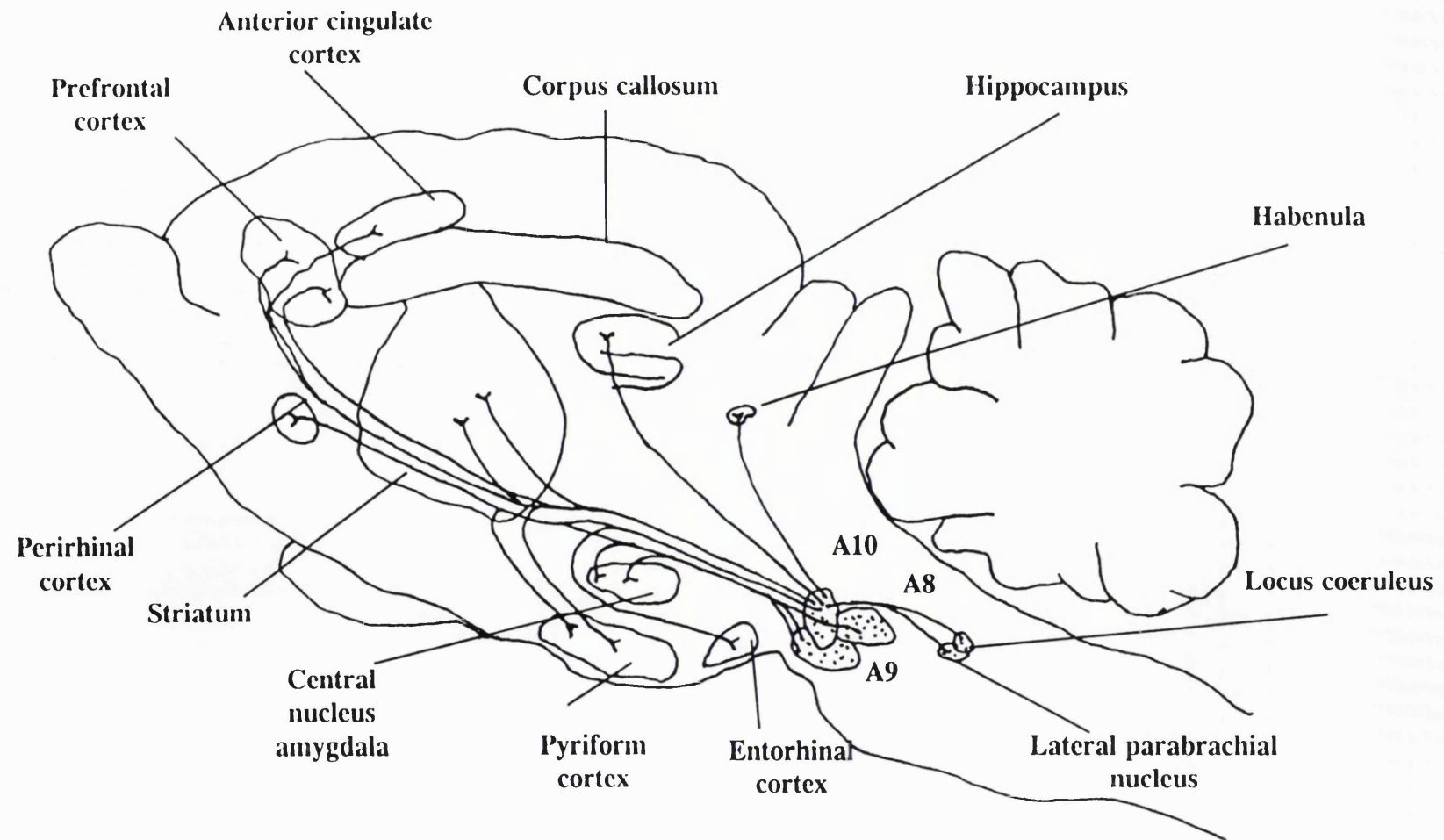


The dopaminergic afferents to the cortex show a select and restricted pattern of innervation both during development and in the adult in contrast to 5-HT and NA. In the rat, discrete dopaminergic projections arise from cell groups in the ventral tegmental area and the substantia nigra (Lindvall and Björklund, 1984) (See Fig. 5). Cells of these nuclei differentiate between E11-E15 and their projections reach the anterior frontal cortex by E16 (Voorn et al., 1988). Upon reaching the cortex they are restricted to the intermediate zone until birth where they extend into the cortical plate of the frontal cortex and project caudally to the limbic cingular cortex. By the termination of postnatal weeks 2 and 3, a distribution resembling the adult is apparent in both the motor and limbic areas (Berger and Verney, 1984; reviewed by Parnavelas et al., 1988).

The other neurotransmitter substances- i.e: GABA, GLU, and ACh, are characterized as (either partially or completely) having an *intrinsic* origin from cells of the cerebral cortex. GABA is synthesized by nonpyramidal interneurons of the cerebral cortex and is the major inhibitory neurotransmitter in the CNS. In the embryonic rat, the GABAergic system (localized both in interneurons and fiber tracts) is apparent as early as E13, being present in the brainstem, mesencephalon and diencephalon (Lauder et al., 1986). In the cerebral cortex, studies have shown that GABAergic cells first appear around E13 and are subsequently detected within the subplate, layer I, and throughout layers VI to II (Wolff et al., 1984; Cobas et al., 1991). It is suggested that the dense GABAergic plexuses apparent in layer I by E18 may in part be due to a further extrinsic input by fibers originating from the corticopectal fiber system (Lauder et al., 1986).

**FIG. 5. SCHEMATIC DIAGRAM ILLUSTRATING THE MAIN
DOPAMINERGIC PATHWAYS INNERVATING THE ADULT RAT CORTEX**

(Adapted after Cooper et al., 1991)



The major excitatory neurotransmitter GLU is distributed widely throughout the CNS (Fonnum, 1984). Widespread effects of this neurotransmitter are mediated through activation of ionotropic and metabotropic receptors, and in the adult CNS these receptors are implicated in neurotoxicity (Choi, 1988). The developmental profile of glutamatergic projection neurons during the development of the mammalian cortex, however, has remained limited in comparison to that of the monoaminergic substances, yet evidence for a trophic role of GLU in development has continued to increase (Cohen-Cory et al., 1991; Lindholm et al., 1994; Liu et al., 1995; Meyer-Franke et al., 1995).

ACh is described as having an intrinsic origin within interneurons of the cerebral cortex, as well as an extrinsic component from the cholinergic cell bodies located in the basal forebrain (Mesulam et al., 1983a, b; Parnavelas et al., 1986). The cholinergic system innervates the cortex from the basal forebrain during postnatal ontogeny, with onset of choline acetyltransferase activity (indicative of the synthesis of ACh) increasing during postnatal weeks within all layers of the cortex (McDonald et al., 1987; Dori and Parnavelas, 1989). An interesting result, however, is a transient population of cholinergic neurons observed in all cortical layers in late gestation and early postnatal life (Dori and Parnavelas, 1989). Such transient populations may provide trophic signals in the developing cortex.

2.3 Serotonin in Development

2.3.1 5-HT and early embryogenesis

Studies focusing on the early control of cell division in fertilized eggs of sea urchins and chicks reported regulatory actions for 5-HT in development (reviewed by Buznikov et al., 1996). These investigations showed that various neurotransmitter substances are synthesized and released by yolk granules following egg fertilization. Levels of 5-HT, in particular, consistently showed variation at the start of cell division, while 5-HT receptor antagonists specifically inhibited cleavage in these cells. Such effects by 5-HT on cell proliferation were associated with the regulation of intracellular levels of cyclic adenosine monophosphate (cAMP) and Ca^{2+} . Thus, it is inferred that the regulation of these secondary messengers may be an important aspect in the control of cell proliferation (reviewed by Buznikov et al., 1996). Interestingly, 5-HT is associated with the regulation of proliferation in other studies, where 5-HT was found to stimulate meiosis in bivalve molluscs (Deguchi and Osanai, 1995). The important mediators in this investigation were observed to be Ca^{2+} and pH.

5-HT has been implicated in other early events during development such as regulation of morphogenetic cell movements of the neural tube and gut of the PNS (reviewed by Buznikov et al., 1996), as well as participation in craniofacial and cardiac morphogenesis in the mouse (Shuey et al., 1992, 1993; Yavarone et al., 1993). Inhibition of 5-HT uptake in craniofacial regions showed an effect on cell death and proliferation in mesenchymal tissues and malformation. It is suggested that the malformations observed may be a consequence of 5-HT effects on cell migration (Moiseiwitsch and Lauder, 1995). Furthermore, 5-HT is shown to regulate gene expression in mesenchymal tissue (Moiseiwitsch and Lauder, 1997). Taken together,

these studies provided evidence for the role of 5-HT as a dose-dependent regulatory signal at these sites.

2.3.2 5-HT and cell proliferation

Studies into the effect of 5-HT on cell proliferation are primarily on non-neuronal cells. *In vitro*, bovine smooth muscle cells increase in proliferation during exposure to concentrations of 5-HT between 1nM-10μM. Mitogenesis of these cells labelled by ³H-thymidine incorporation required a prolonged exposure time to 5-HT (20-24 hr) for stimulation of DNA synthesis to continue (Nemecek et al., 1986). 5-HT is also shown to have a synergistic action, potentiating the mitogenic effects of PDGF. Further studies on the mitogenic effect of 5-HT have been conducted using fibroblast cells (Seuwen et al., 1988). These investigations reported an interaction between 5-HT and other growth factors (in this case bFGF) on mitogenicity, although 5-HT was observed to have no significant effect on its own. Regulation of proliferation by 5-HT is proposed to be mediated by specific surface receptors and secondary transduction pathways. Interestingly, NIH 3T3 fibroblasts transfected with cDNA clones encoding the 5-HT_{1c} receptor gene, could transform fibroblasts into actively proliferating tumor cells (Julius et al., 1989).

2.3.3 5-HT and the regulation of differentiation and growth

The role of 5-HT in the process of differentiation of neuronal cells has been investigated using embryonic cells of the rat raphe nuclei. 5-HT was depleted in target regions during neurogenesis by application of the neurotoxin *p*-chlorophenylalanine (*p*CPA) to raphe nuclei *in vivo* (Lauder and Krebs, 1976, 1978). Within target regions (i.e: the hippocampus, cerebral cortex, striatum, thalamic nuclei and superior colliculus), these studies reported a delay in neurogenesis compared to controls, indicating that 5-

HT is required as a differentiation signal. Experiments on the role of 5-HT in the cerebral cortex and hippocampus, however, have focused on the postnatal period of development. In studies that utilized organotypic cultures of the rat neocortex, 5-HT was observed to promote morphofunctional development such as stimulation of glia proliferation, axon myelination, neuronal differentiation and synaptogenesis (Chubakov et al., 1986). A similar stimulatory effect by 5-HT was observed in postnatal hippocampal cultures (Gromova et al., 1983). A recent report defining a differentiative action of 5-HT on postnatal GABAergic interneurons of the cerebral cortex correlated this action with stimulation of specific serotonergic receptors (Kostner and Hornung, 1995).

Influences of 5-HT on cellular fate have also been reported by studies that used serotonergic neurons of the midbrain raphe nuclei in the snail *Helisoma*. Data from *in vivo* and *in vitro* experiments point to a role of 5-HT in the autoregulation of raphe neuronal development. This is dependent on the concentration of 5-HT applied (Whitaker-Azmitia and Azmitia, 1986; Shemer et al., 1991). Low concentrations of 5-HT *inhibit* neuronal differentiation and neurite outgrowth, whereas high concentrations activate serotonergic glial receptors. In turn, the glia growth factor S-100 is released which *stimulates* growth of the serotonergic neurons (Whitaker-Azmitia et al., 1990). Thus, the effects of 5-HT appeared dependent on the cell type present and class of receptor expressed. Further studies reported an additional survival action as well as regulation of somal size by 5-HT on serotonergic neurons (Liu and Lauder, 1991). The effect of 5-HT on growth cones and neuritic elongation was demonstrated, however, in *in vitro* and *in vivo* preparations of the *Helisoma* buccal ganglia (McCobb et al., 1988a, b; Goldberg et al., 1991). Using both adult and embryonic identified neurons these investigations reported that individual neurons react to specific neurotransmitters. One

class of neurons was observed to withdraw filopodia in response to low concentrations of 5-HT, while other neurotransmitter substances such as DA induced inhibition of mobility of a different subset of neurons. Further results showed that 5-HT induced withdrawal of neuronal axons could be reversed by ACh. These results indicate the specificity of action of neurotransmitter substances and the interactions that may occur in regulating growth and differentiation of neurons. In the adult CNS, there are many situations showing the interactions between monoaminergic systems in growth and maintenance. Perhaps the most interesting of these studies is the interaction of monoamine systems in the pathophysiology of affective disorders and schizophrenia (Hsiao et al. 1993; Kahn and Davidson, 1993).

2.3.4 5-HT, cortical plasticity and maintenance

These studies mainly expose monoaminergic nuclei to pharmacological lesions during postnatal or adult stages of life. The effects of these lesions are assessed in the target regions of the depleted monoaminergic terminals. The role of the transient hyperinnervation of layer IV in the rat somatosensory cortex by 5-HT axons during the first postnatal week has been investigated using this technique (Fujimiya et al., 1986; D'Amato et al., 1987). Such studies reported that the disruption of 5-HT afferents resulted in decrease in monoaminergic and non-monoaminergic synapses within the cerebral cortex (Okado et al., 1993; Chen et al., 1994). 5-HT afferents which normally formed periphery-related patterns termed "barrels" in somatosensory areas (Rhoades et al., 1990) are also modulated and delayed. The above studies, however, concluded that 5-HT was not *essential* in pattern formation in the somatosensory cortex (Loeb et al., 1987; Blue et al., 1991; Bennett-Clarke et al., 1994; Osterheld-Haas et al., 1994).

Recently, new data indicate that the *levels* of 5-HT present during this period are essential to the formation of these barrels (Cases et al., 1996).

The effect of neurotransmitter substances on injured tissue has also been investigated. Normally, within non-injured tissue, levels of neurotransmitters substances are low, however after mechanical injury this increases about 10-100-fold. These induced neurotransmitter substances support the survival and regeneration of embryonic ganglia neurons as well as central neurons of the septum and hippocampus (Björklund and Stenevi, 1981; Gage and Björklund, 1986) and appear to come from the target tissue (Azmitia and Whitaker, 1983; Holet and Cotman, 1984; Zhou et al., 1995). A specificity by 5-HT for the enhancement of growth of serotonergic neurons as opposed to fibres from noradrenergic neurons has been documented (Zhou et al., 1987).

3. Serotonin Receptors and Intracellular Pathways

5-HT has been shown to have profound and diverse actions also in the adult CNS; effects on mood, sleep, arousal, vomiting, cardiovascular regulation, thermoregulation, pain, anxiety and depression have so far been reported (Wilkinson and Dourish, 1991; Leonard, 1996). How can a single neurotransmitter exert multiple actions is indicated by the presence of an extreme diversity of pharmacologically and physiologically identified receptor subtypes. Originally, three main groups of 5-HT receptors were classified, each recognising different synthetic ligands (Bradley et al., 1986). However, cloning of 5-HT receptors has revealed a vast heterogeneity of receptor subtypes. At present there are 13 different mammalian receptor subtypes which are divided into seven distinct families (5-HT₁-5-HT₇) based on the sequence of their amino acids and coupling to secondary messengers. These receptors have been found in both the adult and developing CNS to display regional heterogeneity, however, an additional indication as to the importance of these receptors in ontogeny is the appearance of transient patterns and over-expression. The *adult* distribution of 5-HT receptors and their coupling to secondary messengers is briefly reviewed in Table 1. The appearance of 5-HT receptors during *development* and in particular those associated with the cerebral cortex (namely 5-HT₁, 5-HT₂ and 5-HT₃), are further considered in the following two sections.

3.1 5-HT receptors in the developing nervous system

At early stages of development within the proliferative zones of the primate cortex high densities of 5-HT₁-serotonergic, D₁-dopaminergic, α_1 - and α_2 -adrenergic, and high affinity kainate receptors are observed (Lidow and Rakic, 1995). In addition, a

synchronized overproduction of neurotransmitter receptors has been documented (Lidow et al., 1991). These studies indicate that dividing or newly generated cortical neurons are capable of receiving specific signals from neurotransmitters located within their environment. Interestingly, studies that have investigated the synaptic properties of serotonergic growth cones in the embryo show that at E15 and E20, before synapses are formed, the growth cones display features of mature serotonergic synapses (Ivgy-May et al., 1994). Other studies have observed the release of neurotransmitters prior to the development of synaptic contacts (Young and Poo, 1983; Sun and Poo, 1987).

Many studies have focused on the appearance of serotonergic receptors during development. *Functionally active* embryonic serotonergic receptors were shown by using the 5-HT neurotoxin *pCPA* and agonist 5-methoxytryptamine (Whitaker-Azmitia et al., 1987). These fetal 5-HT receptors (5-HT_{1a}) in the cortex and brain stem responded to changing levels of 5-HT similar to adult receptors. 5-HT_{1a} receptors also autoregulate the effects of 5-HT on the serotonergic neurons of the raphe mesencephalon (Whitaker-Azmitia and Azmitia, 1986; Sotelo et al., 1990). Interestingly 5-HT_{1a} receptors are proposed to mediate neuritic branching in the embryonic cortex (Sikich et al., 1990). *In situ* hybridization studies also reported the existence of both 5-HT₂ and 5-HT₃ receptor mRNA transcripts within the developing cortical plate (Roth et al 1991; Hellendall et al., 1993; Johnson and Heineman, 1995). The 5-HT₂ receptor mRNAs show a specificity in the developmental distribution of its subtypes. In addition, generally three potentially important periods are identified in expression that correspond to the onset of receptor signal, followed by a period of accelerated expression, and then a later period of regression in the intensity of signal (Roth et al., 1991) as is also the case for 5-HT₁ receptors (Daval et al., 1987; Whitaker-Azmitia et al., 1987, 1990).

Table 1. Characteristics and distribution of adult 5-HT receptors

Receptor	Subtype	Effector Mechanism	mRNA Distribution
5-HT ₁ (G-protein coupled)	1a	inhibits adenylate cyclase	Hippocampus, raphe nuclei, amygdala, septum
	1b/1d β		Striatum, hippocampus, ganglion cells, subthalamic nuclei, entorhinal and cingulate cortex, cerebellum, spinal cord, raphe nuclei
	1d α		Striatum, nucleus accumbens, dorsal raphe nuclei, hippocampus
	1e		Caudate putamen, parietal cortex, frontoparietal motor cortex, olfactory tubercle
	1f		Hippocampus, spinal cord, uterus, mesentery
5-HT ₂ (G-protein coupled)	2a	activates PLC	Cerebral cortex, hippocampus, striatum, spinal cord, olfactory bulb
	2b		Stomach fundus, intestine, heart, kidney, lung, brain
	2c		Choroid plexus, medulla, pons, striatum, hippocampus, hypothalamus, spinal cord
5-HT ₃ (ligand-gated ion channel)			Cerebral cortex, hippocampus, amygdala, spinal cord, olfactory bulb, dorsal root ganglia
5-HT ₄ (G-protein coupled)		activates adenylate cyclase	Striatum, brain stem, thalamus, hippocampus, olfactory bulb, heart, gastrointestinal tract
5-HT ₅ (unknown linkage)	5a	?	Hippocampus, cerebral cortex, cerebellum, olfactory bulb, habenula, spinal cord
	5b		Hippocampus, dorsal raphe nuclei, habenula
5-HT ₆ (G-protein coupled)		activates adenylate cyclase	Caudate putamen, olfactory tubercle, nucleus accumbens, cerebral cortex, hippocampus
5-HT ₇ (G-protein coupled)		activates adenylate cyclase	Hippocampus, hypothalamus, superior colliculus, raphe nuclei, intestine, heart

(Adapted after Tecott and Julius, 1993; Sandou and Hen, 1994; Lucas and Hen, 1995)

The 5-HT_{2c} and 5-HT_{2a} receptors both display an early pattern of onset within the rat CNS with expression beginning at E14. The highest signal for 5-HT_{2c} receptors is found to be localized in the choroid plexus with this signal increasing throughout brain stem areas at later ages. There are no or undetectable signals in the cortical plate. 5-HT_{2a} receptor transcripts, conversely, were undetectable in the choroid plexus with expression shown in the cortical plate, subplate, and other areas by E18 (Hellendall et al., 1993). Other investigations report a relative over-expression of this receptor, reaching a peak at the end of the second postnatal week followed by a gradual decline to adult levels (Morilak and Ciranello, 1993). These studies implicate a role for 5-HT in the cortex in the late embryonic and early postnatal period. One may speculate that this role is in regulating an aspect of differentiation since much of the period of proliferation is over. Interestingly, however, in the neuroepithelium of the cerebral cortex, hippocampus, amygdala, strionucleus, hypothalamus and stria terminalis, 5-HT₃ receptor mRNA transcripts are detected from E14 to E18 (the last stage analyzed) (Johnson and Heineman, 1995).

3.2 Intracellular pathways

Environmental factors activate secondary transduction pathways which in turn mediate the transcription of important genes. Key second messengers in these pathways are shown to be adenylate cyclase, phospholipase C (PLC) and intracellular Ca²⁺ levels (reviewed in Fig. 6), which directly can influence such decisions as proliferation, cell death, neurite extension and differentiation in a number of cell types (reviewed by Lauder et al., 1993). Each of these second messenger pathways are activated by a particular receptor (reviewed for 5-HT in Table 1).

Interactions of secondary messengers in developmental decisions, for example, is shown by the effect of GLU on the neuroarchitecture of hippocampal pyramidal neurons (Mattson et al., 1988a, b). High levels of GLU cause cell death in certain mammalian central neurons (Rothman, 1985; Choi, 1992) and are thus implicated in neurodegenerative diseases. This group reported the regression of hippocampal neuronal dendrites in response to low levels of GLU (10-100 μ M), while high concentrations (1mM) caused cell death. They noted that the effects of GLU on neurite outgrowth and cell survival were mediated by parallel intracellular pathways, i.e: neuritic outgrowth was inhibited by low levels of Ca^{2+} or protein kinase C while high levels of activators for these substances caused cell death. In addition stimulation of cAMP by forskolin could increase dendritic outgrowth (Mattson et al., 1988a, b). A similar study (Meyer-Franke et al., 1995) noted the effects of neurotransmitters and growth factors in the activation of *cAMP* and proposed that they together with *electrical activity* acted to regulate cell survival. Other studies which implicate, in particular, *intracellular Ca^{2+}* levels in the spectrum of modulatory growth effects by neurotransmitters (in this study 5-HT, DA and ACh), are documented using identified neurons of *Helisoma* (Lipton and Kater, 1989; Goldberg et al., 1991).

3.3 Regulation of proliferation and differentiation

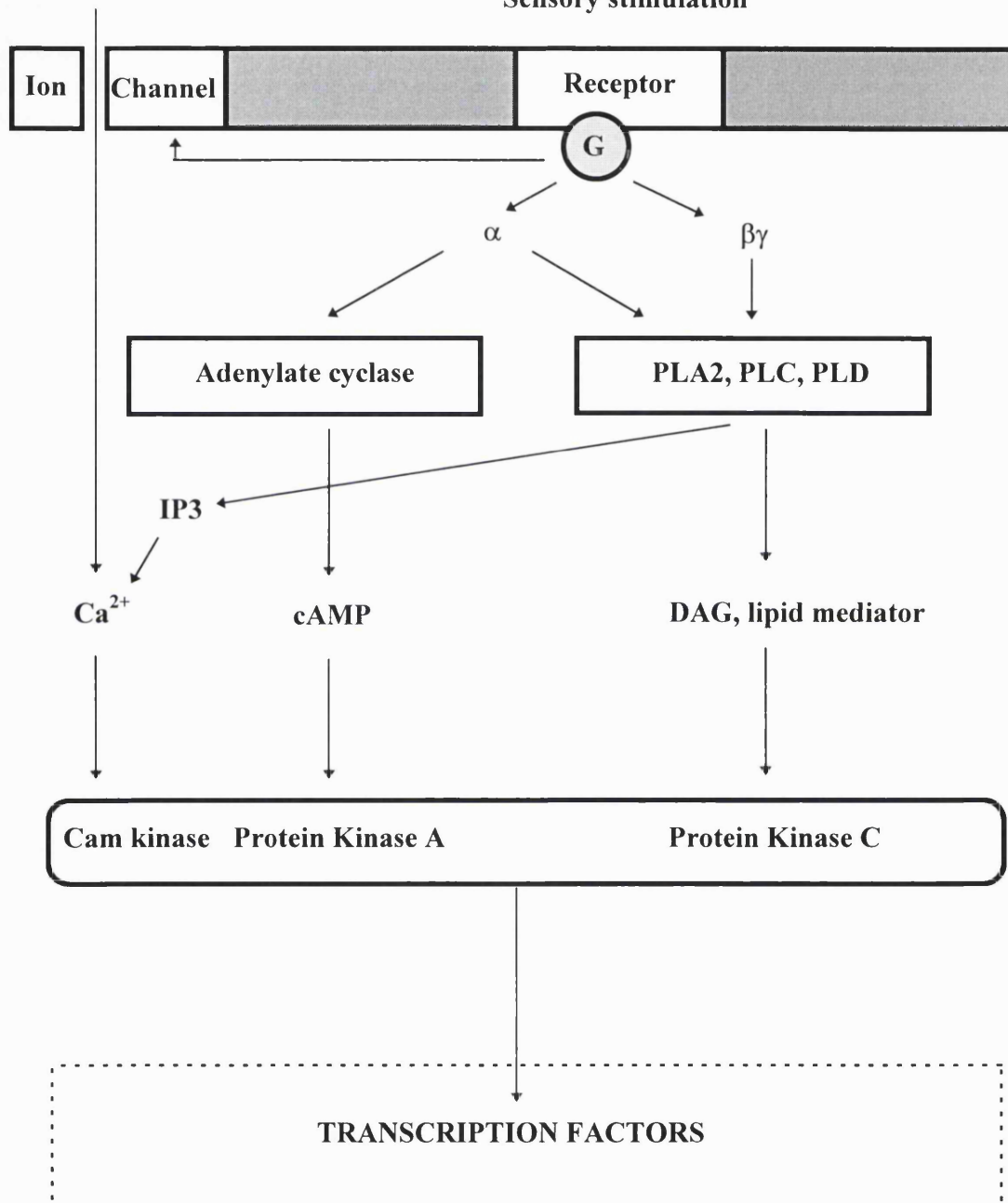
In general, it has been observed that neurotransmitter receptors that stimulate phosphatidylinositol hydrolysis (i.e: 5-HT₂, α_1 -adrenergic, and muscarinic) act to regulate proliferation, while receptors that activate adenylate cyclase (i.e: 5-HT₁, α_2 -adrenergic, and D₂-dopaminergic) may play a role in cellular differentiation. However, there are a variety of exceptions to this simple correlation (Lauder, 1993).

**FIG. 6. INTRACELLULAR PATHWAYS ACTIVATED BY
NEUROTRANSMITTERS**

Neurotransmitter signals are mediated through ion channels or specific receptors coupled to G-proteins (G). A cascade of effector systems are activated which result in the induction of transcription factors within the nucleus of the cell. PLA2, PLC, PLD, phospholipase A2, C, and D; DAG, 1,2-diacylglycerol; cAMP, 3', 5'-adenosine monophosphate; IP3, inositol 1,4,5-triphosphate; Cam kinase, calcium-calmodulin-dependent protein kinase; Protein kinase A, cAMP-dependent protein kinase; Protein kinase C, calcium-lipid-dependent protein kinase (Adapted after Bhat, 1995).

**Depolarization
Neurotransmitter**

**Hormones
Transmitters
Sensory stimulation**



4. Other Neurotransmitters in Development

4.1 Noradrenergic actions, receptors and intracellular pathways

Noradrenergic actions in the rat CNS have been documented to include the regulation of survival, proliferation, growth and differentiation of neurons and glia, and plasticity of regional structures during development. Noradrenergic receptors at present are subdivided into 4 main categories (α_1 , α_2 , β_1 , and β_2) the adult distribution of which is summarized in Table 2 (Nicholas et al., 1996).

NA is reported to induce neurotoxicity (at levels of 25 μ m and upwards) within embryonic cortical cultures *in vitro* (Rosenberg, 1988). This neurotoxicity, in contrast to that caused by high concentrations of Ca^{2+} by GLU in hippocampal neurons, is mediated by the generation of oxygen free radicals formed by the instability of NA, while death is prevented by free radical scavengers (Rosenberg, 1988). Modulation of proliferation by NA has been shown in neuronal and glial cells in the CNS (Ducan et al., 1990; Hodges-Savola et al., 1996). β -adrenergic receptors, for example, regulate astrogliosis and cell proliferation after optic nerve injury, while both α - and β -adrenergic receptors were shown to control DNA synthesis during critical periods in development. In addition to the regulation of survival and proliferation, effects on neurochemical differentiation (Konig et al., 1986) and morphology (Shain et al., 1987; Kwon et al., 1997) have been documented in which cAMP dependent mechanisms are implicated.

Table 2. Distribution of Adult Noradrenergic Receptors

Receptor	Subtype	Effector Mechanism	mRNA Distribution
α (G-protein coupled)	1b	activates PLC	Cerebral cortex, thalamus, dorsal raphe, pineal gland, medulla, spinal cord, olfactory bulb, hypothalamus, intermediolateral cell column
	1d		Olfactory bulb, hippocampus, inferior olivary nucleus, cerebral cortex, spinal cord, cerebellum
	2a	inhibits adenylate cyclase	Locus coeruleus, intermediolateral cell column, cerebral cortex, medulla, nucleus tractus solitarius, spinal cord
	2b		Thalamus
	2c		Olfactory bulb, hippocampus, striatum, sympathetic ganglia, cerebral cortex, cerebellum, thalamus, hypothalamus, pineal gland, spinal cord
β (G-protein coupled)	1	activates adenylate cyclase	Pineal gland, cerebral cortex, thalamus, sympathetic ganglia
	2		Olfactory bulb, cerebral cortex, thalamus, hypothalamus, pineal gland, hippocampus, spinal cord

(Adapted after Nicholas et al., 1996).

Many actions of NA within the cerebral cortex have been reported in postnatal animals. These studies have shown that the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA), which ablates NA axons, produces extensive and severe morphological alterations to pyramidal neurons (Felten et al., 1982). Pyramidal neurons were found to exhibit decreased length and branching of basolateral dendrites, loss of dendritic spines and an increased number of cells with rounded somatic contours. However, other studies using 6-OHDA neurotoxicity or ablation of the locus coeruleus by electrolytic lesions, have reported a less severe influence (Maeda et al., 1974; Wendlant et al., 1977) or no effect on pyramidal dendrites (Ebersole et al., 1981; Lidov and Molliver, 1982c). Further investigations have documented effects of 6-OHDA toxicity on serotonergic sprouting and the morphological organization of the somatosensory cortex (Blue and Molliver, 1987; Loeb et al., 1987).

4.2 Dopaminergic actions, receptors and intracellular pathways

DA has a relatively restricted innervation in the cerebral cortex being primarily localized to the anterior prefrontal and cingulate regions (See Fig. 5), and as such there are few studies relating to the trophic actions of DA within these regions in development. Investigations, however, have focused on the regulation of the biosynthetic enzyme for DA tyrosine hydroxylase (TH). TH is synthesized, stored and released by dopaminergic neurons of the brain stem, and expression of this enzyme is governed in an autoregulatory manner by dopaminergic neurons (De Vitry et al., 1991). A similar mechanism of regulation has been described for serotonergic neurons in development (Whitaker-Azmitia and Azmitia, 1986). However, in cortical embryonic cultures DA regulates TH expression in neurons which normally *in vivo* do not contain TH (Iacovitti et al., 1987). This observation suggests that differentiation of cortical

neurons is influenced by local environmental factors. Such phenotypic plasticity is well characterized in neurons of the PNS (Patterson et al., 1978; Teitelman et al., 1985; Landis et al., 1988).

DA in other systems is shown to regulate growth cone motility acting through the D₁ receptor in cultured retinal neurons (Lankford et al., 1988). This action has been hypothesized to be mediated by intracellular levels of cAMP, although other studies have pointed to a coupling of the D₁ receptor not related with adenylate cyclase mechanisms (Surmeier et al., 1995; Yang and Seamans, 1996). In addition, DA has been shown to have toxic effects on cortical and dopaminergic neurons in culture (Rosenberg, 1988; Michel and Hefti, 1990), modulation of neuritic outgrowth (McCobb et al. 1988b; Todd, 1992), as well as regulation of glutamic acid mRNA (Retaux et al., 1994). Interestingly, DA itself is regulated by noradrenergic terminals (Pozzi et al., 1994). The distribution of dopaminergic receptors in the adult rat CNS is shown in Table 3. During cortical *development*, however, the appearance of three dopaminergic receptors (D₁, D₂, and D₃) has been reported (Schambra et al., 1994; Diaz et al., 1997).

Table 3. Distribution of adult dopaminergic receptors

Receptor	Subtype	Effector Mechanism	mRNA Distribution
D ₁ -like	D ₁	can activate adenylate cyclase and PLC	Neostriatum, nucleus accumbens, Islands of Calleja
	D ₅	activates adenylate cyclase	Hippocampus, parafascicular nucleus
D ₂ -like	D ₂	inhibits adenylate cyclase and activates PLC	Neostriatum, core of nucleus accumbens, substantia nigra and ventral tegmental area
	D ₃	inhibits adenylate cyclase	Nucleus accumbens, Islands of Calleja, cerebellum, substantia nigra and ventral tegmental area, hippocampus
	D ₄	inhibits adenylate cyclase	Frontal cortex, hippocampus, cerebellum

(Adapted after Sokoloff and Schwartz, 1995).

5. Summary and Aims of Study

This thesis aims to investigate the actions of 5-HT during the embryonic period of cortical development. Specifically, utilizing dissociated cultures of E14, E16 and E18 cortices we established the effects of 5-HT on cortical cell survival and proliferation. 5-HT is established as an instructive environmental factor in the development of the PNS and CNS (see sections 2.3 and 3). Studies have so far examined the effects of 5-HT predominantly in *target* areas in the CNS such as the hippocampus, cerebral cortex, and craniofacial regions, while there are few regional embryonic studies. The *origin* of serotonergic innervation the brainstem raphe nuclei, has also been a focus of many investigations.

Progress in the definition of specific actions for growth factors within the developing cerebral cortex is apparent. Our *in vitro* studies (Cavanagh et al., 1997; Pappas and Parnavelas, 1997) have respectively highlighted the roles of bFGF and BDNF in regulating the division of embryonic cortical progenitor cells and inducing the expression of calbindin positive interneurons. Such studies have also complemented lineage analyses of cell fate specification, cell death and proliferation in the developing cortex *in vivo* (Mione et al., 1994, 1997; Lavdas et al., 1996; Thomaidou et al., 1997).

The presence of local environmental factors such as 5-HT, DA, and GABA at critical periods during cortical development have led us to ask the questions: are these neurotransmitters involved as specific environmental signals at this stage?, and, if so, how are these effects mediated? Recent evidence for the role of GABA, for example, denotes regulation of aspects of the proliferative cycle during the embryonic period (LoTurco et al., 1995). Our *in vitro* studies have also confirmed the action of GABA on cortical progenitor cells, this effect mediated by the GABA_a receptor (Antonopoulos et

al., 1997). A previous study has indicated the involvement of 5-HT in the establishment of differentiated cortical embryonic neurons *in vivo* (Lauder and Krebs, 1978, Lauder, 1990). Our studies, using dissociated and organotypic cell cultures, have sought to further characterize the actions of 5-HT within the developing cortex (Dooley et al., 1997; Lavdas et al., 1997). Part of the results of this investigation appear in Dooley et al., 1997.

The following chapters cover the *dissociated* cortical culture investigations involving 5-HT and focuses on three main areas as follows:

- Chapter 3- Serotonin and the survival of embryonic cortical cells.

We characterized the effect of 5-HT on embryonic cortical cells using defined environmental conditions. We reasoned that due to the appearance of 5-HT at a time well into the period of neurogenesis, it would be unlikely for it to influence neuronal proliferation, but rather, affect neuronal survival and differentiation.

- Chapter 4- An analysis of the survival of cortical cells by serotonin.

We identified the receptors through which 5-HT mediates its actions on developing cortical cells. *In situ* hybridization, receptor binding and immunohistochemical studies have jointly identified the presence of three different serotonergic (5-HT₁, 5-HT₂ and 5-HT₃) receptors during these early stages of cortical development (Whitaker-Azmitia et al., 1987; Hellendal et al., 1993; Morilak and Ciranello, 1993; Johnson and Heinemann, 1995). As such, either of these receptors were ideally placed to regulate the observed effects.

- Chapter 5- Serotonin and comparison with other neurotransmitters.

We compared the actions of 5-HT in the developing cortex with those of other monoamines (i.e: NA and DA), as well as GABA and ACh.

CHAPTER 2

METHODS AND MATERIALS

6.1 Preparation of Cortical Cultures

Cerebral cortices dissected from embryonic day 14 (E14), E16, or E18 Sprague-Dawley rats (E1= day of vaginal plug), were pooled, cleared of meninges and enzymatically dissociated by incubation in Dulbecco's Modified Eagle's Medium (DMEM; ICN) containing 0.1% trypsin (ICN) and 0.001% DNAase 1 (Boehringer Mannheim) for 30 min at 37° C. After washing in Ca²⁺/Mg²⁺ free Hanks Balanced Salt Solution (HBSS; ICN), treatment was continued by 0.05% trypsin, 0.002% DNAase I, and 0.5mM EDTA (Sigma) in HBSS for 12 min. Trypsinization was inhibited by 10% active fetal calf serum (FCS; Gibco) and cells totally dissociated by gentle pipette trituration. The resulting cell suspension was centrifuged and resuspended in DMEM/F12 (Sigma). Cells were plated on poly-L-lysine (10µg/ml; Sigma) and laminin (5µg/ml; Sigma) coated 96-well plates, or larger glass coverslips (13mm) in 24-well plates, at the appropriate density of 5 x 10⁴ cells/well or 2 x 10⁵ cells/coverslip. Culture plates were kept in a humidified 95% air/5% CO₂ incubator at 37°C, and cells allowed to attach in this medium for 30 min after which cells were maintained in DMEM/F12 containing 10% heat inactivated FCS (HI-FCS), 2mM L-glutamine (ICN), and penicillin/streptomycin mixture (PS; ICN) for 24 hr. After this stabilization period, cultures were washed in DMEM and placed in N-2 defined medium (Gibco) supplemented with 1% HI-FCS, PS, and 2 mM glutamine in DMEM/F12 for up to 7 days in the presence or absence of chemical reagents. Media and reagents were partially

replaced every 2-3 days and are listed in Tables 5 and 6 (see section 6.7). (Modified after Bottenstein and Sato, 1979).

Experiments which involved the switching of medium (i.e: from control to 5-HT-treated medium, or 5-HT-treated to control) were conducted as follows: cortical cultures (E16) were prepared and maintained for 24 hr in 10% FCS according to the above protocol. Cells were cultured in control or 5-HT-treated medium. At specified time points cells were washed x 3 in warm DMEM for 10 min and culture medium was switched. All cultures were grown up to 7 days.

6.2 MTT Survival Assay

The survival of cortical cells was assessed by the MTT (3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide) assay based on a protocol by Mossmann (1983). MTT which is incorporated and metabolized by living cells only, was kept in stock solutions of 5mg/ml at -20°C in light protected aliquots. It was added to cells grown in 96-well plates (as described in Preparation of Cultures) at a final concentration of 0.5 mg/ml for 2 hr. Thereafter, the MTT solution was removed, and the blue precipitate (produced via cleavage of the tetrazolium ring) was dissolved in dimethyl sulfoxide (DMSO) at 50 µl/well. Solutions were further diluted 1:10 with DMSO and measured with a spectrophotometer at a wavelength of 540 nm. Survival was, therefore, defined in terms of optical density units reflecting the absorbance values of precipitate produced. 3-4 wells per treatment at each time point was measured and experiments repeated 2-3 times. All survival values were expressed as a percentage of 1 DIV controls.

6.3 Immunohistochemistry

Immunohistochemistry was used to assess the phenotypic features of cells within the cultures using standard procedures as follows: culture preparations after 1 or 7 DIV were fixed with 4% paraformaldehyde for 10 min in phosphate buffered saline (PBS). After this period, cells were rinsed once for 10 min in PBS and placed in a blocking solution containing 5% normal goat serum in PBS for 20 min. Primary antibodies were then applied (an overnight 4°C incubation in PBS/0.5% Triton X-100) as follows: neurons were labelled with a monoclonal antibody to microtubule-associated protein (MAP-2; Boehringer Mannheim, 1:500), astrocytes with a polyclonal antibody to glial fibrillary acidic protein (GFAP; DAKO, 1:200), and progenitor cells with a polyclonal antibody to nestin (kindly donated by Dr McKay, NIH, 1:1000). Further sub-characterization of neuronal cells was provided by primary polyclonal antibodies to GABA (Sigma; 1:500) and GLU (Sigma; 1:1000). Following incubation in primary antibody, cells were rinsed (3x 10 min) in PBS and the corresponding biotinylated secondary antibody (Vectastain, 1:200) applied for 2 hr at room temperature. After a further rinse (3x 10 min) in PBS, they were incubated in the final layer consisting of the avidin-streptavidin horseradish peroxidase complex (Vectastain, 1:200) for 2 hr at room temperature, and developed using diaminobenzidine (DAB) as a substrate. Coverslips were mounted in PBS/glycerol (1:1). A Leica D-20 microscope using bright-field and phase objectives was used to analyze cells.

6.3.1 *BrdU staining*

BrdU (Bromodeoxyuridine; 10 μ M) was added to cultures for 2 hr during days 1-5 and 7, or 16 hr at 7 DIV to determine the BrdU labelling index among progenitor cells. Briefly, after the incubation period, cultures were fixed in 4% paraformaldehyde and then treated for 1 hr with 2N hydrochloric acid, washed in PBS, neutralized with 0.2 M borate buffer (pH 8.5; 15 min) and incubated in 5% normal goat serum in PBS (20 min). A monoclonal anti-BrdU antibody (Sigma; 1:500) in PBS/0.5% Triton X-100 was used overnight at 4°C and cells visualized by using the peroxidase coupled avidin-biotin staining kit (Vectastain) with DAB as a substrate. Coverslips were mounted in PBS/glycerol (1:1).

6.4 Tunel Labelling

Terminal transferase (TdT) nick end labelling was used, according to a modification of the method described by Gavrieli et al. (1992), to detect dying cells in the cultures. Briefly, cultures were fixed with 4% paraformaldehyde for 10 min at 1, 2, 4, and 7 DIV and processed for DNA nick end labelling as follows: coverslips were washed twice with dH₂O and pre-incubated for 15 min at room temperature in TdT buffer (30 mM trisma base (pH7.2), 140 mM sodium cacodylate, and 1 mM cobalt chloride in dH₂O). Coverslips were then incubated with 1 unit/100 μ l TdT and 1 unit 100 μ g/ml biotinylated UTP for 60 min at 37°C. The reaction was terminated by washing sequentially with dH₂O followed by PBS for 5 min. Cultures were then blocked with 5% normal goat serum in PBS for 20 min, washed twice in PBS (10 min) and staining accomplished by incubation in streptavidin-conjugated FITC (1:75) for 90

min. Coverslips were mounted in Citifluor and examined using a Leica D-20 microscope equipped with the appropriate fluorescein filter.

6.4.1 Double immunolabelling

After 1, 4, or 7 DIV cultures were fixed using 4% paraformaldehyde in PBS for 10 min and processed for double immunolabelling using standard fluorescence methods. Briefly, cultures were first incubated with a monoclonal anti-MAP-2 (1:500) primary antibody at 4°C overnight. Following incubation in a second layer of goat anti-mouse antibody conjugated to TRITC (rhodamine; 1:50) for 2 hr at room temperature, cultures were processed using the TUNEL protocol as described above in the dark. A similar double-labelling procedure using a primary polyclonal anti-BrdU (1:500) antibody following TUNEL staining was also applied. BrdU staining was accomplished using a second layer consisting of goat anti-mouse conjugated to TRITC (rhodamine; 1:50) for 2 hr at room temperature. All coverslips were mounted in Citifluor and analyzed using the Leica D-20 microscope equipped with the appropriate filters.

6.5 Cell Counts and Statistical Analysis

Ten fields per coverslip were counted at 2 mm intervals along the vertical and horizontal axes using a 40 X objective lens with the aid of a 500 µm graticule. 5-6 coverslips per treatment at each time point were analyzed in 2 experiments. Data are indicated as mean \pm SEM. The statistical significance of variations was evaluated using the unpaired Student's *t* test; $p < 0.05$ considered significantly different compared to controls.

6.6 Morphological Analysis of MAP-2 Labelled Cells

Cultures were fixed in 4% paraformaldehyde at 7 DIV and labelled for MAP-2. Only cells with clearly delineated processes were analyzed and drawn using a 40 X objective lens with the aid of a camera lucida attachment to a Leica D-20 bright field microscope. Neuronal images were traced onto a computer screen with a digitalizing mouse and then measured utilizing the TABLYT computer program (Dr. J. Cook, UCL, London) which automatically calculates cell body area, and length of neurites. 50 cells per coverslip were drawn, and 3 coverslips per treatment group assessed. Experiments were repeated twice.

6.7 Antibodies, Chemical Reagents, and Culture Media

The following tables (4, 5, and 6) contain a summary and additional information as to the antibodies used in visualizing specific cell types, chemical reagents added to cultures during 1-7 DIV, and the culture medium. All reagents added to culture media were sterilized by passage through a 0.2 μm filter (Millipore).

Table 4: Antibodies and histochemistry

Antibody	Raised In	Sub-class	Source	Cat. No.	Stock	Dilution	Notes
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Primary antibodies

Anti-MAP-2	Mouse	IgG ₁	Boehringer Mannheim	1284959	1:10	1:500	Neuronal intermediate filament
Anti-GLU	Rabbit	-----	Sigma	G-6642	1:10	1:1000	Neuro-transmitter
Anti-GABA	Rabbit	-----	Sigma	A-2052	1:10	1:500	Neuro-transmitter
Anti-GFAP	Rabbit	-----	Dako	Z 334	Undil.	1:200	Glial intermediate filament
Anti-Nestin	Rabbit	130	Dr. McKay	-----	1:10	1:1000	Progenitor cells
Anti-BrdU	Mouse	IgG ₁	Sigma	B-2531	1:10	1:500	Dividing cells

Secondary antibodies (or 2nd layer)

Anti-mouse (conjugated to TRITC)	Goat	IgG	Sigma	T-6528	Undil.	1:50	
Anti-mouse (biotinylated)	Goat	IgG	Vector Labs	BA-9200	Undil.	1:200	
Anti-rabbit (biotinylated)	Goat	-----	Vector Labs	BA-1000	Undil.	1:200	

Tertiary antibodies (or 3rd layer)

Streptavidin (conjugated to FITC)	-----	-----	Amersham	RPN-1232	Undil.	1:75	
Streptavidin (conjugated to horseradish peroxidase)	-----	-----	Vector Labs	P-4000	Undil.	1:200	

Miscellaneous

Biotinylated-16-dUTP	-----	-----	Boehringer Mannheim	1093070	Undil.	0.5µl/100µl	
Terminal transferase (TdT)	-----	-----	Boehringer Mannheim	220582	Undil.	0.2µl/100µl	
DAB tablets	-----	-----	Sigma	D-4293	-----	0.03%	

Table 5: Chemical reagents added to cultures

Name	Receptor Activated	Source	Cat. No.	Stock	Dilution	Notes
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Serotonin (5-HT) and associated derivatives

5-HT or (5-hydroxytryptamine)	5-HT ₁₋₇	Sigma	H-7752	10mM	25μM	
α-methyl-5-HT (maleate)	5-HT _{2a/c} agonist	Tocris Cookson	0557	10mM	25μM	
Cinanserin	5-HT _{2a} antagonist	Tocris Cookson	0460	10mM (in methanol)	5μM	
8-OH-DPAT (HBr) or 8-hydroxy-2-(di-n-propylamino) tetralin	5-HT _{1a} agonist	Sigma	H-8520	10mM	25μM	
Nan 190 (HBr) or 1-(2-methoxyphenyl)-4-(4-phthalimidobutyl) piperazine	5-HT _{1a} antagonist	Tocris Cookson	0553	10mM	10μM	
1-(3-Chlorophenyl) biguanide (HCl)	5-HT ₃ agonist	Tocris Cookson	0380	10mM	25μM	
Y-25130 (HCl) or -N-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-m-3-oxo-3,4-dihydro-2H-1,4-2H-1,4-benzoxazine-8-carboxamide	5-HT ₃ antagonist	Tocris Cookson	0440	10mM	10μM	

Dopamine (DA) and associated derivatives

DA or 3-HT (HCl) or 3-hydroxytryamine	D ₁ -like and D ₂ -like	Sigma	H-8502	10mM	10μM	
Dihydroxidine (HCl)	D ₁ agonist	Tocris Cookson	0884	10mM (in ethanol)	10μM	
Bromocriptine mesylate	D ₂ agonist	Tocris Cookson	0427	10mM	10μM	

Table 5. Chemical reagents added to cultures (cont.d)

Name	Receptor Activated	Source	Cat. No.	Stock	Dilution	Notes
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Noradrenaline (NA) and associated derivatives

NA or Arterenol (bitartrate salt)	NA α and β	Sigma	A-9512	10mM	10 μ M	
M-6434 or (2-[(5-chloro-2-methoxyphenyl)azo]-1H-imidazole)	NA α_1 agonist	Tocris Cookson	0461	10mM (in DMSO)	10 μ M	
UK14,304 or (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline)	NA α_2 agonist	Tocris Cookson	0425	10mM	10 μ M	
Dobutamine HCl	NA β_1 agonist	Tocris Cookson	0515	10mM	10 μ M	
Clenbuterol	NA β_2 agonist	Tocris Cookson	0688	10mM (in methanol)	10 μ M	

Miscellaneous

GABA or γ -aminobutyric acid	GABA _A and _B	Sigma	A-2129	10mM	10 μ M	
ACh or acetylcholine	muscarinic and nicotinic	Sigma	A-2661	10mM	10 μ M	
MTT (thiazolyl blue)	-----	Sigma	M-5655	5mg/ml	0.5mg/ml	Incorporated by living cells
6-acetyl-7-deacetyl forskolin	-----	Sigma	A-9420	10mM (in DMSO)	10 μ M	Increases cAMP formation
U-73122 or 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)-amino)-hexyl]-1H-pyrrole-2,5-dione	-----	Calbiochem	662035	4.3mM (in ethanol)	0.4 μ M	Inhibits PLC formation

Table 6: Culture media and associated materials

Name		Source	Cat. No.	Stock	Dilution
DMEM or Dulbecco's modified eagle's medium		ICN	12-332-54	-----	-----
DMEM/F12		Sigma	D-6421	-----	-----
HBSS or Hank's balanced salt solution		ICN	18-104-54	-----	-----
L-Glutamine		ICN	16-801-49	200mM	10µl/ml
Dnase I or deoxyribo-nuclease 1		Boehringer Mannheim	1284932	10mg/ml	10µl/ml or 20µl/ml
Trypsin		ICN	16-893-49	2.5%	40µl/ml or 20µl/ml
Penstrep. (penicillin/streptomycin)		ICN	16-700-49	5,000iu/ml + 5,0 µg/ml	10µl/ml
FCS or fetal calf serum (active)		Gibco	10106-060	Undil.	100µl/ml or 10µl/ml
HI-FCS (inactivated)		Gibco	10108-074	Undil.	100µl/ml or 10µl/ml
N-2 medium supplement		Gibco	17502-014	Undil.	10µl/ml
Laminin		Sigma	L-2020	1mg/ml	5 µl/ml
Poly-L-lysine (HBr)		Sigma	P-1524	1mg/ml	10 µl/ml
Coverslips (glass, 13mm diameter)		BDH	406/0189/12	-----	-----
Prefilters (0.2µM)		Millipore	AN3 H02500	-----	-----

CHAPTER 3

SEROTONIN AND THE SURVIVAL OF EMBRYONIC CORTICAL CELLS

7.1 Introduction

Although 5-HT is known to be present at an early stage during the development of the cerebral cortex and to enhance the differentiation of cortical neurons, little is known as to whether it exerts additional actions. Using the technique of dissociated culture of neural cells as described in the methods (see section 6.1), we first set up and cultured embryonic (E14, E16 and E18) cortical cells over a period of 1-11 days *in vitro* (DIV). The main question which we sought to answer in chapter 3 was what effect 5-HT was exerting on these cells within this prenatal period? We reasoned that due to the appearance of serotonergic afferents midway during neurogenesis in the cerebral cortex (Lidov and Molliver, 1982a, b; Wallace and Lauder, 1983), that 5-HT may play a role in the survival and differentiation of these cells rather than proliferation. This section thus identified and described the initial cell populations found in our cultures, standardized the conditions under which cells were grown, and examined whether 5-HT participated in the survival of these cells. Our main finding was that 5-HT increased the survival of cortical cell cultures in an age and concentration dependent manner.

7.2 Cell Types in Cultures Prepared from E14, E16 and E18 Cortices

As a first step to developing an *in vitro* system in which we could analyze the events of proliferation, survival and differentiation, we assessed whether a similar pattern of neurogenesis would be present in our culture conditions compared to that

observed *in vivo*. In the rat, the generation of neurons begins as early as E13-E14 and proceeds up to E20-E21 with the majority of neurons produced during the later periods.

Dissociated cultures under our conditions were assessed on 13 mm coverslips in 24-well plates. The relative proportions of cell types present in cultures of different ages were characterized after 1 day incubation in 10% FCS before the introduction of 5-HT. This period allowed cells to properly settle and attach to the coverslips. Cells in our cultures were characterized by using immunohistochemistry with cell-specific markers. We found that BrdU, a marker for dividing progenitor cells (Takahashi et al., 1992) which was added 16 hr prior to fixation, labelled a large proportion of cells (82%) in E14 cultures after 1 DIV (Figure 7A). A corresponding decrease was found in E16 cultures with 38% of cells expressing BrdU and a smaller 20% in E18 preparations after 1 DIV (Figures 8A, and 9A respectively).

Microtubule-associated protein-2 (MAP-2; Matus et al., 1981), a marker of differentiated neurons, stained 19% of cells in E14 cultures after 1 DIV (Figure 7B). By E16 this proportion had increased to 26% and in E18 cultures 53% after 1 DIV (Figures 8B, and 9B respectively).

Astrocytes, identified by the presence of glial fibrillary acidic protein (GFAP; Bignami et al., 1972), were seen only rarely throughout this stage. Nestin, a marker of undifferentiated neuroepithelial cells was also localized in some cells (Lendahl et al., 1990) (Figure 8C, and 9C; Table 7). This initial characterization of our cultures was sufficient to suggest that the appropriate proportions of cell types were present in line with their correct developmental stages after 1 DIV.

Figure 7: Photomicrographs of E14 cortical cells

Shown are cells grown after 1 DIV in 10% FCS. Micrographs illustrate cells immunostained for (A) BrdU, and (B) MAP-2. Scale bar = 20 μm .

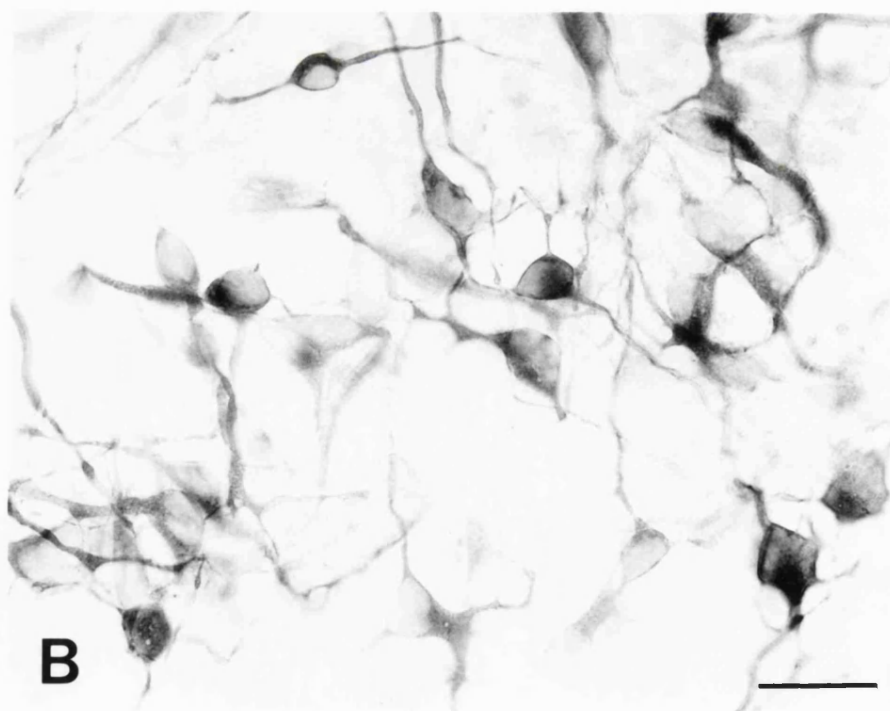
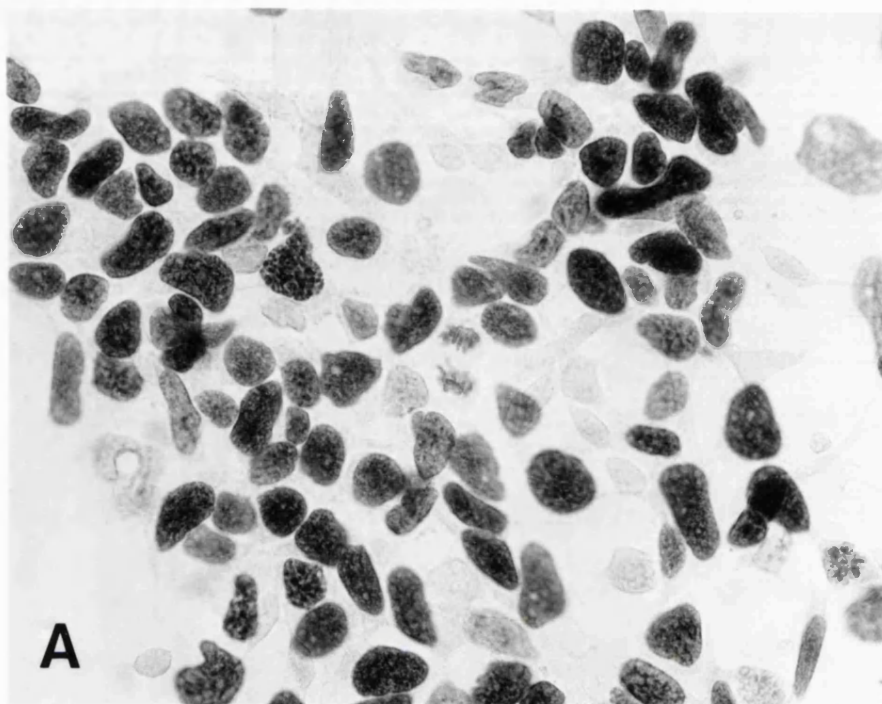


Figure 8: Photomicrographs of E16 cortical cells

Shown are cells grown after 1 DIV in 10% FCS. Micrographs illustrate cells immunostained for (A) BrdU, (B) MAP-2, and (C) Nestin. Scale bar = 20 μm .

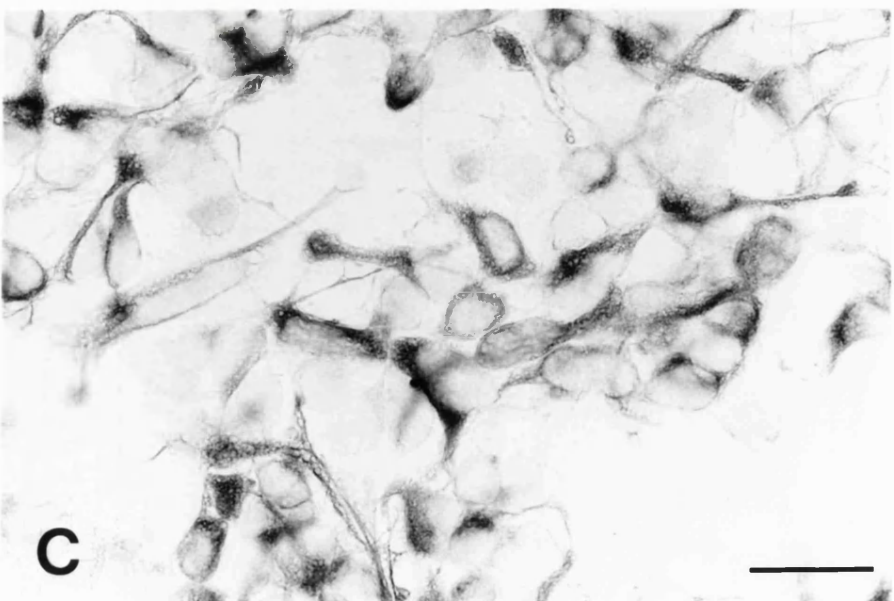
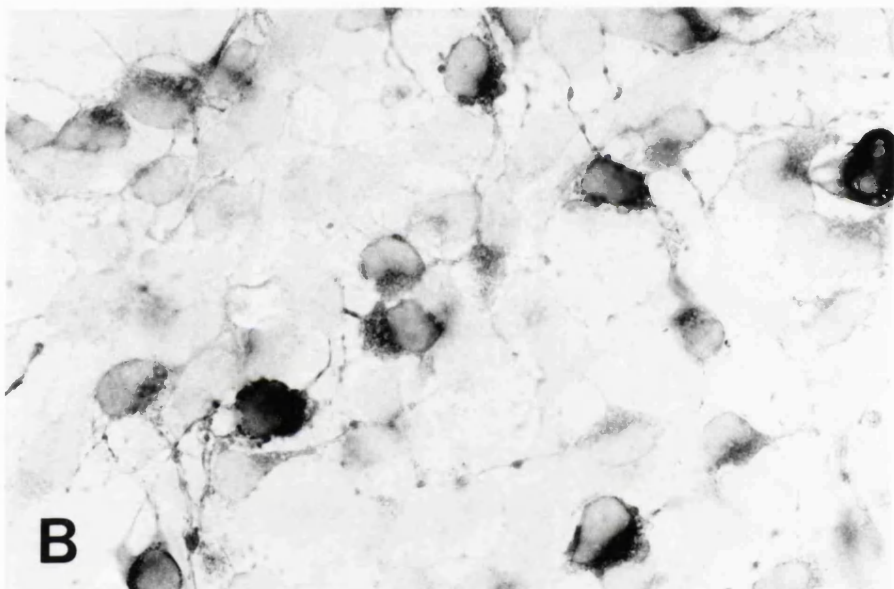
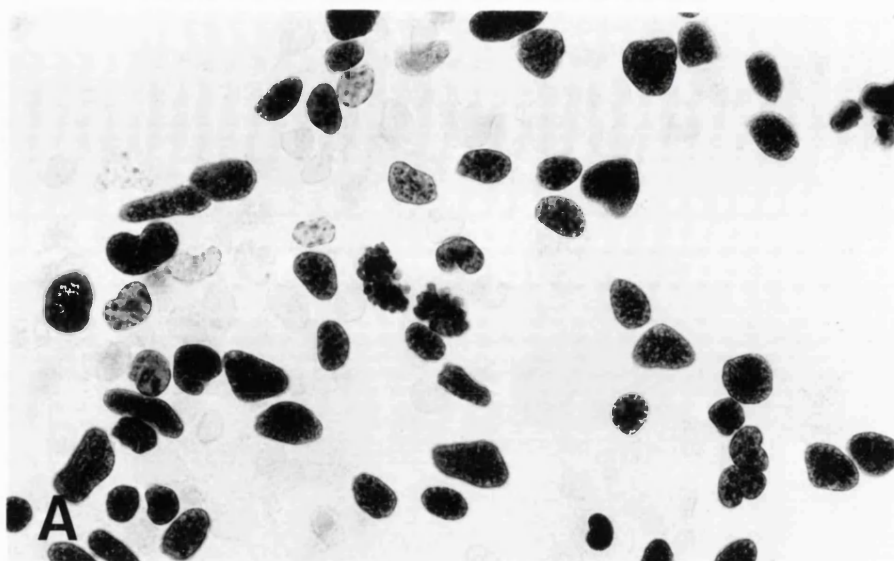


Figure 9: Photomicrographs of E18 cortical cells

Shown are cells grown after 1 DIV in 10% FCS. Micrographs illustrate cells immunostained for (A) BrdU, (B) MAP-2, and (C) Nestin. Scale bar = 20 μm .

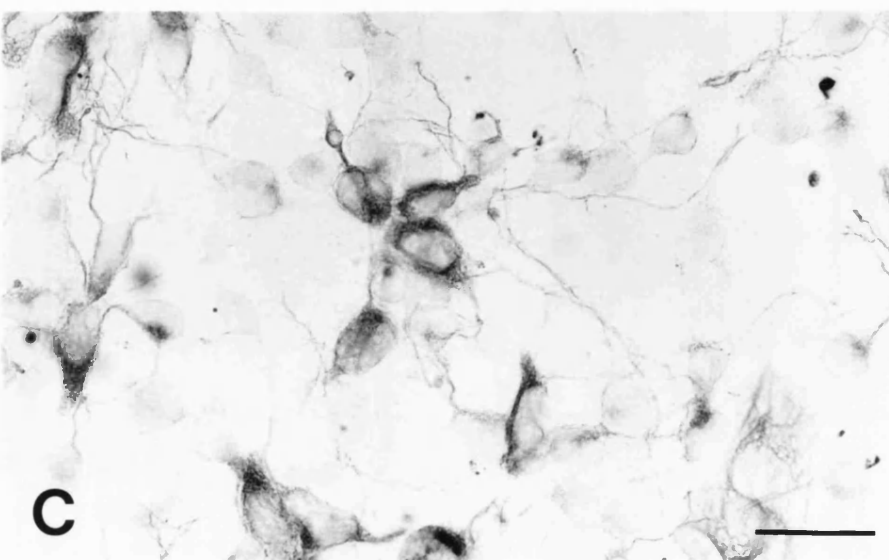
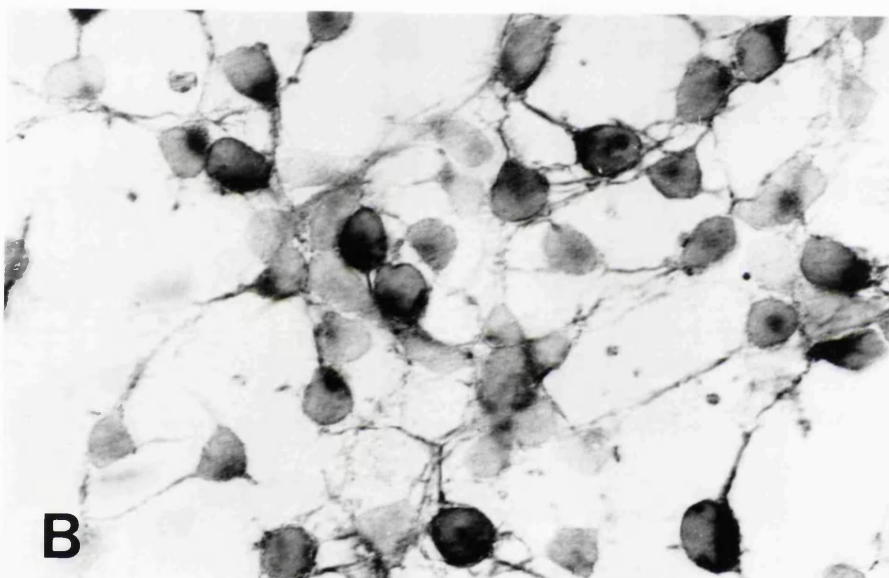
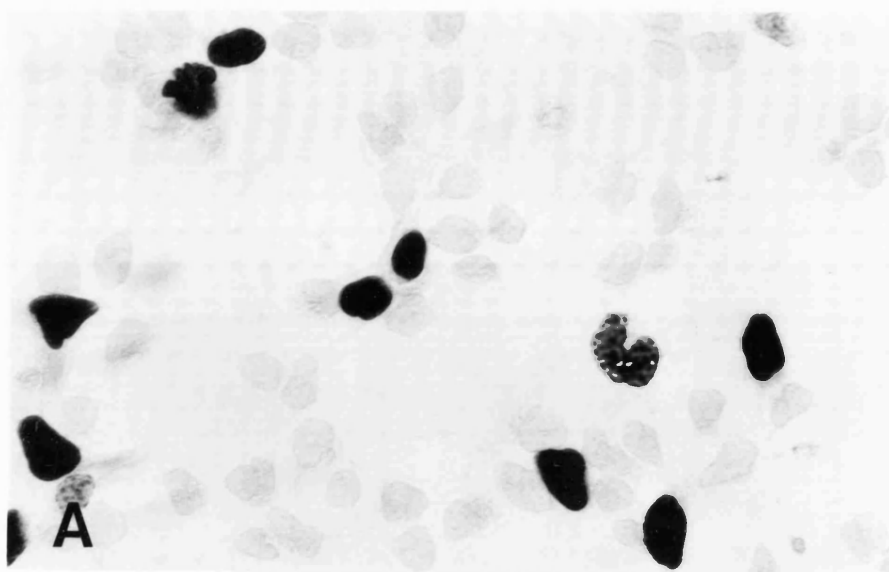


Table 7. Antigen expression in cortical cells *in vitro*

% Labelled Cells at 1 day (\pm SEM)			
Markers	E14	E16	E18
Map-2	18.7 ± 2.2	25.8 ± 1.3	53.1 ± 1.3
GFAP	0.2 ± 1.2	1.5 ± 0.8	1.2 ± 0.5
BrdU	82.2 ± 1.2	37.5 ± 2.0	20.1 ± 0.8
Nestin	-----	30.4 ± 1.3	22.9 ± 0.9

E14, E16, and E18 cultures were grown in 10% FCS in 24-well plates on glass coverslips for 24 hr and immunostained to various cell-specific antigens as indicated above. The percentage of labelled cells was analyzed in 5-6 coverslips from 2 separate experiments and expressed as mean \pm SEM. Numbers of nestin positive stem cells at E14 were not determined.

7.3 Standardizing Culture Conditions

7.3.1 *Fetal calf serum*

We reduced the level of FCS in our cultures in order to create an environment in which we could easily assess the effects of 5-HT on survival, proliferation or differentiation of these cells. Thus, E16 cultures after the required 1 DIV in 10% FCS, were changed to conditions where their basic medium was either serum-free or supplemented with 1% FCS or 10% FCS (Bottenstein and Sato, 1979).

Cultures containing 1% FCS or 10% FCS typically survived well for 4 DIV, with the cells under these conditions adhering well to the coverslip and extending many neuritic processes (Figure 10A). After this time point, cultures with 1% FCS gradually declined in cell viability, while cultures containing 10% FCS had survived to 7 DIV (Figure 10A). We chose 1% FCS containing cultures as an appropriate environment in which to assess the effect of 5-HT particularly on cell survival. Such an effect would become evident after 4 DIV in 1% FCS cultures.

Under conditions of serum-free medium, we observed an immediate and steady decline in cell viability, with a morphologically poor appearance to cells by 4 DIV (Figure 10A). Most cells at this stage showed small, shrunken and rounded nuclei with broken and retracted dendritic processes of a torturous nature indicative of cell death.

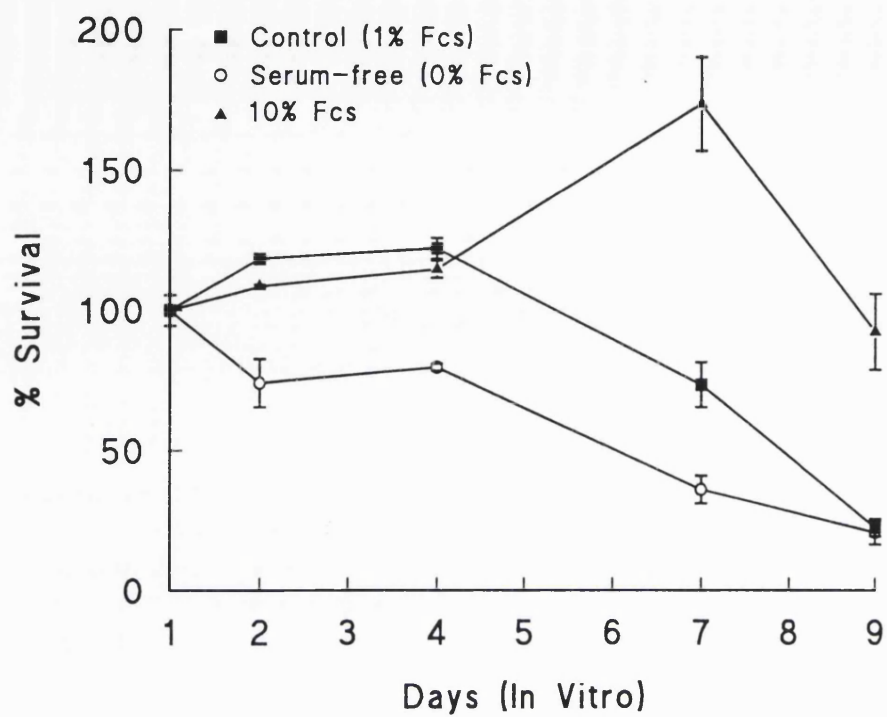
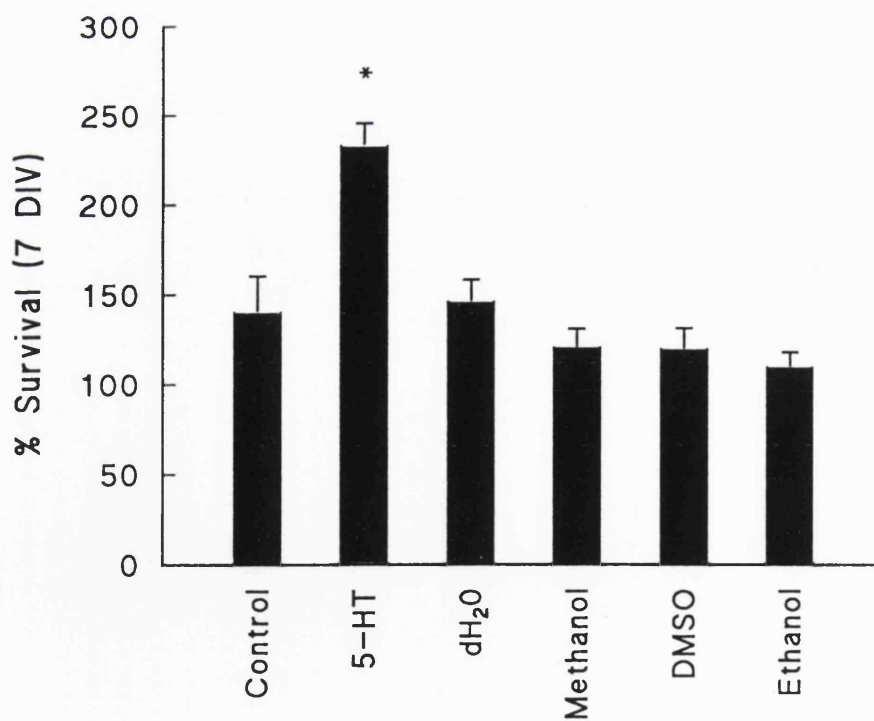
**Figure 10: Graphs showing the survival of E16 cortical cell cultures
under various environmental conditions**

Cells were grown for up to 7 DIV in medium treated with (A) serum, or (B) vehicles as indicated. The MTT assay was used to quantify survival at the specified time points.

(A) Graph showing the effect of serum on survival. The percentage of surviving cells was assessed at 1, 2, 4, 7, and 9 DIV. Media contained 1%, 10% or no serum. Data is derived from 1 of 2 separate experiments yielding similar results. n=3 wells at each time point per treatment.

(B) Graph showing the effect of vehicles on survival. The percentage of surviving cells was assessed at 7 DIV, in medium treated with distilled H₂O, methanol, dimethyl sulfoxide (DMSO), ethanol, or control. Data is derived from 1 of 2 separate experiments yielding similar results. n=3-4 wells per treatment.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

A**B**

We next assessed the effect 5-HT had on the survival of these cultures under *both* conditions of serum-free medium and inclusive of 1% FCS. We observed that inclusion of 25 μ M 5-HT in E16 cortical cultures could prolong the period of survival of these cells under *both* types of conditions. Serum-free cultures with 5-HT now survived for 4 DIV or longer, whereas 1% FCS containing cultures with 5-HT survived for up to 7 days or longer (data not shown).

We concluded that the inclusion of 5-HT in our cultures affected the survival of these cells regardless of the inclusion of serum. The observation that 5-HT affects the survival of cortical cells, *independent* of the presence of serum, suggested a unique regulation of survival not dependent on factors contained within the serum.

7.3.2 Vehicles used and culture density

In order to further standardize and explore the effects of other possible environmental influences on cell survival, we first tested the vehicles used to make up stock solutions of reagents which were frequently added to the medium (see Tables 5 and 6, section 6.7). Such vehicles were added to cultures in non-toxic concentrations of 0.01% for up to 7 DIV. All the vehicles tested (i.e: dH₂O, methanol, DMSO, and ethanol) produced no significant effect on cell survival compared to control cultures where no vehicle was added (Figure 10B). An additional control, in which 25 μ M of 5-HT was added to a culture, produced a significant increase in cell survival (Figure 10B).

It was also interesting to us to investigate the effect of cell density on the survival of these cultures and with the added inclusion of 5-HT. Temple and Davis (1994) reported that the close association of cortical cells appeared to be a factor in the

regulation of cell proliferation, while other studies have also noted the effects of a high density of cells in the maintenance of cell survival.

Our studies noted the effects of an increasing concentration of E16 cortical cells over a 9 day period *in vitro* (see Figures 11 and 12). We observed that increasing the density of control cortical cultures from 5×10^4 cells per well up to 4×10^5 cells per well particularly seemed to increase the number of surviving cells between 4 and 7 DIV (Figures 11 and 12). Additionally, we found that the survival effect exerted by 5-HT became more pronounced and significant amongst the higher cell densities at the later stages of culture (Figure 12A, and B).

In future experiments, cultures were maintained in a medium containing 1% FCS. Cells were plated at a density of 2×10^5 cells per well as further described in section 6.1.

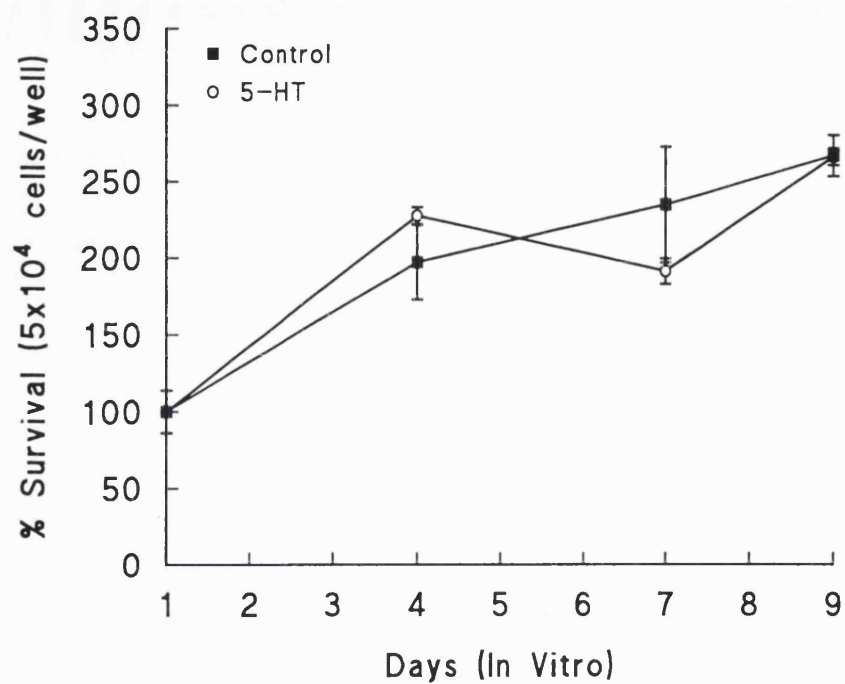
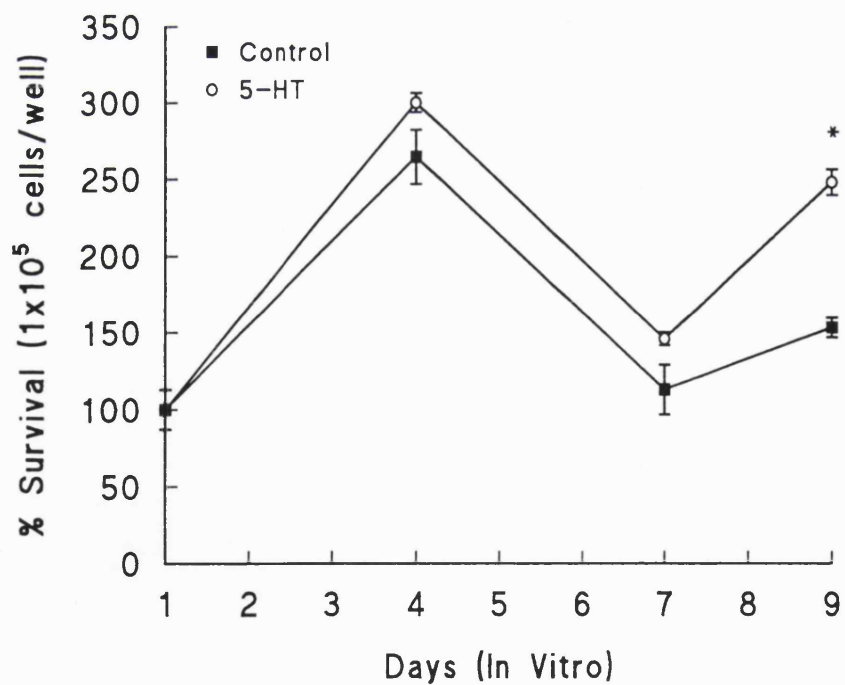
**Figure 11: Graphs showing the survival of E16 cortical cell cultures
plated at various densities**

Cells were grown for up to 9 DIV at densities of (A) 5×10^4 , or (B) 1×10^5 cells per well. The MTT assay was used to quantify survival at the specified time points.

(A) Graph showing the effect of cell density (5×10^4 cells/well) on survival. Cultures were grown in control or 5-HT-treated medium and the percentage of surviving cells assessed at 1, 4, 7, and 9 DIV. Data is derived from 1 of 2 separate experiments yielding similar results. n=3-4 wells at each time point per treatment.

(B) Graph showing the effect of cell density (1×10^5 cells/well) on survival. Cultures were grown in control or 5-HT-treated medium and the percentage of surviving cells assessed after 1, 4, 7, and 9 DIV. Data is derived from 1 of 2 separate experiments yielding similar results. n=3-4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

A**B**

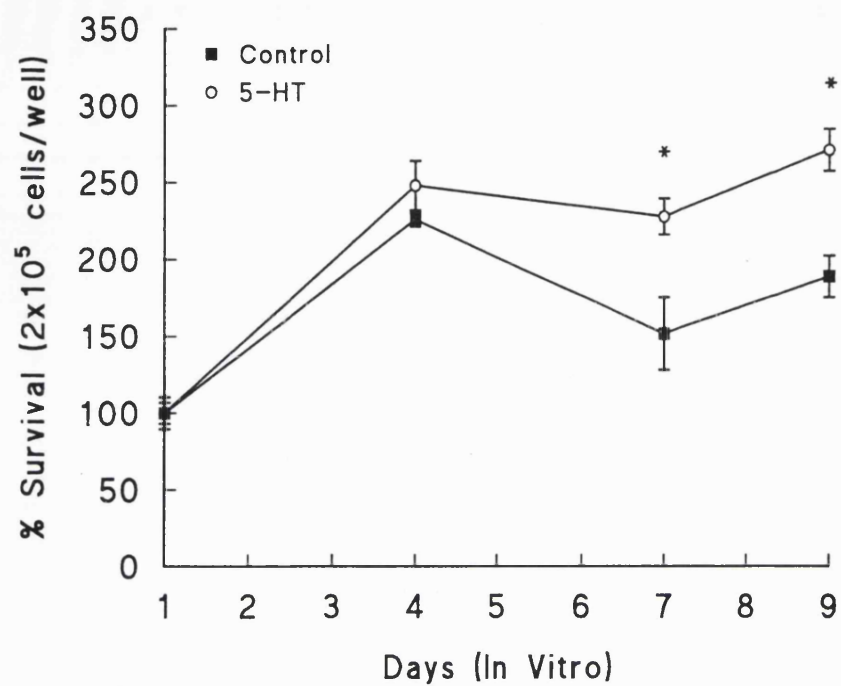
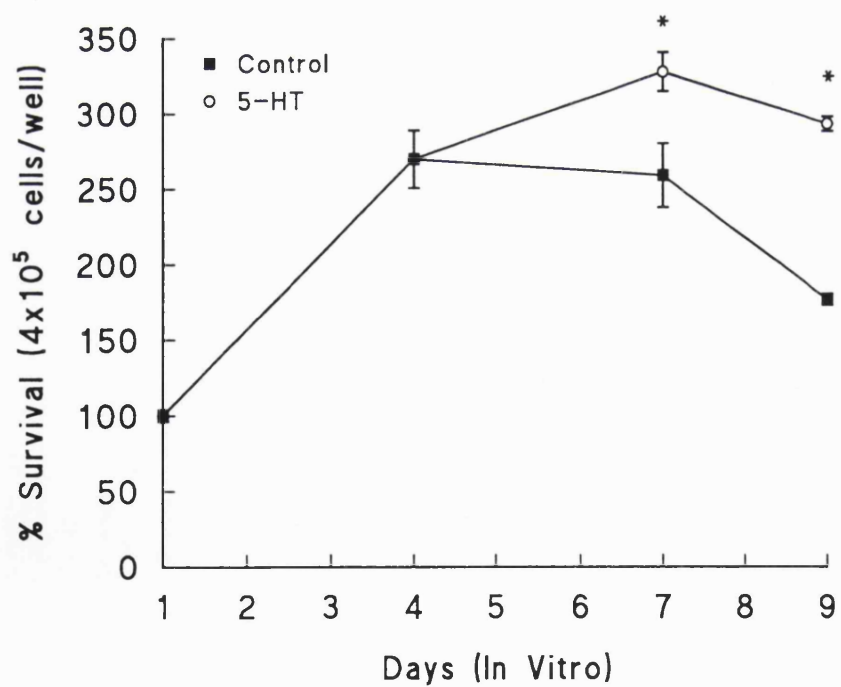
**Figure 12: Graphs showing the survival of E16 cortical cell cultures
plated at various densities (cont.d)**

Cells were grown for up to 9 DIV at densities of (A) 2×10^5 , or (B) 4×10^5 cells per well. The MTT assay was used to quantify survival at the specified time points.

(A) Graph showing the effect of cell density (2×10^5 cells/well) on survival. Cultures were grown in control or 5-HT-treated medium and the percentage of surviving cells assessed at 1, 4, 7, and 9 DIV. Data is derived from 1 of 2 separate experiments yielding similar results. n=3-4 wells at each time point per treatment.

(B) Graph showing the effect of cell density (4×10^5 cells/well) on survival. Cultures were grown in control or 5-HT-treated medium and the percentage of surviving cells assessed at 1, 4, 7, and 9 DIV. Data is derived from 1 of 2 separate experiments yielding similar results. n=3-4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

A**B**

7.4 5-HT Promotes the Survival of Developing Cortical Cells

7.4.1 5-HT promotes cortical survival in an age dependent manner

To determine whether 5-HT promoted cell survival in the developing cortex, we prepared and maintained cultures from E16 rats (E16 cultures) in a defined medium with or without 5-HT for a period of up to 9 DIV. We first assessed survival in 96-well tissue culture plates by the MTT assay (see section 6.2). Under these conditions, control cultures survived well for the first 4 days, after which there was a steady decline in cell viability through to 9 DIV (Figure 13B). However, cultures exposed to 25 μ M 5-HT showed approximately a 2-fold increase in cell survival compared to controls by 7 DIV; a significant increase was maintained up to 9 DIV (Figure 13B). This result confirmed earlier observations of the action of 5-HT on promoting cortical cell survival.

We next examined whether the enhanced cortical cell survival *in vitro* was a developmentally regulated process. First, we studied E14 cortical cultures which *in vivo* would not normally receive 5-HT innervation. Control cultures generally survived well for up to 7 DIV after which cell numbers progressively declined, indicating no additional need of trophic factors during this period (Figure 13A). Application of 5-HT only slightly enhanced (1.3-fold) the survival of these cells, and the difference became significant after 9 DIV (Figure 13A). This was in contrast to the earlier and greater effect of 5-HT on the survival of E16 cortical cultures, and suggested that there was a specific period during which 5-HT could exert its actions.

This hypothesis was reinforced by survival assessments on E18 cultures which revealed a different picture. Cell numbers in these cultures showed a rapid decline

Figure 13: Graphs showing the survival of 5-HT-treated cortical cell cultures during development

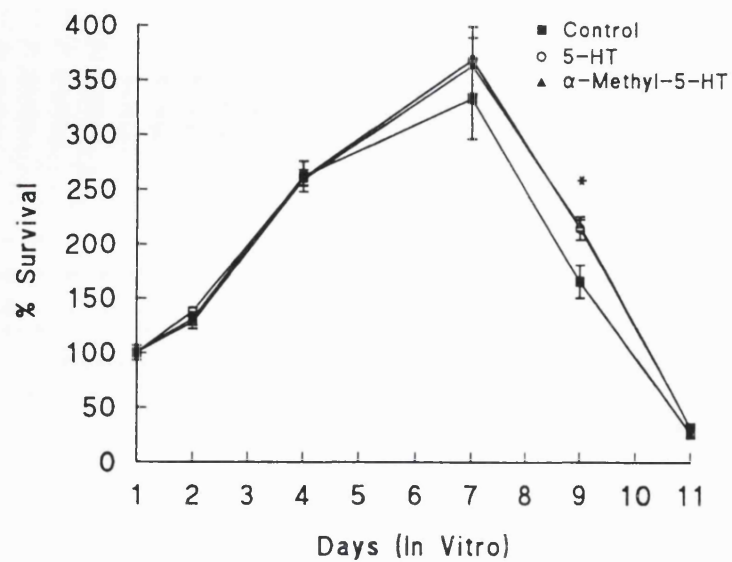
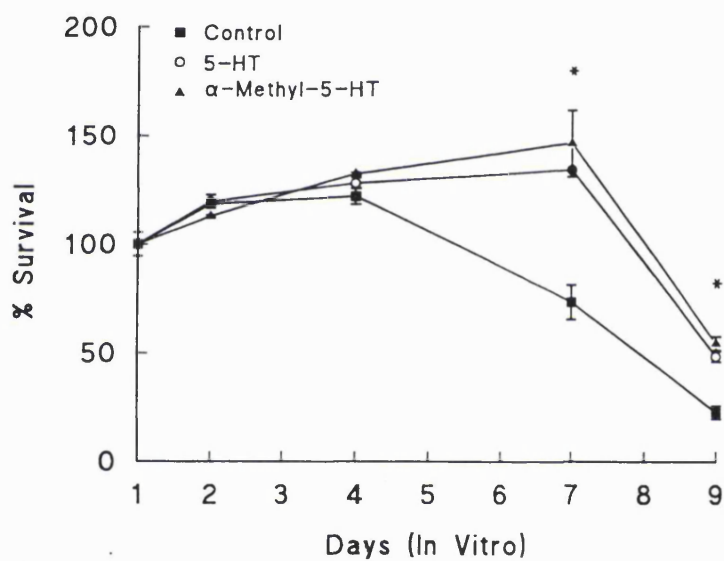
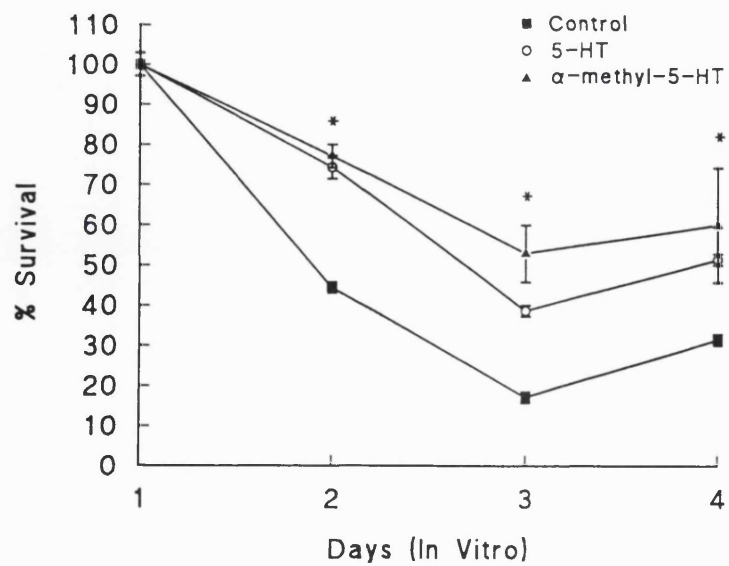
E14 (A), E16 (B), and E18 (C) cortical cells were cultured in 1% HI-FCS control medium or in the presence of 5-HT, or α -methyl-5-HT as indicated. The MTT assay was used to quantify survival.

(A) The percentage of surviving E14 cortical cells in 5-HT (25 μ M) compared to controls, assessed at 1, 2, 4, 7, 9 and 11 days of culture. Data is derived from 2 separate experiments, n=6 wells at each time point per treatment.

(B) The percentage of surviving E16 cortical cells in 5-HT (25 μ M) compared to controls, assessed at 1, 2, 4, 7, and 9 days of culture. Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells at each time point per treatment.

(C) The percentage of surviving E18 cortical cells in 5-HT (25 μ M) compared to controls, assessed at 1, 2, 3 and 4, days of culture. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. P<0.05 was considered significant compared to controls and where indicated by an asterisk represents both 5-HT and α -methyl-5-HT.

A**B****C**

immediately from 1 DIV, but 5-HT promoted cell survival (1.6-2.2-fold) throughout the early 4 day culture period (Figure 13C). These results indicate that the effect of 5-HT on cortical cell survival is a developmentally regulated process.

7.4.2 5-HT promotes cortical survival in a concentration dependent manner

The effect of 5-HT on cortical cells appeared to be dose-dependent at 7 DIV (Figure 14A and B). Specifically, the maximal effective concentration was 50 μ M; concentrations greater than 100 μ M both at 4 and 7 DIV caused cytotoxicity that eventually resulted in cell death. Toxicity at such high doses has also been reported by other investigators for 5-HT and for other monoamines (Rosenberg, 1988). In order to minimize the cytotoxic effects, we used a concentration of 25 μ M throughout this study.

7.4.3 Time period of 5-HT action

To determine when during the culture period 5-HT exerted its survival effects, the indoleamine was removed from the medium at first after 1-24 hr (Figure 15A), or at later time points of 2, 3 and 4 days (Figure 15B). These experiments showed that incubation with 5-HT for a period of up to 2 days did not increase cell survival (Figure 15A, and B). However, longer exposures of 3 and 4 days progressively increased the survival of cells in culture (Figure 15B). Exposures greater than 4 days produced no additional significant increase in cell number (data not shown).

7.4.4 5-HT decreases cortical cell death in E16 cultures

The level of apoptotic cell death was also investigated in control and 5-HT-treated cultures. This was assessed on coated glass coverslips in 24-well plates by *in situ* end labelling of DNA fragmentation [terminal deoxynucleotidyl transferase dUTP-

Figure 14: Graphs showing cortical cell survival at various concentrations of 5-HT and α -methyl-5-HT

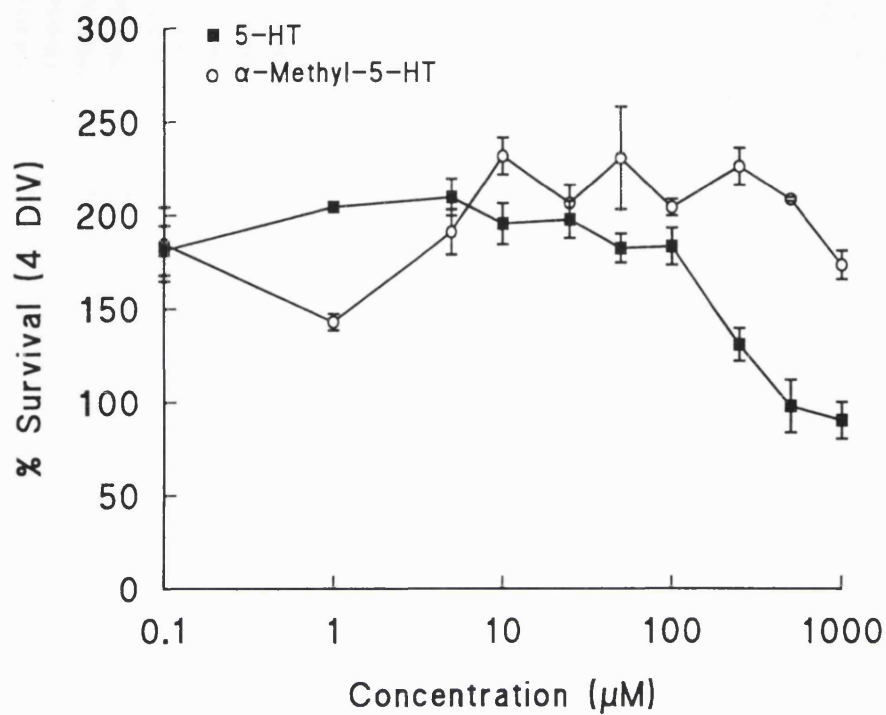
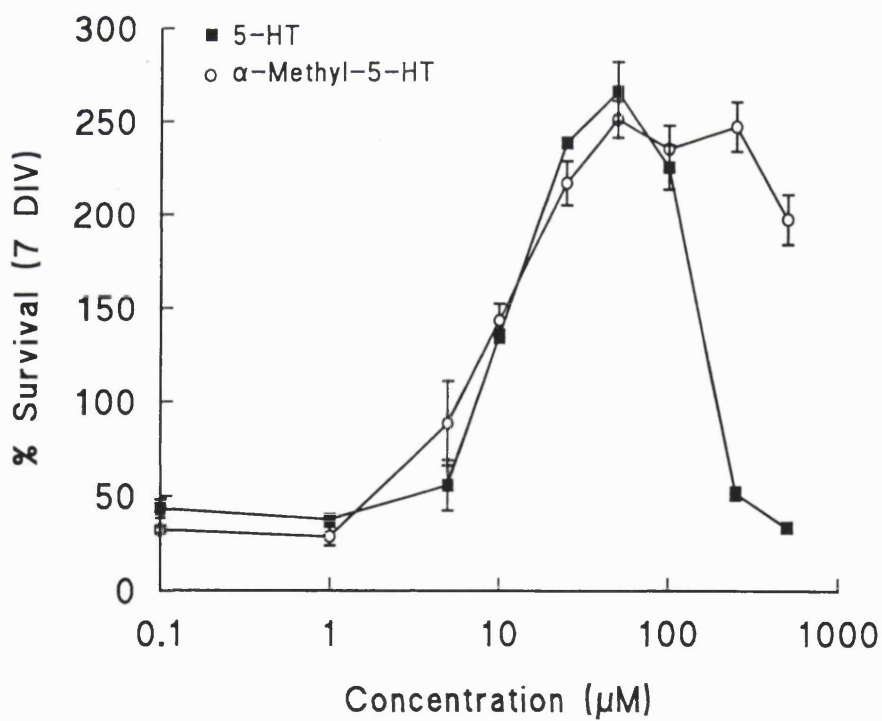
E16 cultures were grown in control, 5-HT, or α -methyl-5-HT treated medium for 4 DIV

(A) or 7 DIV (B). Cell survival was determined by using the MTT assay.

(A) 4 DIV, showing percentage cell survival in 5-HT (0.1-1000 μ M) or α -methyl-5-HT (0.1-1000 μ M). Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells per time point per treatment.

(B) 7 DIV, showing percentage cell survival in 5-HT (0.1-500 μ M) or α -methyl-5-HT (0.1-500 μ M). Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells per time point per treatment.

All results are expressed as mean \pm SEM.

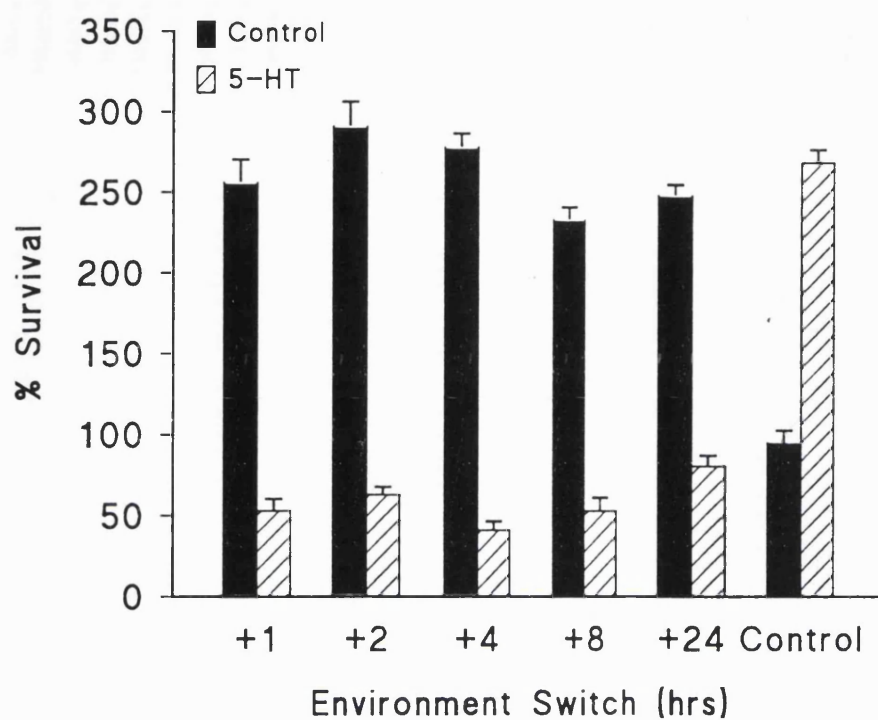
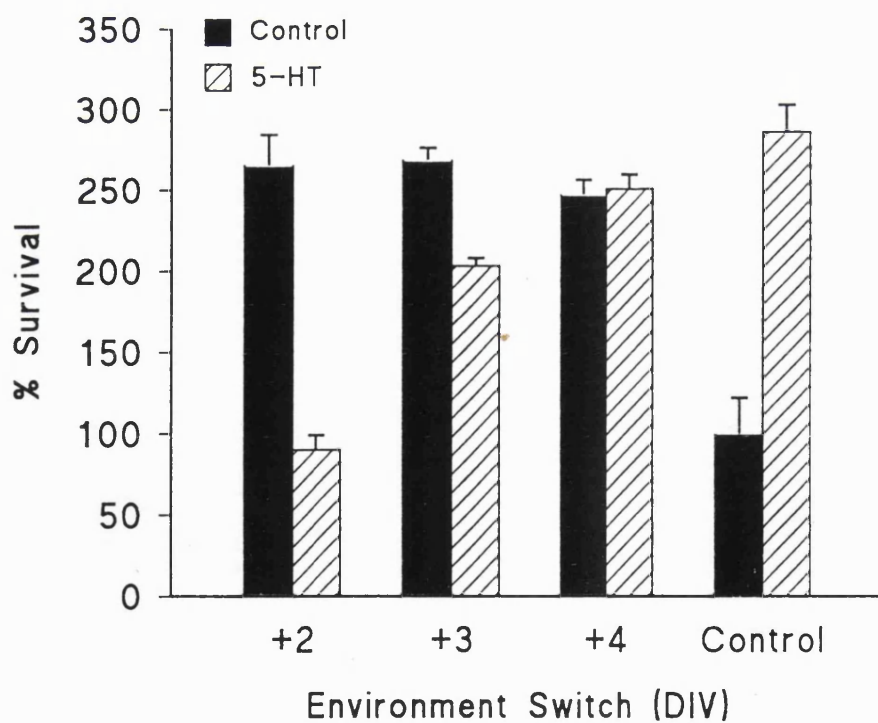
A**B**

**Figure 15: Graphs showing cortical cell survival after switching
culture conditions**

E16 cortical cells were grown for 1 DIV in HI-FCS, and cultured in control or 5-HT-treated medium. At specified time points culture medium was switched, and cells incubated up to 7 DIV. Survival was assessed using the MTT assay.

(A) Graphs showing the effect on cortical survival of cultures whose medium was switched at +1, +2, +4, +8, or +24 hr *in vitro*. The additional control shown represents cultures where medium was not switched throughout the 7 DIV. Data represents 1 of 2 separate experiments yielding similar results. n=4 wells per time point per treatment.

(B) Graphs showing the effect on cortical survival of cultures whose medium was switched at +2, +3, or +4 DIV. The additional control shown represents cultures where medium was not switched throughout the 7 DIV. Data represents 1 of 2 separate experiments yielding similar results. n=4 wells per time point per treatment. All results are expressed as mean \pm SEM.

A**B**

biotin nick end labelling (TUNEL)]. Apoptotic cells, identified by intense labelling of their nucleus with streptavidin conjugated to biotinylated d-UTP, were visible with the use of fluorescent second layers (see section 6.4; Figures 16A and B). In these preparations, 5-HT was found to significantly decrease the number of dying cells by 7 DIV (Figure 17A). Taken together, these results clearly show that 5-HT promotes cell survival in the developing cortex by impeding cell death. This effect is more pronounced at the later stages of neurogenesis than at the onset of corticogenesis.

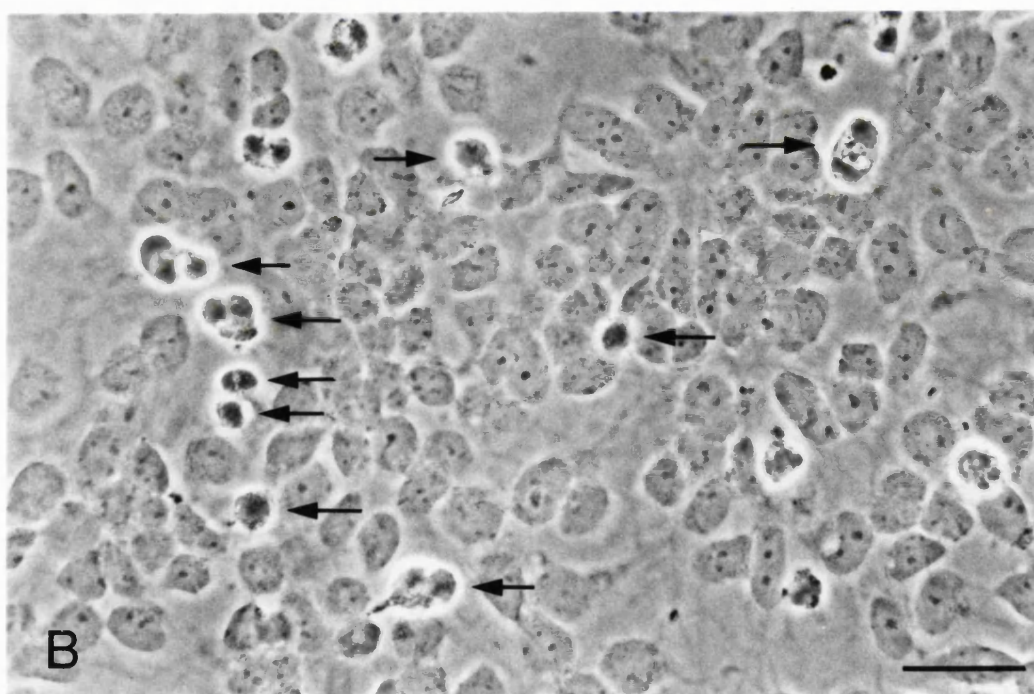
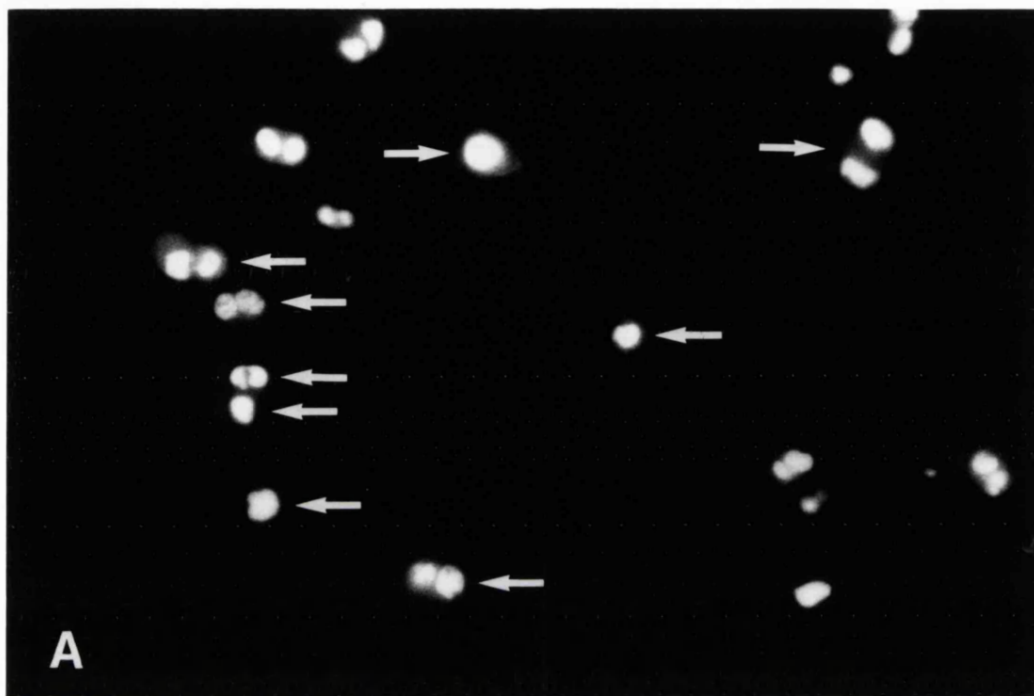
7.5 5-HT Does Not Affect Cortical Cell Proliferation

In order to reveal possible changes in proliferation brought about by culturing E16 cells in 5-HT containing medium, we exposed different cultures to BrdU for 2 hrs at each of the 7 DIV and then fixed them. Cells that had incorporated BrdU were identified immunohistochemically (see section 6.3.1). Counts of BrdU labelled cells showed that 5-HT did not increase the *proportion* of replicating cells throughout 7 DIV (Figure 17B), and confirmed that the observed increase in cell numbers following application of 5-HT was indeed due to increased survival rather than increased cell production.

Figure 16: Photomicrographs showing dying cortical cells

Cortical cells (E16) were cultured for 1 DIV in 10% FCS. (A) shows TUNEL immunolabelling indicative of dying cells. Arrows indicate dying cells which in their corresponding phase contrast image (B) have a slightly rounded appearance.

Scale bar = 20 μm .



**Figure 17: Graphs showing cortical cell death and proliferation in
5-HT-treated cultures**

Cortical cells (E16), were grown for up to 7 DIV in either control or 5-HT-treated medium. At specific time points, cultures were assessed for the amount of cell death

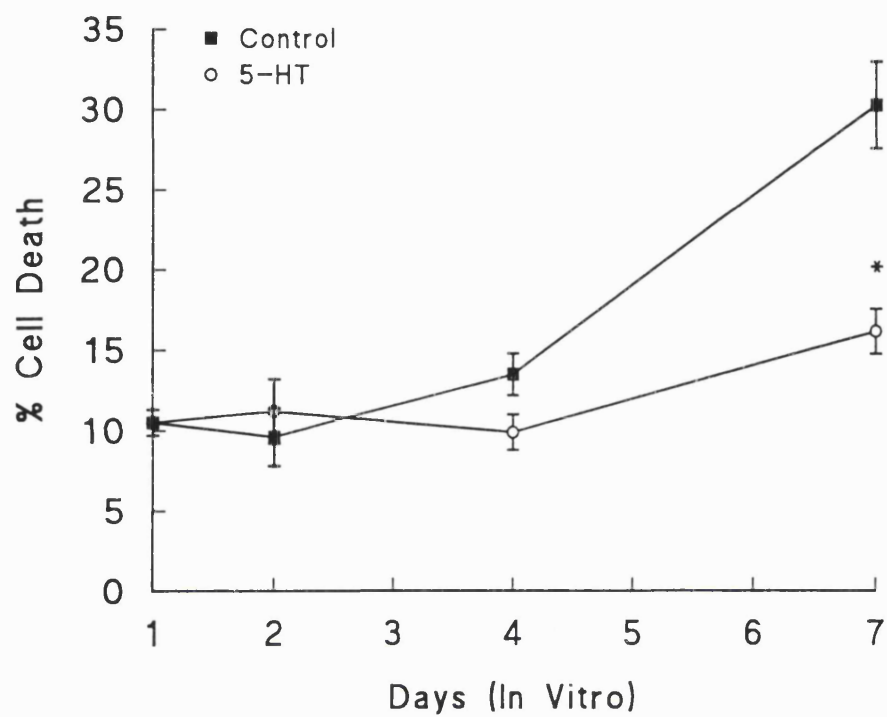
(A), or division (B) shown in each treatment.

(A) The effect of 5-HT on cortical cell death. Cultures were assessed using the TUNEL method at 1, 2, 4, and 7 DIV. Data is derived from 5-6 coverslips of 2 separate experiments.

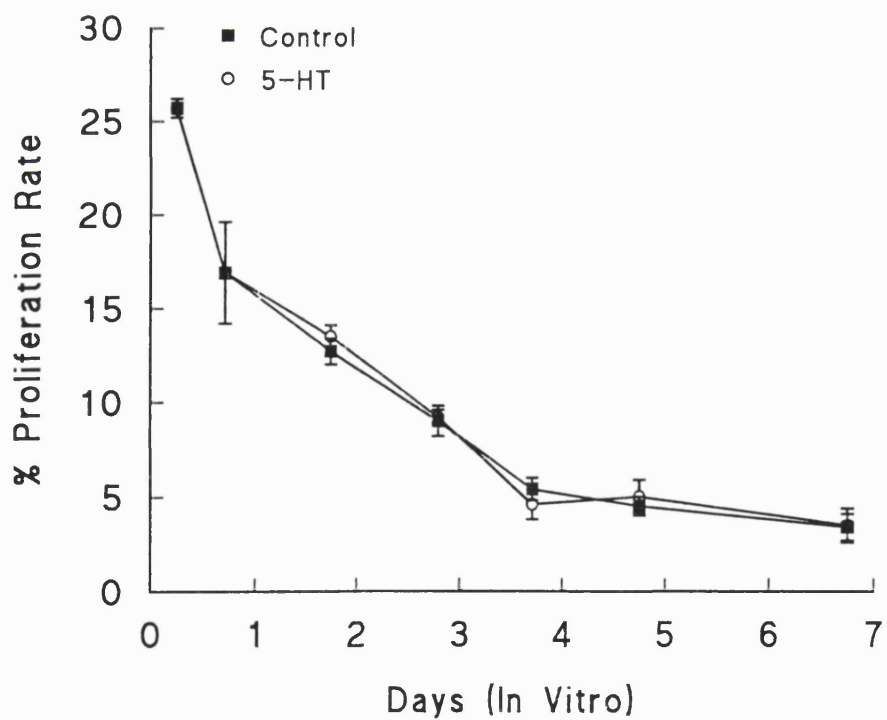
(B) The effect of 5-HT on the rate of proliferation. BrdU was added to cultures for 2 hr and the percentage of BrdU immunopositive cells assessed at 6 hrs after plating and at 1, 2, 3, 4, 5, and 7 DIV. n= 5-6 coverslips from 2 separate experiments.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls.

A



B



7.6 Discussion

7.6.1 *Standardizing culture conditions*

Survival of E14-E18 cortical cultures was assessed in the first instance under conditions of a serum-free environment (Bottenstein and Sato, 1979). Although 5-HT was observed to promote survival in these conditions, we chose to supplement our cultures with 1% FCS which extended the period of survival of cortical cells and allowed for their differentiation. As a result, a small amount of 5-HT was present in all cultures (Argez et al., 1984), but this was not directly measured in these experiments.

An additional finding was that similar to other investigators we revealed a increased cell survival with increased cell density. It would thus appear that cell to cell contact or factors released by neighbouring cells may well play a crucial role in regulating these processes. 5-HT was also able to promote the survival of higher density cultures where cell death became apparent. Such cell death may have occurred due to the high numbers of cells surviving and proliferating. It has been postulated by some reporters that there is a close link between cell proliferation and subsequent cell death (Evan et al., 1995; Thomaidou et al., 1997).

7.6.2 *5-HT promotes the survival of cortical cells in vitro*

We found that addition of 25 μ M 5-HT promoted cell survival, an effect that was more pronounced in the E16 and E18 cultures compared to those prepared from E14 rats. This finding suggests that older cultures, containing predominantly differentiated neurons, depends more on 5-HT for survival than E14 cultures which initially contain mostly undifferentiated neuroepithelial cells. 5-HT has also been shown to promote the survival of midbrain serotonergic neurons *in vitro* (Liu and Lauder, 1991). Although the

effect in this case was greater in earlier cultures, it suggested the existence of a critical period in the regulation of cell survival by 5-HT.

The increase in cell survival observed here was apparent for a period of a few days (depending on the age of the culture), after which both 5-HT-treated and control cultures did not survive. Further application of 5-HT did not prevent the eventual decline (data not shown), suggesting the need for other trophic factors. Additional studies that examined the survival and growth actions of 5-HT *in vitro* have indicated the importance of glia in mediating these effects. In these studies (Whitaker-Azmitia and Azmitia, 1989; Liu and Lauder, 1992), 5-HT was observed to stimulate astrocytes (of both cortical and brainstem origin) to produce factors which, in turn, promoted the survival and growth of serotonergic neurons. This is not surprising since 5-HT receptors and uptake mechanisms have long been shown to be present on glial cells (Katz and Kimelberg, 1985; Hansson et al, 1987). However, our cultures contained only a small population (<2%) of glial cells, and as such suggest that these cells did not participate in the mediation of the observed survival effect.

Other aspects which must also be considered is the relative instability of 5-HT, being oxidised within hours after addition to the culture medium. However, the use of stable 5-HT agonists such as α -methyl-5-HT, which also produced a similar pattern of survival, suggested that breakdown products of the oxidation process were not likely to be involved in our survival response. Additionally, cultures were replenished with fresh reagents after 2-3 DIV while further controls in which monoaminergic enzymes were added to vehicles with 5-HT produced no apparent effect on this survival response (data not shown). Other studies have indicated important roles for monoaminergic oxidases in cortical cell toxicity and throughout cortical development (Rosenberg, 1988; Mahler and

Davis, 1996; Cases et al., 1996). Interestingly, our concentration studies with 5-HT in which high doses produced cell toxicity, showed that this toxicity ceased when the stable 5-HT agonist α -methyl-5-HT was used.

Incubating cells with 5-HT at time points between a few hours to seven days showed a progressive increase in cell survival. These experiments indicated that the action of 5-HT was on a developmentally changing population of cells. A critical time point appeared to be 4 DIV after which 5-HT would save the majority of the populations contained within the cultures. Thus, 5-HT may be acting directly on a population of cells or in combination with other local and regulated factors to influence their survival. Such combinations of neurotransmitters and growth factors in regulating developmental processes are now becoming increasingly apparent (Du and Iacovitti, 1995; Antonopoulos et al., 1997).

7.6.3 5-HT does not regulate cortical cell proliferation

We next addressed the question of whether the enhanced survival observed was in fact due to an increased rate of proliferation of progenitor cells. We found no difference between 5-HT-treated and control cultures in BrdU incorporation during 7 DIV, which suggested that 5-HT was not involved in the regulation of proliferation of this mixed cortical population. These conclusions cannot be drawn lightly, however, since observed reports *in vivo* have shown that target depletion of 5-HT lengthened the proliferation period and consequently delayed differentiation indicating an involvement of this indoleamine in cell-cycle mechanics (Lauder and Krebs, 1978). Our results still do not preclude this idea as the *in vivo* situation may provide additional residual factors which can act in cohort with 5-HT and affect proliferation. Additionally, our studies

have not investigated the effects of 5-HT on specific *subpopulations* of cells. Alternatively, however, 5-HT may be perceived to act as a differentiation signal in these *in vivo* studies (Lauder and Krebs, 1978). Our results support this hypothesis by indicating 5-HT as primarily promoting the survival of postmitotic neurons as will be considered in subsequent chapters.

CHAPTER 4

AN ANALYSIS OF THE SURVIVAL OF CORTICAL CELLS BY SEROTONIN

8.1 Introduction

We had previously assessed the developmental stage of cells of E14, E16 and E18 cultures at 1 DIV (Figures 7, 8 and 9). These cultures were fixed and immunostained with the following cell-specific markers: MAP-2, GFAP, nestin, and BrdU. The earlier (E14) cultures, where 5-HT had been shown to have a small effect on cell survival after 9 DIV (Figure 13A), comprised approximately 80% BrdU- and 20% MAP-2-labelled cells; there was only a very small number of GFAP-positive cells (<2%). In contrast, later (E18) cultures, which had displayed an immediate dependency on 5-HT for survival (Figure 13C), contained about 20% BrdU-, 20% nestin- and 50% MAP-2-immunolabelled cells (Table 7). These results and others, had suggested that 5-HT was exerting its effects on a predominantly *postmitotic* population of cells.

The first part of this section seeks to further characterize the phenotypes of the surviving populations of cells in cultures treated with 5-HT. Additionally, we analyzed their morphological characteristics after 7 DIV. Earlier studies have indicated that 5-HT is involved in the regulation of neurite outgrowth and branching (Sikich et al., 1990; Goldberg et al., 1991; Liu and Lauder, 1991; Kostner and Hornung, 1995).

The second part of this section examined the *mechanisms* by which 5-HT exerted its survival effects on embryonic cortical cells *in vitro*. At present, fourteen mammalian 5-HT receptors are known, which have been divided into seven distinct families (5-HT₁-5-HT₇) on the basis of signal transduction pathways and amino acid

homology (Martin and Humphrey, 1994; Sandou and Hen, 1994; Lucas and Hen, 1995). In the adult rat brain, both the 5-HT_{1a}, 5-HT_{2a} and 5-HT_{2c} receptor subtypes reveal a heterogeneous pattern of distribution (Pazos et al, 1985a, b; Mengod et al, 1990a; Pompiano et al, 1992) with the 5-HT_{2a} receptor showing a particularly high distribution in the neocortex as revealed by in-situ hybridization and receptor binding studies (Pazos et al, 1985; Mengod et al, 1990b). The ontogeny of this receptor also revealed a relative over-expression in the cortex during the early postnatal period compared to adult levels (Roth et al, 1991; Morilak and Ciaranello, 1993). Such investigations suggest a specificity of action by 5-HT, whereby its effects may be mediated by developmental and regional distributions of the receptor subtypes present. Finally, we further considered the intracellular mechanisms involved in this survival effect.

8.2 The Effect of Serotonin on Cell Phenotype

8.2.1 5-HT promotes the survival of glutamatergic neurons

In order to further investigate the survival effect of 5-HT, we focused on E16 cortical cultures and assessed the population of surviving cells under control and 5-HT containing conditions. In experiments involving double-labelling for MAP-2/TUNEL and BrdU/TUNEL (see section 6.4.1; Figure 18), we found an increase in the number of MAP-2 labelled cells at 7 DIV in 5-HT-treated cultures, with a corresponding decrease of dying cells (Figure 19A and B). Specifically, these cultures contained a significantly ($p<0.05$) higher proportion of MAP-2 labelled neurons ($43.0\pm2.9\%$) compared to controls ($34.5\pm3.6\%$) (Table 8). Further cell counts at 7 DIV revealed that the proportions of other cell types (GFAP, BrdU, nestin) remained similar in the two conditions (Table 8). An additional analysis of the neuronal population, using antibodies

to the neurotransmitters GLU and GABA (see section 6.3; Figure 20C and D), indicated that the increase in the proportion of neurons in the 5-HT-treated cultures was due to a significant ($p<0.05$) increase in GLU containing cells (Table 8).

8.2.2 5-HT increased cell body area

Morphologically, cells in the control and treated cultures appeared fairly well differentiated, often giving rise to multiple processes. Analysis of the number and lengths of the processes of cells in the control and 5-HT-treated cultures indicated no difference between the two groups (see section 6.6; Table 9). However, measurements of cell body cross-sectional areas showed a 10% increase in the 5-HT-treated group (29.9 ± 0.8 vs $33.5\pm0.6 \mu\text{m}^2$; $p<0.05$) (Table 9). This increase in cell body size may be accounted for by the increased number of GLU-containing neurons, the largest group of cells in the rat cerebral cortex (Parnavelas et al., 1989).

Figure 18: Photomicrographs showing double immunolabelling of E16 cortical cell cultures

Shown are cortical cells cultured for 4 *DIV* in 5-HT-treated medium. Cultures were immunostained for dying and neuronal cell types as follows: (A) Phase contrast, (B) TUNEL, (C) MAP-2, (D) TUNEL and MAP-2. Straight arrows represent identical dying cells while arrowheads show MAP-2 negative cells. Cultures were also immunostained for dying and dividing cell types. Shown are (E) Phase contrast, (F) BrdU. Curved arrows show identical replicative cells. Scale bar = 20 μm .

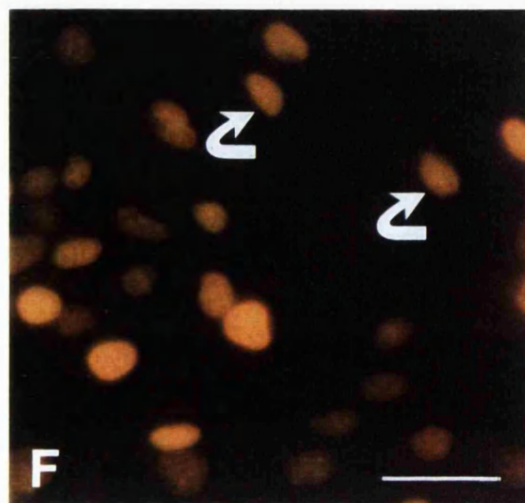
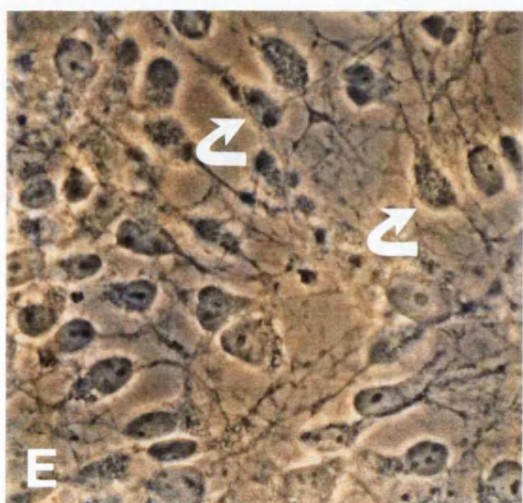
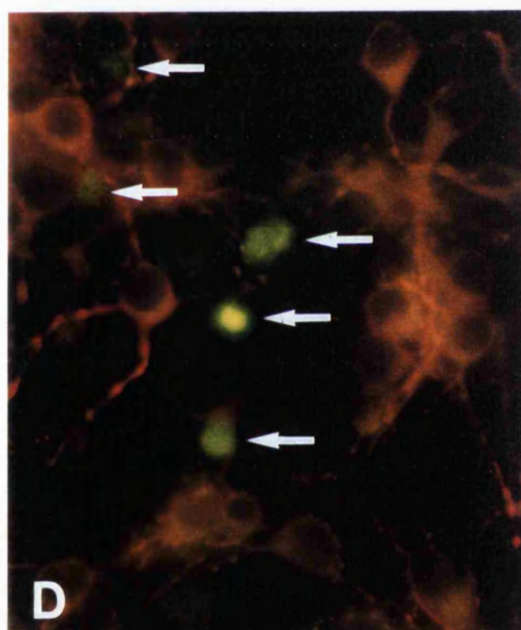
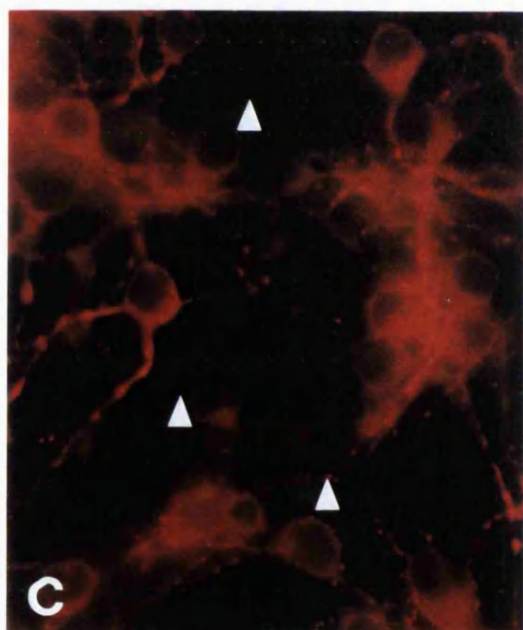
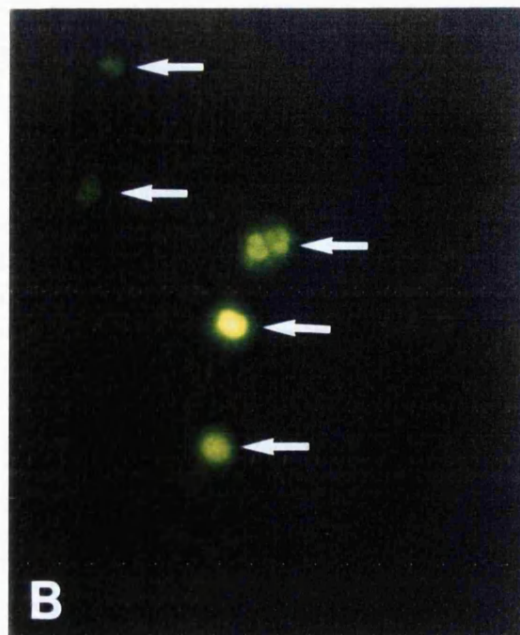
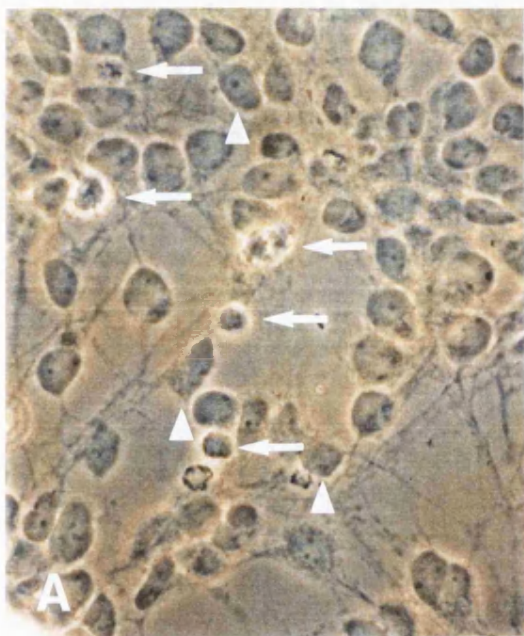


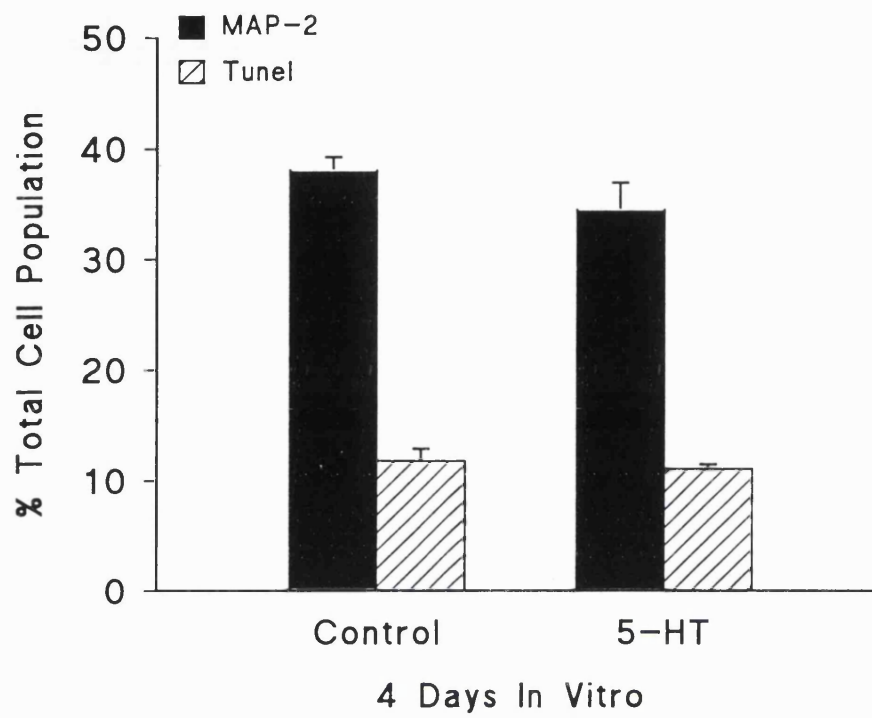
Figure 19: Graphs showing neuronal survival and cell death in 5-HT-treated cultures

Cortical cells (E16) were grown for 4 DIV (A), or 7 DIV (B), in control or 5-HT-treated medium. Cultures were fixed and immunostained for neuronal and dying cell types.

(A) The effect of 5-HT on the number of MAP-2 and TUNEL positive cells at 4 DIV. The percentage of labelled cells was analyzed in n=5-6 coverslips from 2 separate experiments.

(B) The effect of 5-HT on the number of MAP-2 and TUNEL positive cells at 7 DIV. The percentage of labelled cells was analyzed in n=5-6 coverslips from 2 separate experiments. All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls.

A



B

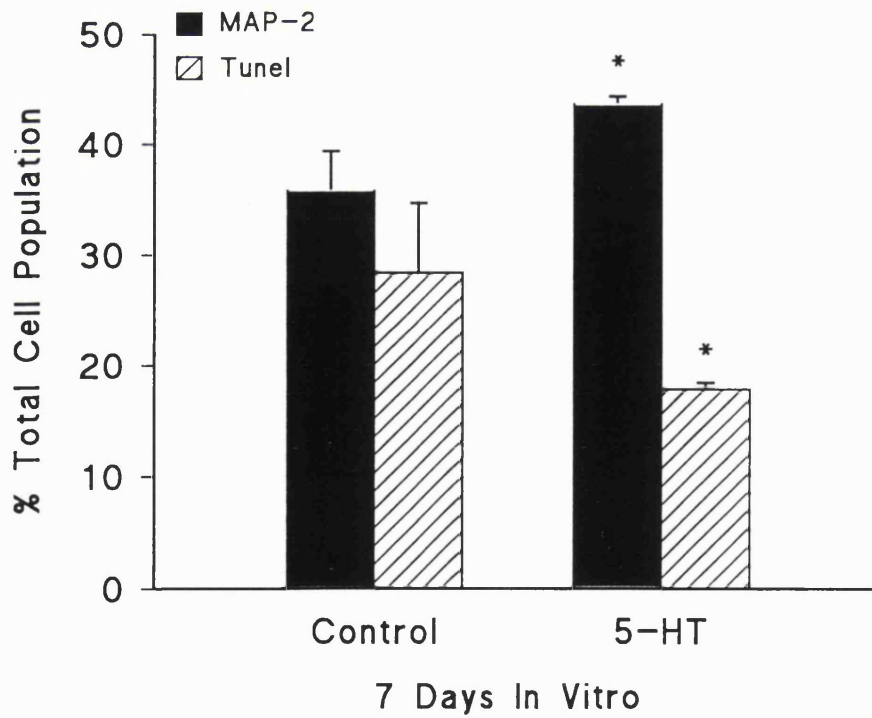


Figure 20: Photomicrographs showing E16 cortical cell types at 7 DIV

Cortical cells were cultured for 7 DIV in 5-HT-treated medium. Cells were labelled for

(A) MAP-2, (B) BrdU, (C) GLU, and (D) GABA. Scale bar = 20 μ M.

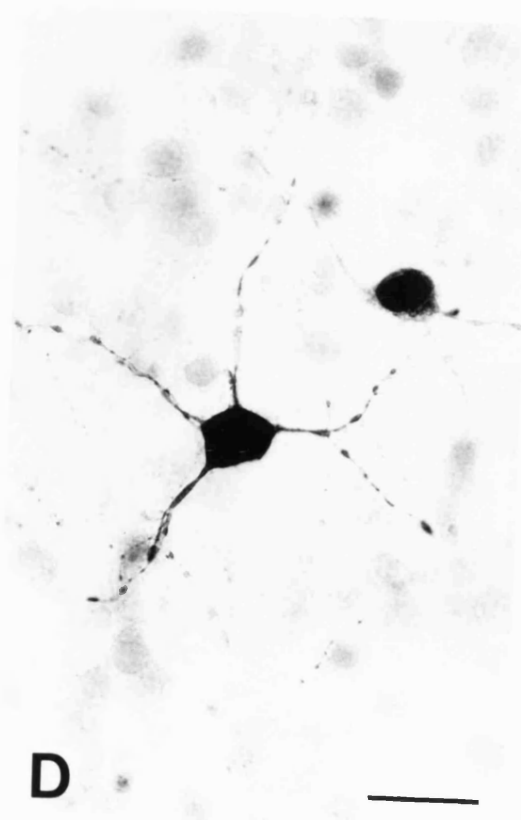
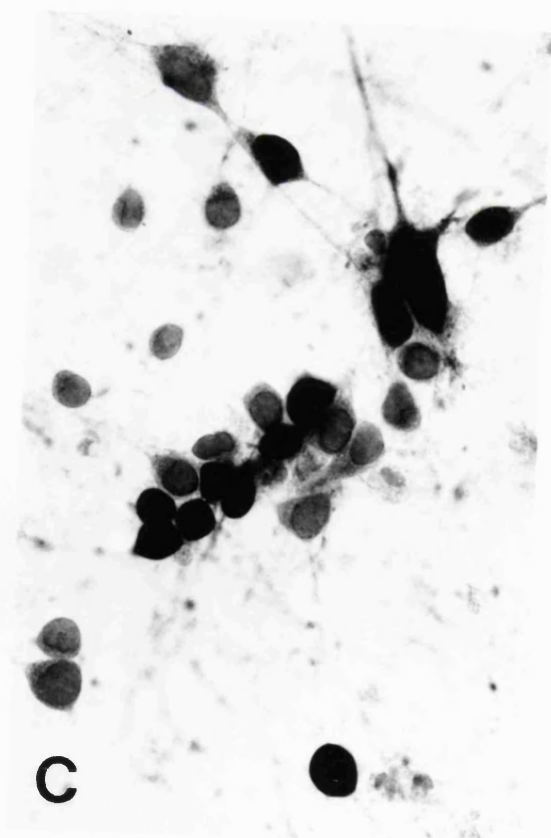
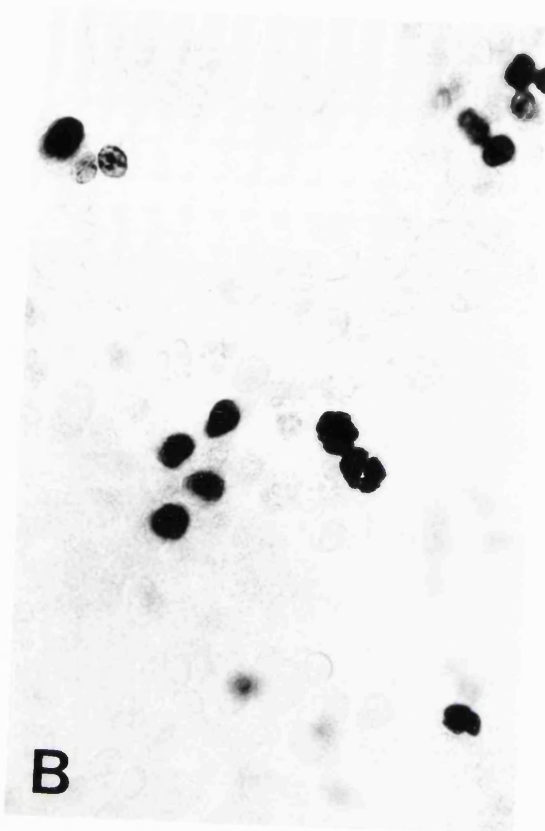
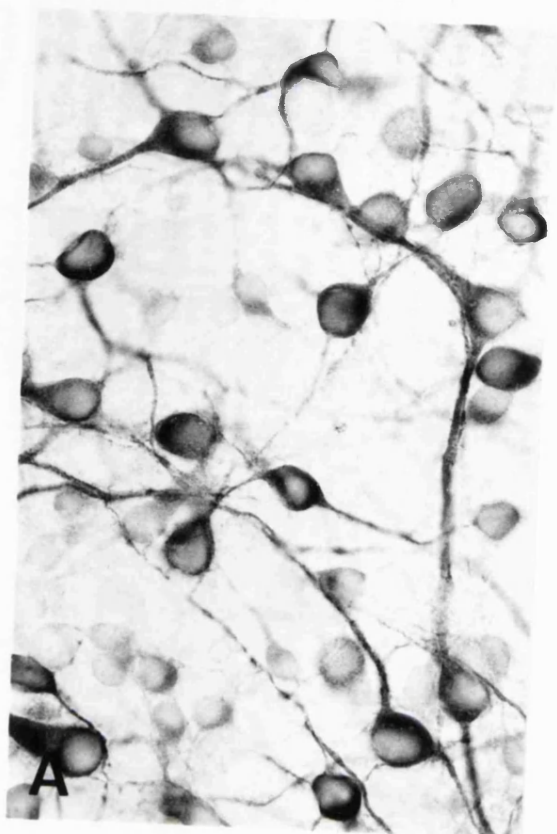


Table 8. Effect of 5-HT on the survival of E16 cortical cells *in vitro*

Markers	% Surviving Cells at 7 days (\pm SEM)	
	Control	5-HT
MAP-2	34.5 \pm 3.6	43.0 \pm 2.9*
GLU	22.6 \pm 1.9	31.4 \pm 3.3*
GABA	1.6 \pm 0.2	1.8 \pm 0.3
GFAP	0.9 \pm 0.1	1.3 \pm 0.3
BrdU	23.8 \pm 0.4	23.9 \pm 1.4
Nestin	20.7 \pm 4.9	21.2 \pm 1.5

E16 cortical cells were cultured in 24-well plates on glass coverslips in 1% HI-FCS control medium or additional 5-HT (25 μ M) for 7 DIV. Cultures were immunostained for a variety of cell-specific markers as indicated above and the percentage of surviving cells assessed. Data is derived from 5-6 coverslips in 2 separate experiments and expressed as mean (\pm SEM). $P < 0.05$ was considered statistically significant compared to controls and is indicated by an asterisk.

Table 9. Effect of 5-HT on cell morphology of E16 cultures

Treatment	Cell Body Area (μm^2)	Number Of Neurites	Length Of Longest Neurite (μm)
Control	29.9 ± 0.8	2.5 ± 0.1	14.1 ± 1.1
5-HT	$33.5 \pm 0.6^*$	2.6 ± 0.1	13.7 ± 1.3

E16 cortical cells were cultured in 1% HI-FCS control medium or 5-HT (25 μM) for 7 DIV and immunolabelled for MAP-2. Isolated MAP-2 positive cells were drawn and analyzed using a computer assisted program (see section 6.3). Results are from 1 of 2 separate experiments yielding similar results. Data is expressed as means \pm SEM, and $P < 0.05$ was considered statistically significant compared to controls.

8.3 Mechanism of Serotonin Action on Cortical Cultures

8.3.1 5-HT_{2a} receptors mediate the effects of 5-HT

To investigate the mechanism through which 5-HT mediated its survival effect on cortical cells, E16 and E18 cultures (which showed greater survival responses) were exposed to a panel of 5-HT receptor agonists and antagonists at previously determined non-toxic concentrations of 5-25 μ M (data not shown) (Figures 21 and 22 respectively). The 5-HT_{2a/2c} agonist, α -methyl-5-HT (25 μ M), mimicked the effects of 5-HT in the promotion of cortical cell survival (Figures 14, 21A and 22A). This survival was also dose-dependent but, unlike 5-HT, showed no toxicity towards cortical cells at higher doses (Figure 14B). In contrast, an agonist for the 5-HT_{1a} receptor [8-hydroxy-2-(*n*-dipropylamino) tetralin; 8-OH-DPAT; 25 μ M] did not produce increased survival (Figures 21B and 22B) suggesting that the effect was mediated by the 5-HT_{2a} and 5-HT_{2c} receptor subtypes. However, it may be argued that the 5-HT_{2c} receptor, originally termed 5-HT_{1c} (Martin and Humphrey, 1994), is not involved in the observed effect of 5-HT since this receptor subtype is not expressed in the developing cerebral cortex (Hellendall et al., 1993).

In order to test the hypothesis that the observed response is mediated by the 5-HT_{2a} receptor, control cultures were incubated with its antagonist cinanserin (5 μ M) for 7 DIV. A decrease in survival of cortical cells was observed when cinanserin was added alone; when combined with 5-HT (25 μ M), it blocked the enhanced cell survival observed after addition of the indoleamine (Figures 21A and 22A). Furthermore, an antagonist of the 5-HT_{1a} receptor (Nan 190, 10 μ M) (Figures 21B and 22B), as well as the 5-HT₃ receptor agonist 1,3-chlorophenylbiguanide (25 μ M) and antagonist Y25130

(10 μ M), had no significant effect on cell survival after 7 DIV (Figures 21C and 22C).

These results point to the importance of the 5-HT_{2a} receptor in mediating this response.

**Figure 21: Graphs showing cortical cell survival in *E16* cultures
treated with serotonergic agonists and antagonists**

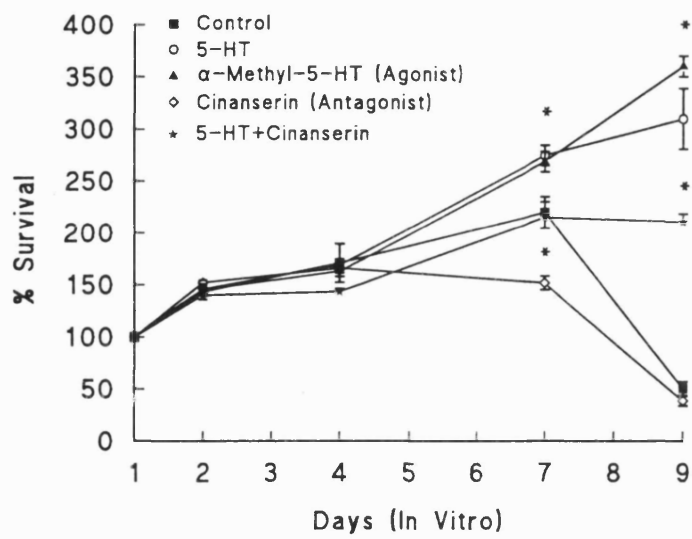
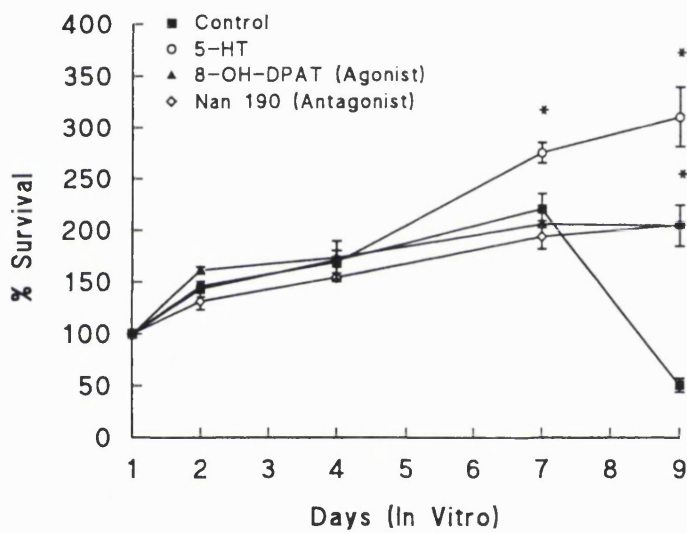
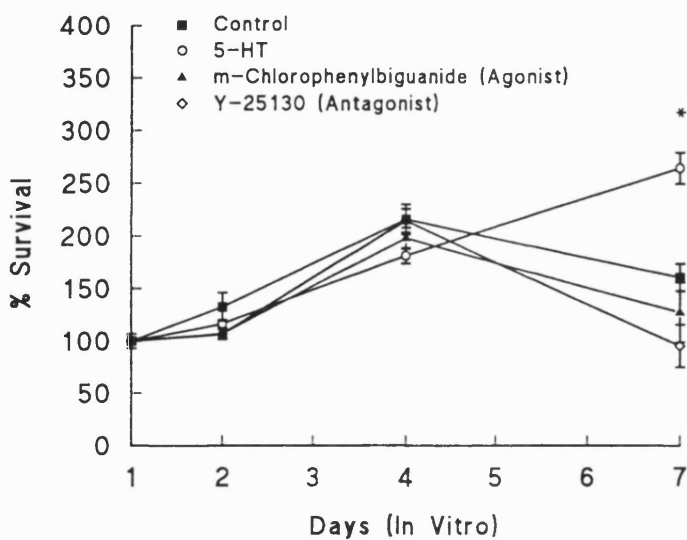
Cortical cultures were grown for 9 DIV in medium treated with (A) 5-HT_{2a/c} receptor agonists and antagonists, (B) 5-HT_{1a} receptor agonists and antagonists, or (C) 5-HT₃ receptor agonists and antagonists. The MTT assay was used to quantify survival at specific time points.

(A) The percentage of surviving cortical cells in cultures treated with 5-HT_{2a/c} receptor agonists and antagonists was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells at each time point per treatment.

(B) The percentage of surviving cortical cells in cultures treated with 5-HT_{1a} receptor agonists and antagonists was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells at each time point per treatment.

(C) The percentage of surviving cortical cells in cultures treated with 5-HT₃ receptor agonists and antagonists was assessed at 1, 2, 4, and 7 days of culture. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. P<0.05 was considered significant compared to controls and is indicated by an asterisk.

A**B****C**

**Figure 22: Graphs showing cortical cell survival in *E18* cultures
treated with serotonergic agonists and antagonists**

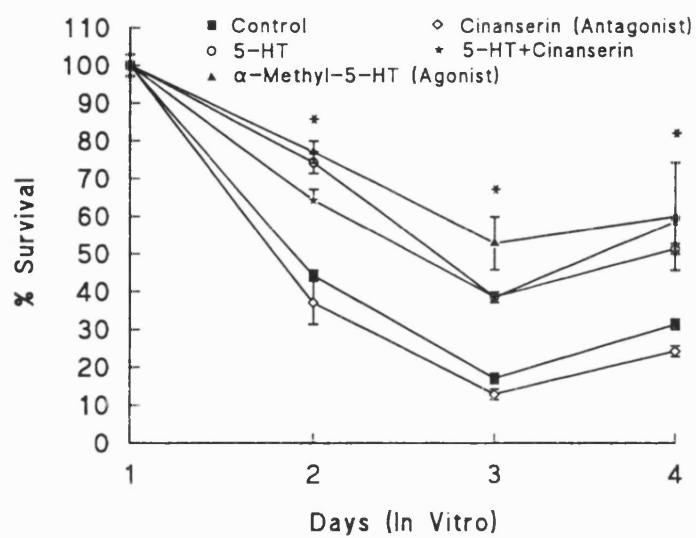
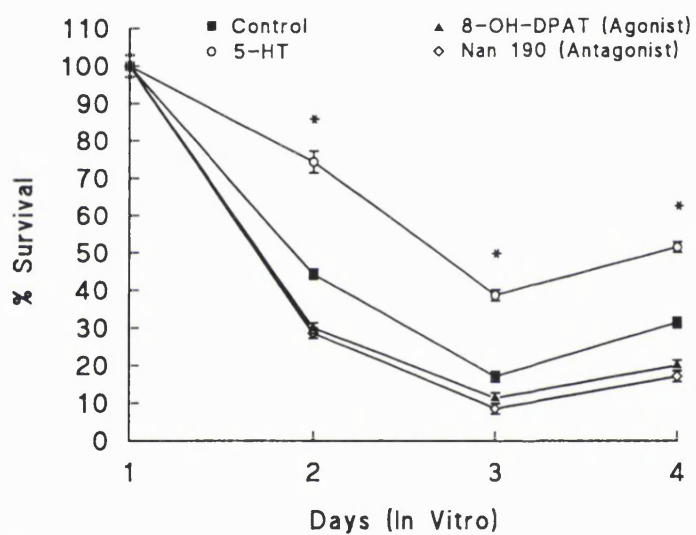
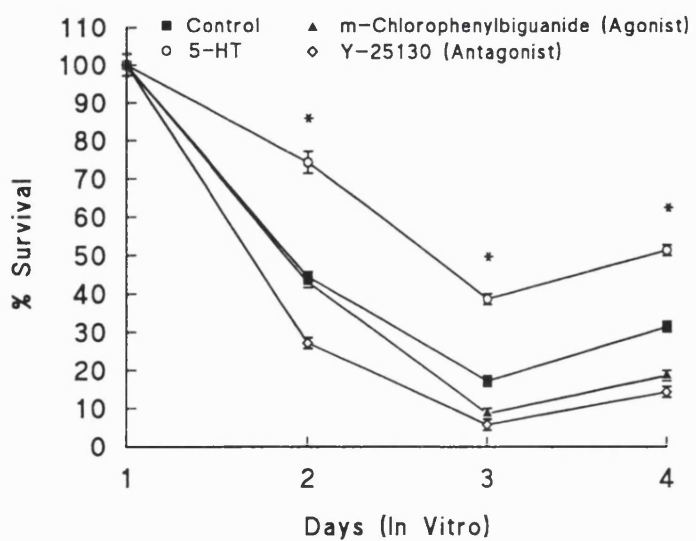
Cortical cultures were grown for 4 DIV in medium treated with (A) 5-HT_{2a/c} receptor agonists and antagonists, (B) 5-HT_{1a} receptor agonists and antagonists, or (C) 5-HT₃ receptor agonists and antagonists. The MTT assay was used to quantify survival at specific time points.

(A) The percentage of surviving cortical cells in cultures treated with 5-HT_{2a/c} receptor agonists and antagonists was assessed at 1, 2, 3, and 4 days. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

(B) The percentage of surviving cortical cells in cultures treated with 5-HT_{1a} receptor agonists and antagonists was assessed at 1, 2, 3, and 4 days. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

(C) The percentage of surviving cortical cells in cultures treated with 5-HT₃ receptor agonists and antagonists was assessed at 1, 2, 3, and 4 days. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. P<0.05 was considered significant compared to controls and is indicated by an asterisk.

A**B****C**

8.4 Discussion

8.4.1 *The effect of 5-HT on cell phenotype*

We addressed the question of which particular sub-populations of cells were surviving in our cultures compared to controls. The finding of increased numbers of neuronal as opposed to mitotic progenitor cells in 5-HT-treated cultures after 7 DIV indicated a primary trophic support of postmitotic neuronal cells. GFAP numbers were also increased in 5-HT-treated cultures, yet this increase was not significant. These results support the general finding of 5-HT action and enhancement of many aspects of neuronal differentiation (Gromova et al, 1983; Chubakov et al, 1986). Further results also were in agreement with these observations, with findings of higher numbers of neuronal cells in E18 cultures which displayed an immediate dependence on 5-HT as opposed to lower neuronal numbers in E14 cultures which exhibited stability and little need for 5-HT. Immunohistochemical analysis with cell-specific markers further revealed that the effect of 5-HT in our cultures was directed specifically to the GLU-containing neuronal population and not to any other cell types. The observed increase in glutamatergic neurons was not due to selective mitogenic action of 5-HT on the progenitors of these neurons as the levels of BrdU incorporation did not differ between the control and 5-HT-treated cultures. Such selective mitogenic action has been suggested by Vaccarino et al. (1995) to account for an increase in GLU-containing neurons following application of fibroblast growth factor in cortical cell cultures.

If cell proliferation is not affected by 5-HT and the proportions of other neuronal and glial cell types were not altered, what is the source of the increased GLU-containing neuronal population? There are two possibilities that may account for this increase: 1) selective survival of glutamatergic neurons and 2) survival and differentiation of newly

generated neurons. Consistent with the increased survival was the observation of reduced cell death in 5-HT-treated cultures compared to controls. While TUNEL histochemistry detected DNA fragmentation indicative of apoptosis, there was also the possibility that necrosis induced cell death in our cultures had occurred. Furthermore, we were unable to determine whether the reduction in cell death was specific to glutamatergic cells or whether it involved undifferentiated neurons that subsequently differentiated along the GLU pathway as suggested by our recent observations in organotypic slice cultures (Lavdas and Parnavelas, 1997). The hypothesis that 5-HT influences the differentiation of prospective target neurons during embryogenesis has been put forth by Lauder and Krebs (1978) in a study involving a number of brain areas. These authors proceeded to suggest that increased differentiation was the result of interaction between 5-HT axons and proliferating cells. However, our present results indicate that 5-HT does not act on dividing cells, but rather on postmitotic neurons. This data could be further consolidated by conclusively identifying all the cell populations contained in our cultures. Cell types identified using a variety of cell specific markers were collectively <100% in total, including relatively low numbers of GFAP and GABA stained cells. Low levels of antigenic expression by cell types or the localization of different antigens not detected by the markers used may account for this deficit. As such it remains possible that the unidentified cell types may be glial progenitors mediating an indirect influence on neuronal cell survival (Whitaker-Azmitia and Azmitia, 1989; Liu and Lauder, 1992).

8.4.2 5-HT increases cell body area

We next investigated the effect of 5-HT on morphological parameters such as cell body area, number of neurites and length of the longest neuritic process of MAP-2 labelled cells. 5-HT increased the cell body area, but not neurite length or number. This agrees with reports of 5-HT influence on cell body size of serotonergic neurons both in *in vitro* and *in vivo* studies (Zhou et al, 1987; Liu and Lauder, 1991). However, an inhibitory effect of 5-HT on neuritic length and number has been reported (Liu and Lauder, 1991). Within the invertebrate system of *Helisoma*, it has been shown that 5-HT can both induce inhibitory or stimulatory effects on neuritic embryonic elongation (Goldberg et al, 1991). These contradictory results are perhaps best explained by findings of invertebrate studies in which intracellular Ca^{2+} levels have been hypothesized to mediate the effect of 5-HT on neurite outgrowth in either a stimulatory or inhibitory manner (Goldberg and Kater, 1989). 5-HT has also been observed to cause increased neuritic branching which has not been investigated in this study (Budnik et al, 1989; Sikich et al, 1990).

8.4.3 5-HT_{2a} receptors mediate the survival effects of 5-HT

We sought to analyze the receptors that mediate the survival effects of 5-HT in cortical cell cultures. We found that the 5-HT_{2a,2c} receptor agonist α -methyl-5-HT promoted the survival of these cells in a similar manner to 5-HT; in contrast, the 5-HT_{1a} receptor agonist 8-OH-DPAT had no effect. 5-HT_{2a} receptors have been localized in the developing rat cerebral cortex at the time of neurogenesis (Roth et al, 1991; Hellendall et al, 1993) and in the adult (Pazos et al, 1985; Mengod et al, 1990a), and recent studies (Marek and Aghajanian, 1996) point to their involvement in the mediation of the excitatory effects of 5-HT on cortical pyramidal neurons. Furthermore, 5-HT_{2c} receptor

subtypes are lacking in this brain region during development and are present in the adult in very low density (Mengod et al, 1990b; Hellendall et al, 1993). This argues against the 5-HT_{2c} receptors participating in the survival effects of 5-HT.

Use of the 5-HT_{2a} antagonist cinanserin in control cultures or in combination with 5-HT appeared to inhibit cell survival. The inhibitory action shown by cinanserin-treated cultures on cell survival suggested that the concentration of 5-HT (nM) contained within serum (Argez et al., 1984) was also effective. We had previously determined, however, that our observed 5-HT increased cell survival was independent of the presence of serum. Various concentrations of 5-HT (ranging between nM-μM) have been used by investigators and as such may additionally account for the diversity of effects sometimes observed (Rosenberg et al. 1988; Sikich, et al., 1990; Yoder et al., 1996).

Application of Nan-190, a 5-HT_{1a} antagonist, produced no significant effect on cell viability. Furthermore, results with an agonist 1-(3-chlorophenyl) biguanide and antagonist Y-25130 of the 5-HT₃ receptor, expressed in the proliferative zones of the developing cortex from E14 (Johnson and Heinemann, 1995), also had no effect on cell survival in cortical cultures. Taken together, these findings strongly suggest that 5-HT, acting through the 5-HT_{2a} receptor, promotes the survival of cortical postmitotic neurons. One has to take into consideration, however, the difficulties of determining the receptor pharmacology of immature cells. While further specific and selective pharmacological agents should be used to conclusively identify the receptors involved, expression of the 5-HT_{2a} receptor mRNA could also be analyzed. Mediation of this survival effect through the 5-HT_{2a} receptor may point to subsequent involvement of the phosphoinositol intracellular signalling pathway in this response.

CHAPTER FIVE

SEROTONIN AND COMPARISON WITH OTHER NEUROTRANSMITTERS

9.1 Introduction

While 5-HT has been shown to regulate many aspects of cell development, both in the CNS and PNS, other neurotransmitters are also becoming important candidates. In primates, for example, neurotransmitter receptors have been shown to display *regional* and *specific* laminar binding properties during pre- and postnatal development (Lidow and Rakic, 1992, 1994). Recently, GABA, which is one of the first neurotransmitter substances to appear in the developing cortex (Van Eden et al., 1989; Parnavelas et al., 1992), has been found to express the GABA_A receptor particularly in the proliferative zones (Van Eden et al., 1989; Araki et al., 1992; Laurie et al., 1992; Ma and Barker, 1995). Our results (Antonopoulos et al., 1997) and others (Meier et al., 1991; Mhatre and Ticku, 1994; Liu et al., 1997) have indicated that GABA_A receptors mediate the trophic effects of GABA during corticogenesis.

Thus, we addressed the effects of other monoamines (i.e: NA and DA), on the survival of cortical cells *in vitro*. The action of GABA and ACh was also characterized. Additionally, we investigated the secondary messenger transduction systems activated by neurotransmitter substances and their receptors in this response. Major intracellular messengers in this process are adenylate cyclase, phospholipase C (PLC), and Ca²⁺ (see section 3), which have been shown to be involved in regulation of neuronal development (Lauder, 1993).

9.2 Noradrenaline and Dopamine Promote E16 Cortical Survival

In order to assess the effects of other neurotransmitters on cortical survival, we adopted similar environmental conditions as described previously (see section 7.3). E16 dissociated cortical cells were cultured for a period of 7 DIV, with or without a neurotransmitter substance. These cultures were then examined for survival using the MTT assay as indicated (see section 6.2). Cultures containing 25 μ M 5-HT promoted cell survival (Figure 23), as described and quantified in chapters 3 and 4. We found that NA (10 μ M) and DA (10 μ M) induced a significant increase in cell viability (Figure 23). GABA (10 μ M) and ACh (10 μ M) however, did not promote survival and appeared to cause a decrease in the number of metabolically active cells (Figure 23). When 5-HT (25 μ M) was added to GABA (10 μ M) or ACh (10 μ M) containing cultures (Figure 24B), a significant increase in survival was observed.

We then reasoned that if 5-HT, NA, and DA were acting on completely different populations, then *additional* increases in survival should be observed when these substances were to be given together. Combining 5-HT (25 μ M) with NA (10 μ M) or DA (10 μ M) in culture, produced no more significant increases in survival than 5-HT alone (Figure 24A). A similar result was observed when NA (10 μ M) was added to DA (10 μ M) in E16 cultures after 7 DIV (Figure 24A).

Figure 23: Graph showing cortical cell survival in cultures treated with various neurotransmitters

Cortical cultures (E16), were grown for 7 DIV in either control or media treated with one of the following neurotransmitter substances: 5-HT, NA, DA, ACh, or GABA. The effect on cortical survival was assessed by the MTT assay at 7 DIV. Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells at each time point per treatment. All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

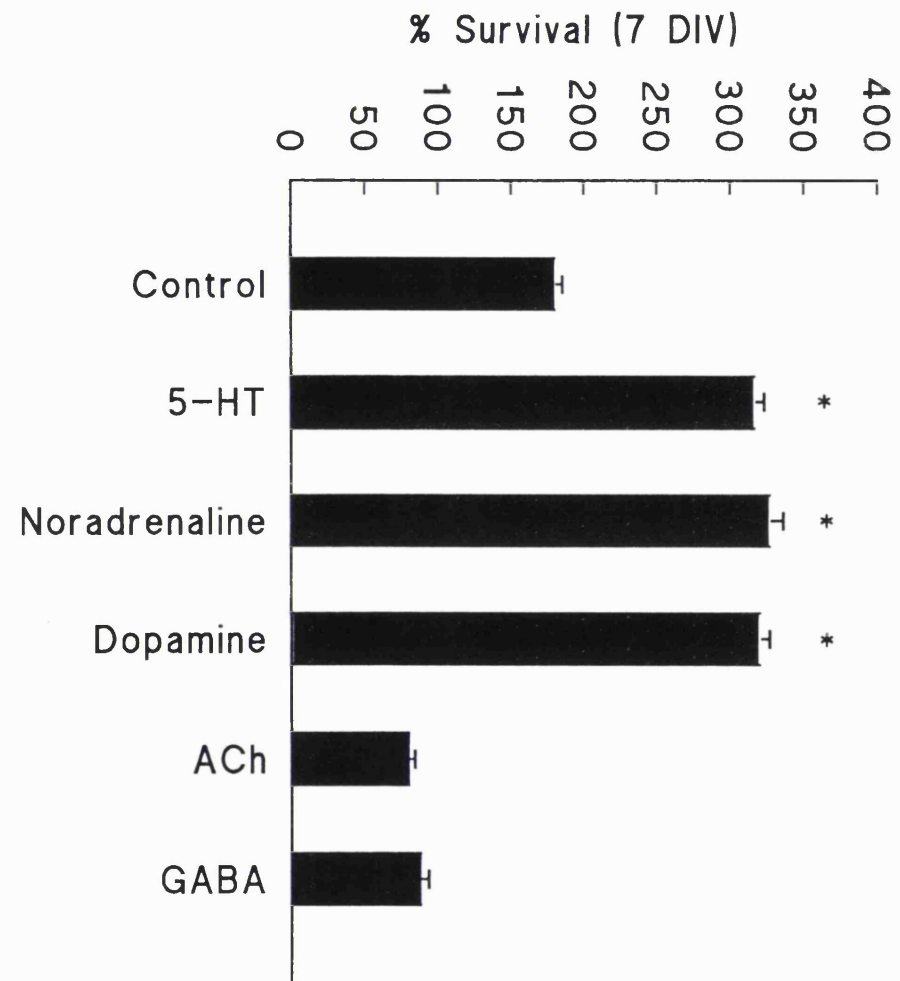
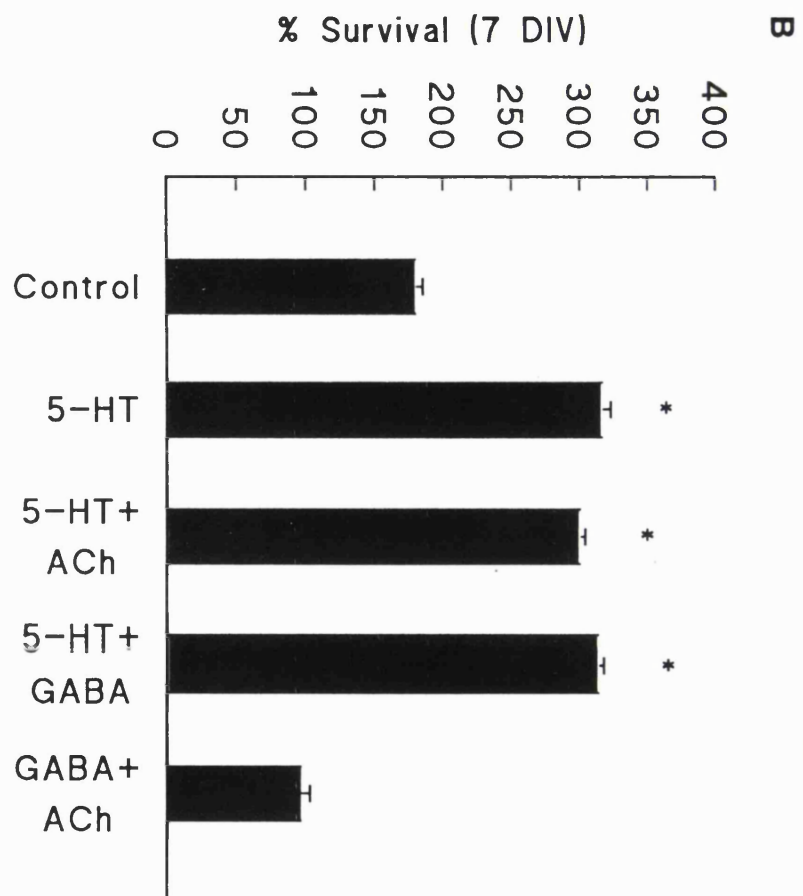
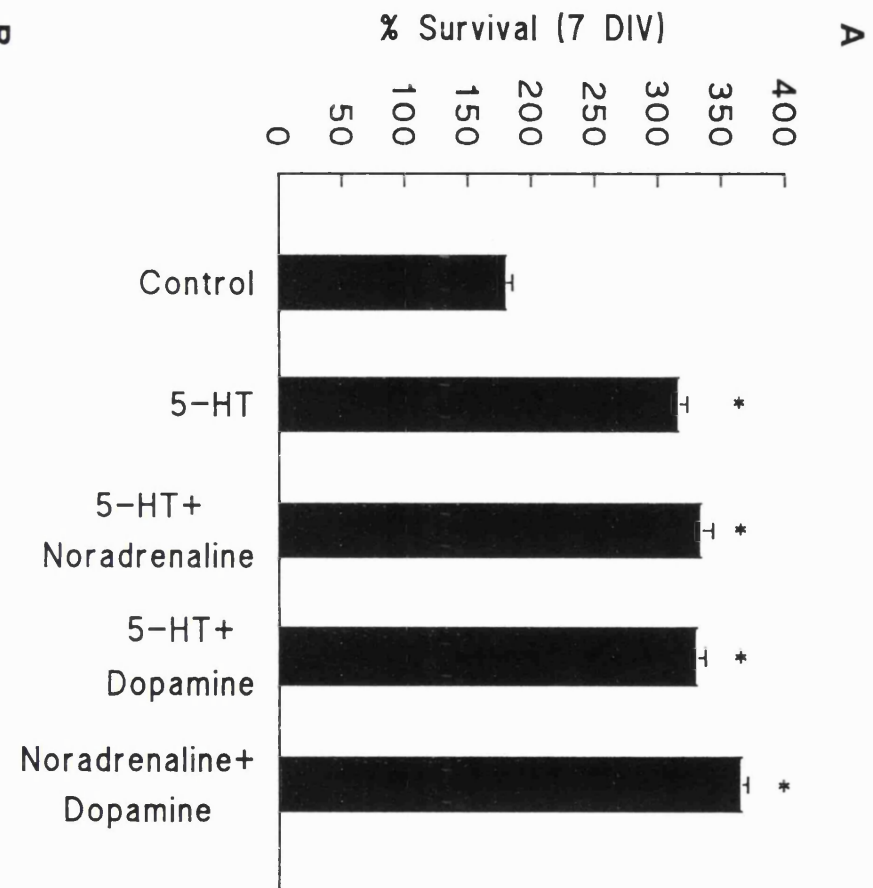


Figure 24: Graph showing cortical cell survival in cultures treated with various combinations of neurotransmitters (cont.d)

Cortical cultures (E16), were grown for 7 DIV in media treated with a *combination* of the following neurotransmitter substances: (A) 5-HT, NA, DA, or (B) 5-HT, ACh, and GABA. The effect on cortical survival was assessed by the MTT assay at 7 DIV. Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells at each time point per treatment. All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.



9.2.1 DA mediated survival is concentration dependent

After 4 or 7 DIV, E16 cultures were assessed for the effect on cell survival of different concentrations of either NA (0.1-500 μM), or DA (0.1-1000 μM), as previously described for 5-HT (see section 7.4.2). High doses of DA ($> 25 \mu\text{M}$), and NA ($>100 \mu\text{M}$) were found to be toxic after 7 DIV (Figure 25B). A similar result was apparent at 4 DIV (Figure 25A). Older cultures containing DA displayed a clear concentration dependent survival increase (Figure 25B).

The neurotransmitter substances GABA and ACh were also applied in a dose-dependent fashion and showed no regulation of survival (Figures 26A and B). After 7 DIV high doses (500 μM) and greater produced significant toxicity (Figure 26B).

Figure 25: Graphs showing cortical cell survival at different concentrations of noradrenaline and dopamine

E16 cultures were grown in control, NA, or DA containing medium for 4 DIV (A) or 7 DIV (B). Cell survival was determined by using the MTT assay.

(A) 4 DIV, showing the percentage of cell survival in NA (0.1-500 μ M), or DA (0.1-1000 μ M). Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells per time point per treatment.

(B) 7 DIV, showing the percentage of cell survival in NA (0.1-500 μ M), or DA (0.1-1000 μ M) at various concentrations. Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells per time point per treatment.

All results are expressed as mean \pm SEM.

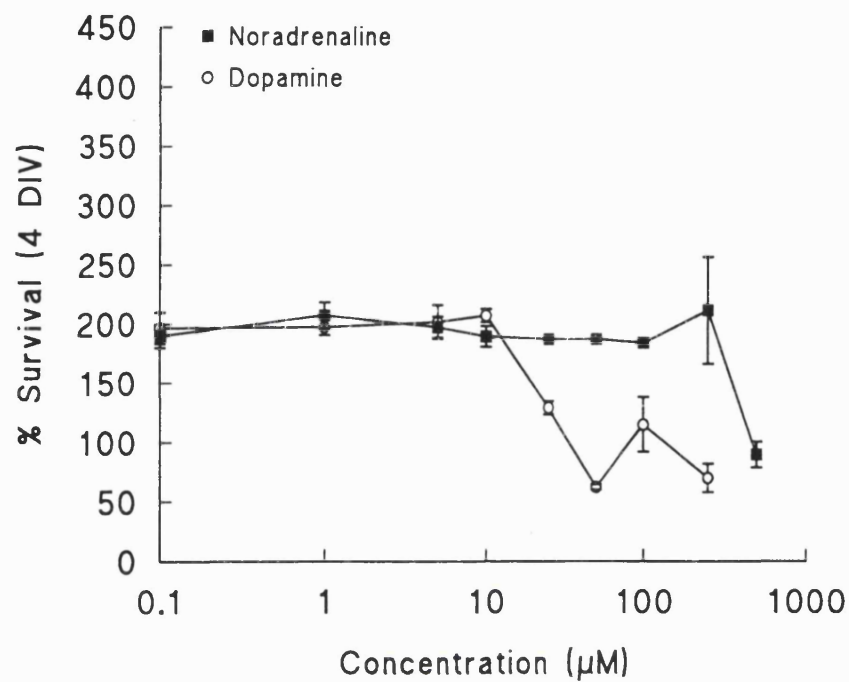
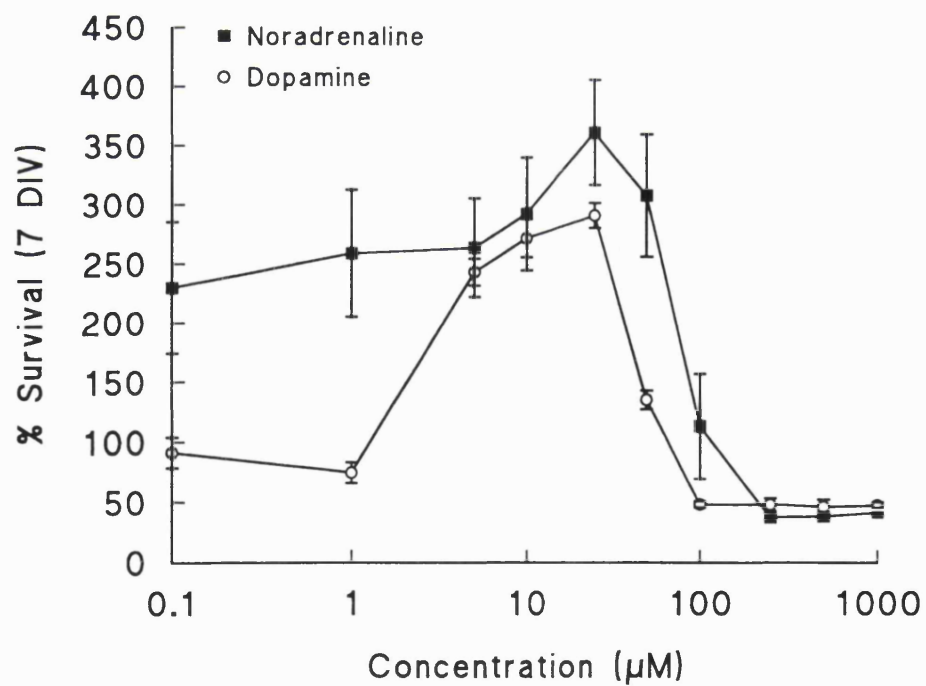
A**B**

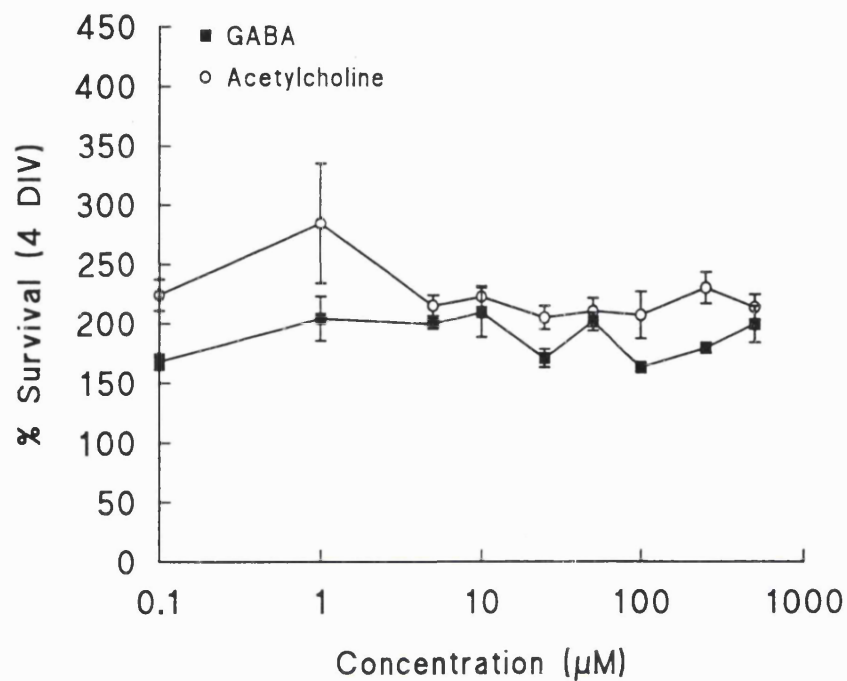
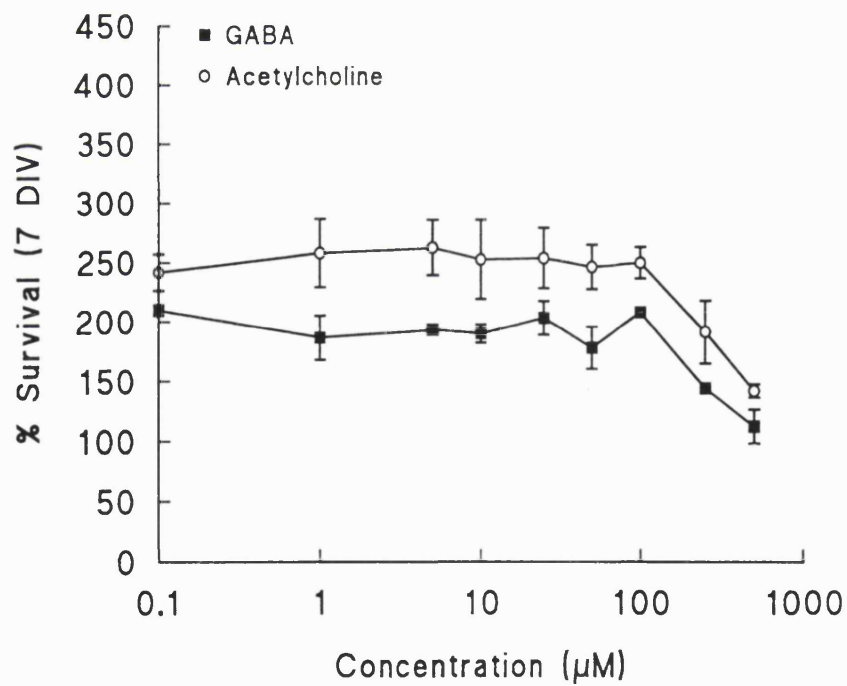
Figure 26: Graphs showing cortical cell survival at different concentrations of GABA and acetylcholine

E16 cultures were grown in control, GABA, or ACh containing medium for 4 DIV (A) or 7 DIV (B). Cell survival was determined by using the MTT assay.

(A) 4 DIV, showing the percentage cell survival in GABA (0.1-500 μ M), or ACh (0.1-1000 μ M). Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells per time point per treatment.

(B) 7 DIV, showing the percentage cell survival in GABA (0.1-500 μ M), or ACh (0.1-1000 μ M). Data is derived from 1 of 3 separate experiments yielding similar results. n=4 wells per time point per treatment.

All results are expressed as mean \pm SEM.

A**B**

9.3 Noradrenergic and Dopaminergic Increased Survival is Mediated by Specific Receptors

We next looked at the question of *how* the neurotransmitters NA and DA were exerting their survival effects on E16 cortical cultures. The dopaminergic receptors D₁, D₂ and D₃, have been reported to be present in the embryonic rat brain including the cerebral cortex (Guenoun and Bloch, 1992; Schlambra et al., 1994; Diaz et al., 1997). Specifically, the D₃ receptor was found to be associated with the proliferative zones from E16 (Diaz et al., 1997). While the D₁ receptor showed a similar early expression from E16, D₂ has been detected slightly later at E18 (Guenoun and Bloch, 1992; Schlambra et al., 1994; Diaz et al., 1997). We analyzed the effect of the dopaminergic D₁ receptor agonist dihydrexidine (10 μ M) and also the D₂ agonist bromocriptine (10 μ M) to assess whether they promoted a similar survival increase as that shown by DA. The D₁ agonist dihydrexidine was observed to mimic the survival effects of DA (Figure 27A), but the D₂ agonist bromocriptine appeared to stimulate a significant decrease in cell viability apparent at 7 DIV (Figure 27B).

The noradrenergic receptor subtypes α_1 -, α_2 -, β_1 -, and β_2 - (see section 4.1) have been shown to exhibit unique developmental profiles in the embryonic cortex (Lidow and Rakic, 1994). Within the adult, adrenergic receptors also show specific distributions in the CNS (Nicholas et al., 1996). Using a panel of receptor agonists to noradrenergic receptors, we found that the β_1 - agonist dobutamine (10 μ M) produced a similar survival increase to NA (10 μ M) (Figure 28B). The β_2 - agonist clenbuterol (10 μ M), and the α_2 - agonist UK 14,304 (10 μ M) (Figures 28A and B) had no significant effect on survival. The α_1 - agonist M-6434 (10 μ M) appeared to influence cell survival but

revealed inconsistent results both at 4 and 7 DIV (Figure 28A). Taken together, these results suggest that survival of embryonic cortical cells can be mediated by a number of neurotransmitter substances, specifically the monoamines 5-HT, NA and DA. These substances exert their effects through specific receptors, however it appears that common intracellular events *downstream* of these receptors may be responsible for the observed actions. Receptor distribution and availability, is also an important consideration. The predominant secondary mechanisms involved in the regulation of these effects are further considered in the following section.

**Figure 27: Graphs showing cortical cell survival in *E16* cultures
treated with dopaminergic agonists**

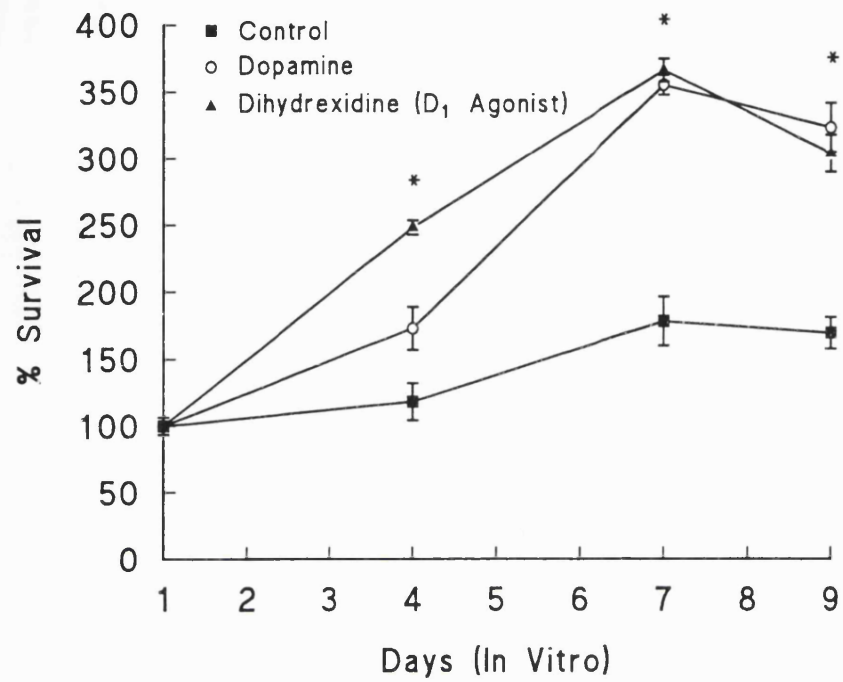
Cortical cultures were grown for 9 DIV in medium containing (A) D₁ receptor agonists, or (B) D₂ receptor agonists. The MTT assay was used to quantify survival at specific time points.

(A) The percentage of surviving cortical cells in cultures treated with D₁ receptor agonists was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

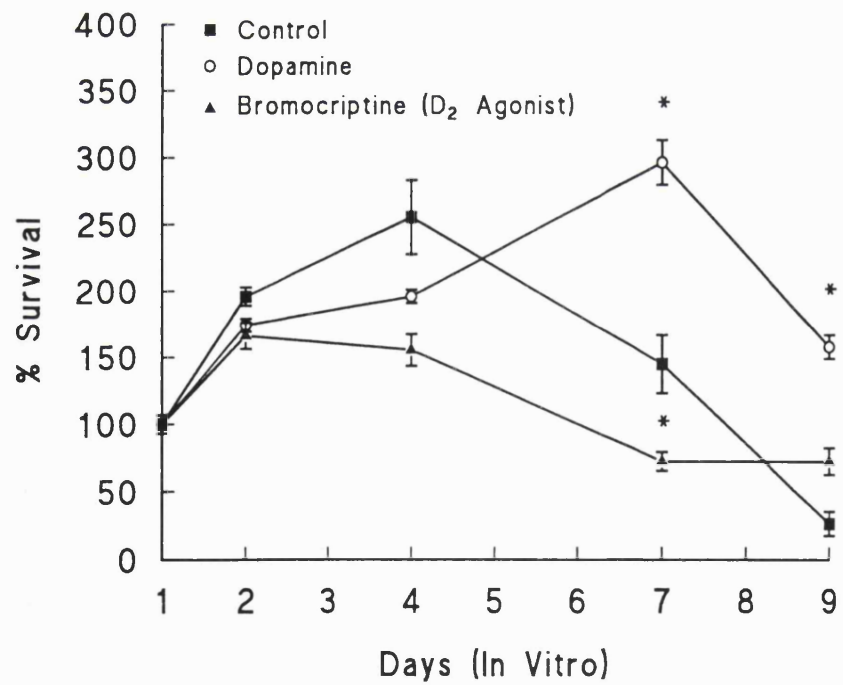
(B) The percentage of surviving cortical cells in cultures treated with D₂ receptor agonists was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. P<0.05 was considered significant compared to controls and is indicated by an asterisk.

A



B



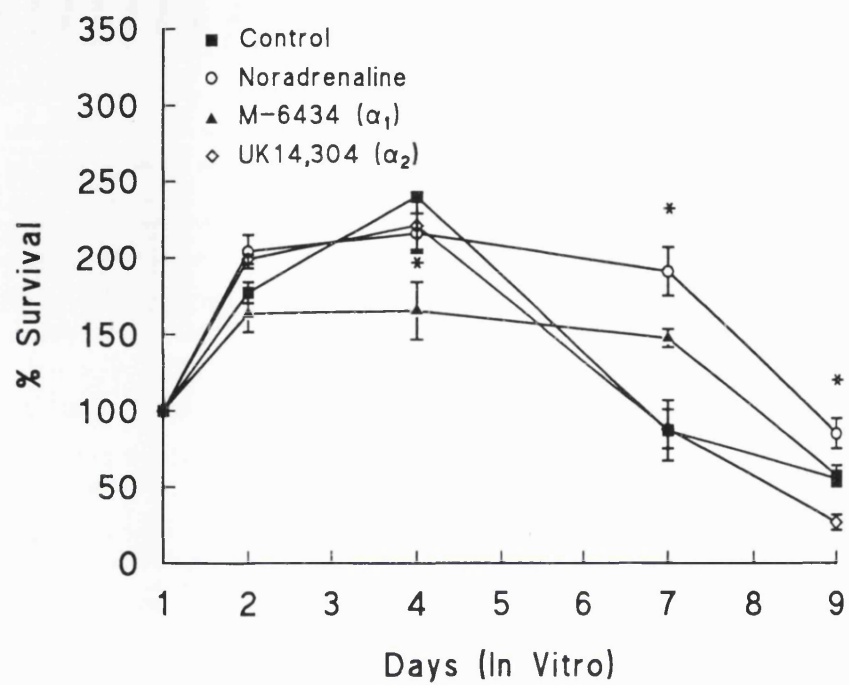
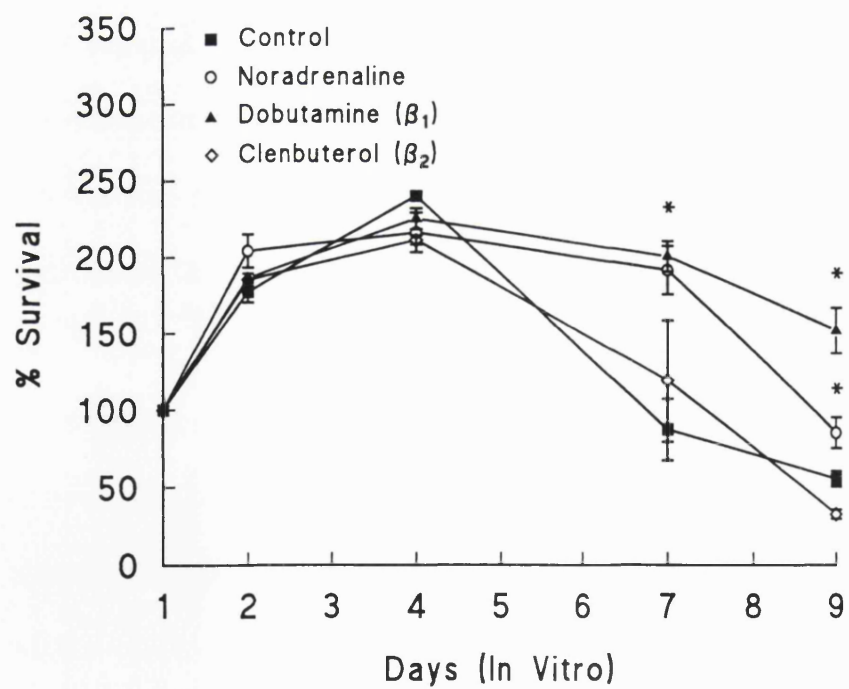
**Figure 28: Graphs showing cortical cell survival in *E16* cultures
treated with noradrenergic agonists**

Cortical cultures were grown for 9 DIV in medium containing (A) NA α - receptor agonists, or (B) NA β - receptor agonists. The MTT assay was used to quantify survival at specific time points.

(A) The percentage of surviving cortical cells in cultures treated with NA α - receptor agonists was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

(B) The percentage of surviving cortical cells in cultures treated with NA β - receptor agonists was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 of 2 separate experiments yielding similar results. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

A**B**

9.4 Intracellular Mechanisms Governing the Increased Survival of Cortical Cells by Serotonin, Noradrenaline, and Dopamine

Our results have so far shown that the survival of embryonic cortical cells can be prolonged by exposure to 5-HT_{2a/c} serotonergic, β_1 - noradrenergic, and D₁- dopaminergic receptor agonists. Each of these receptors induce different secondary messenger transduction systems (Lauder et al., 1993) (see tables 1, 2, and 3). Thus in our cultures, activation of the 5-HT_{2a/c} receptor would increase levels of PLC and Ca²⁺. Stimulation of the D₁- dopaminergic receptor however, would activate the adenylate cyclase pathway, while β_1 - noradrenergic receptors could influence adenylate cyclase, Ca²⁺ levels, as well as PLC if additionally the α_1 receptor was involved (Lauder, 1993).

We thus hypothesized that since these different receptors with differing secondary pathways could increase survival in our cultures, apparently acting on the same population of cells, then a *common* mechanism downstream was responsible for this effect. Other studies have also shown that establishing the exact pathways which mediate such processes, for example that of cell proliferation, is complex due to the participation of multiple mechanisms (Lauder, 1993).

Consistent with this hypothesis was the finding that application of forskolin alone (10 μ M), which increases adenylate cyclase levels, did not increase cell survival (Figure 29A). An inhibitor of the PLC pathway, U-73122 (0.4 μ M) also alone did not appear to influence survival (Figure 29B). Interesting results were observed, however, when forskolin (10 μ M) was added in combination with 5-HT (25 μ M), NA (10 μ M), or DA (10 μ M) respectively. Forskolin at 9 DIV, reduced the 5-HT and NA survival increase, while dopaminergic cultures remained unaffected (Figures 29A, 30A, and

31B). This appeared to be indicative of an imbalance in a common mechanism mediating survival.

A similar picture was obtained when the PLC inhibitor U-73122 (0.4 μ M) was applied in combination with 5-HT (25 μ M), NA (10 μ M), or DA (10 μ M). This time the survival increase by DA at 9 DIV was abolished (Figure 31B), while 5-HT and NA mechanisms were unaffected (Figures 29B and 30B). In summary, therefore, we suggested that regulation of *both* pathways, i.e: PLC and adenylyate cyclase could affect survival. Thus, a mechanism common to both pathways, such as the induction of immediate early genes, protein kinase activation, or threshold Ca^{2+} levels would be the critical factor in the regulation of cell survival.

Figure 29: Graphs showing cortical cell survival in *E16* cultures treated with 5-HT and intracellular messenger reagents

Cortical cultures were grown for 9 DIV in medium treated with (A) 5-HT and forskolin (an activator of adenylate cyclase), or (B) 5-HT and U-73122 (an inhibitor of PLC). The

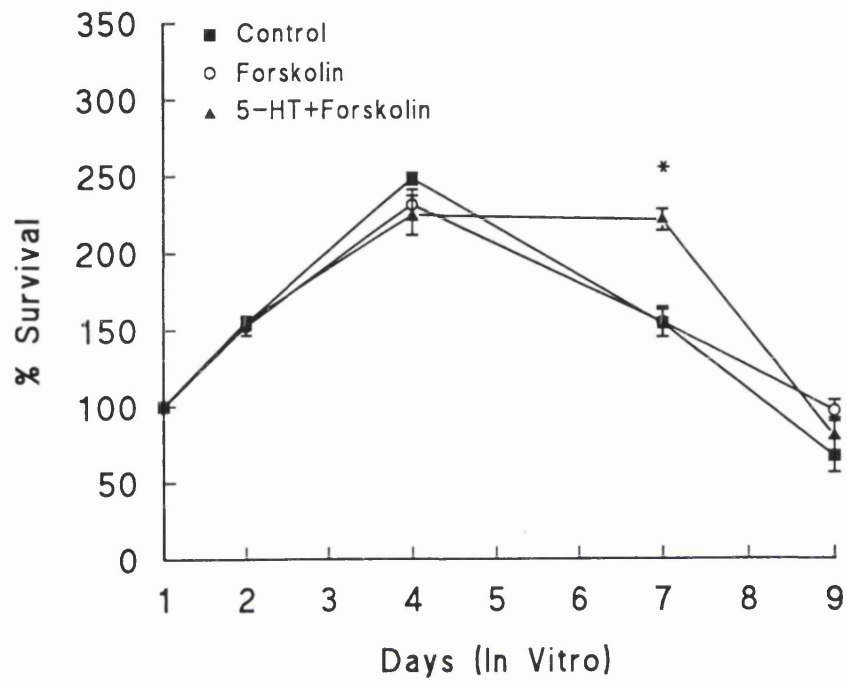
MTT assay was used to quantify survival at specific time points.

(A) The percentage of surviving cortical cells in cultures treated with 5-HT and forskolin was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 experiment. n=4 wells at each time point per treatment.

(B) The percentage of surviving cortical cells in cultures treated with 5-HT and U-73122 was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 experiment. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

A



B

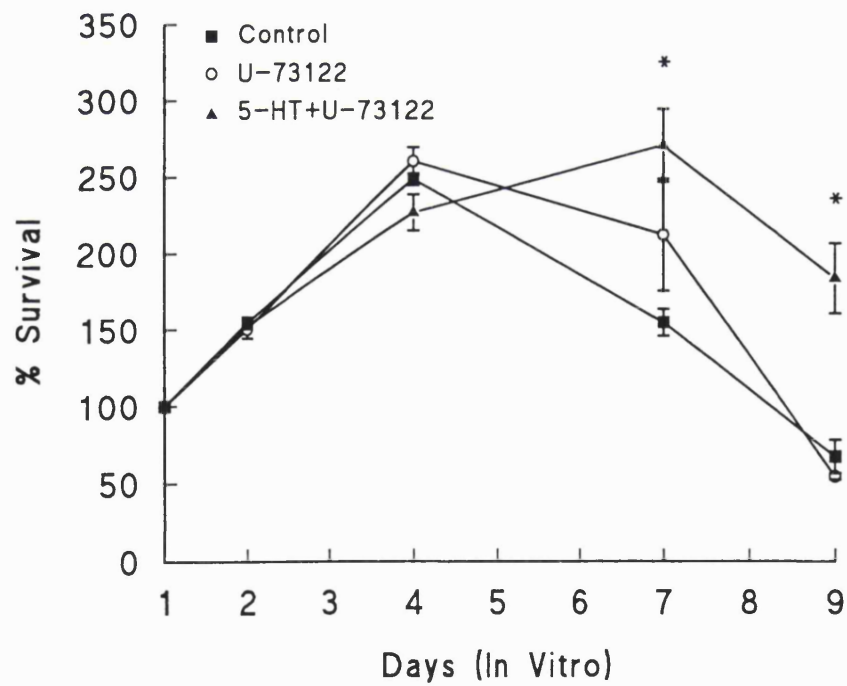


Figure 30: Graphs showing cortical cell survival in *E16* cultures treated with noradrenaline and intracellular messenger reagents

Cortical cultures were grown for 9 DIV in medium treated with (A) NA and forskolin (an activator of adenylate cyclase), or (B) NA and U-73122 (an inhibitor of PLC). The

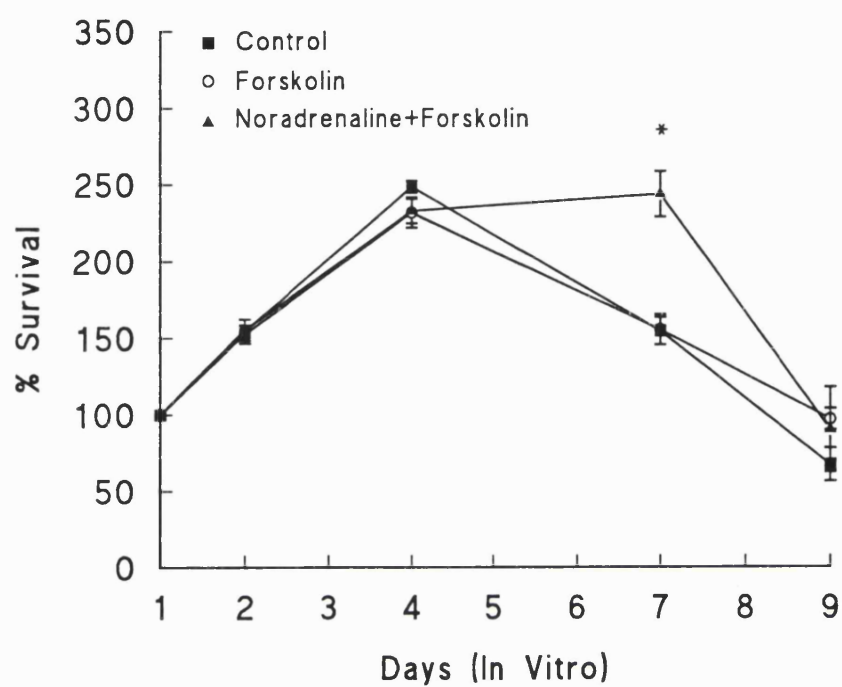
MTT assay was used to quantify survival at specific time points.

(A) The percentage of surviving cortical cells in cultures treated with NA and forskolin was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 experiment. n=4 wells at each time point per treatment.

(B) The percentage of surviving cortical cells in cultures treated with NA and U-73122 was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 experiment. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

A



B

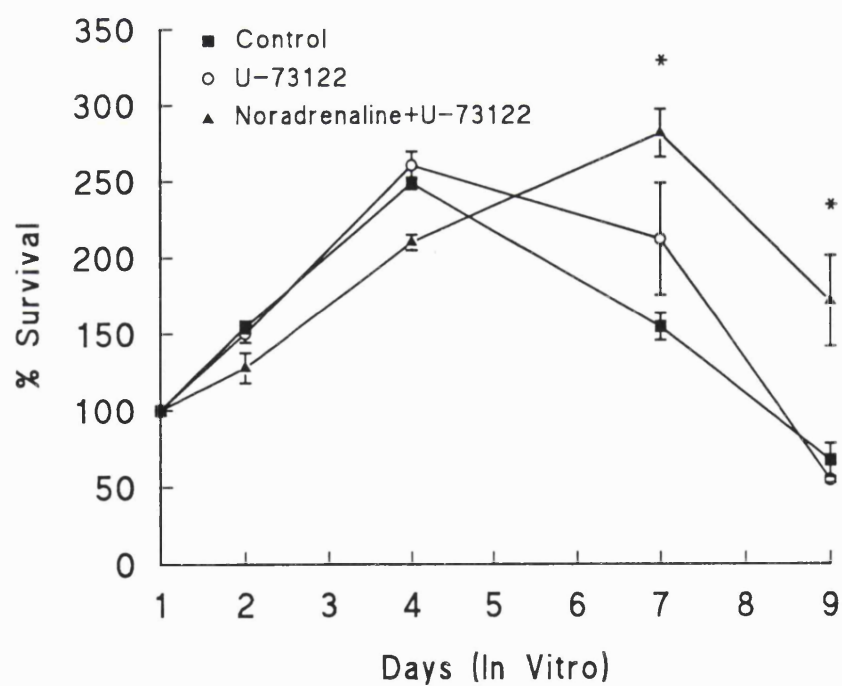


Figure 31: Graphs showing cortical cell survival in *E16* cultures treated with dopamine and secondary intracellular messenger reagents

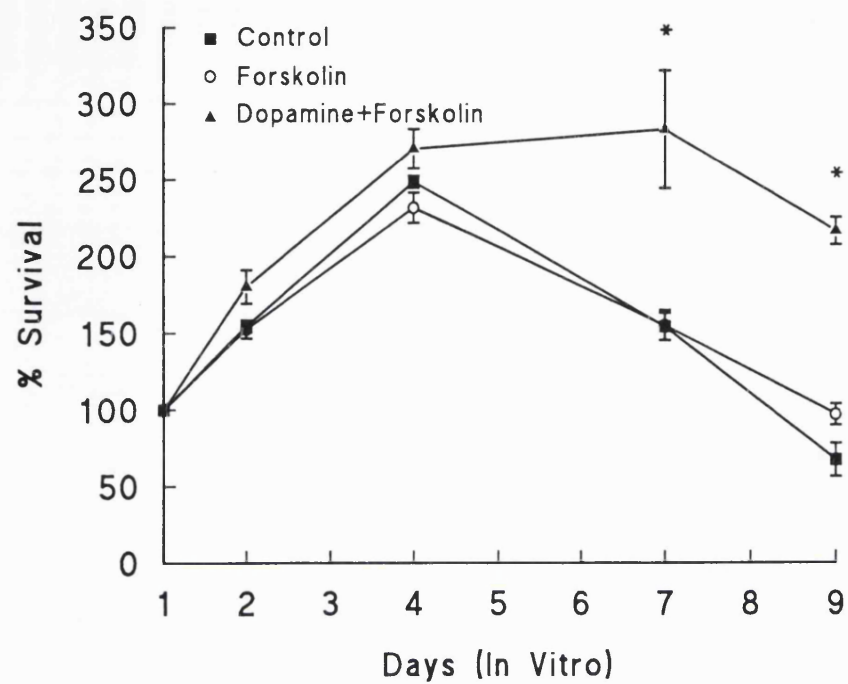
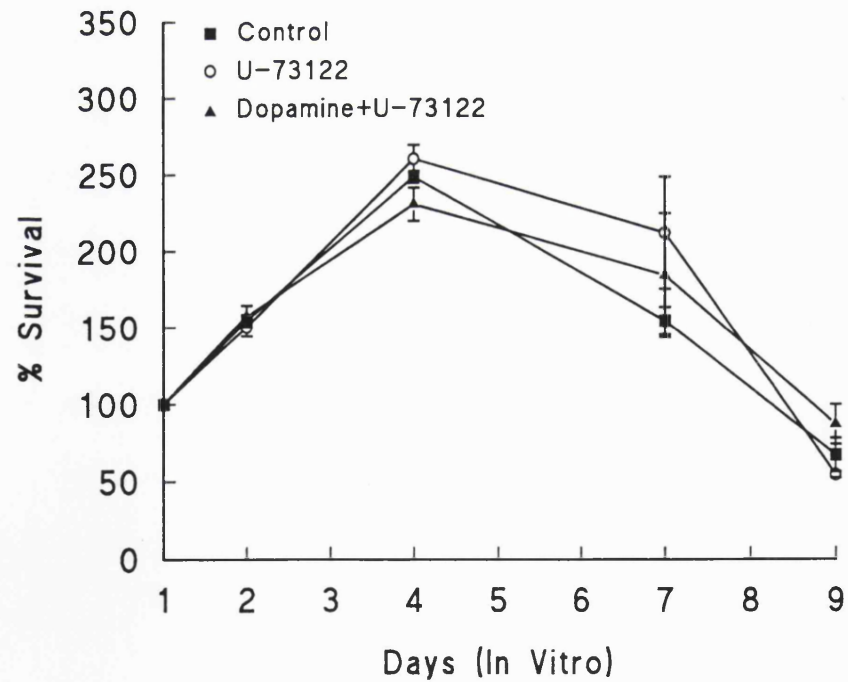
Cortical cultures were grown for 9 DIV in medium treated with (A) DA and forskolin (an activator of adenylate cyclase), or (B) DA and U-73122 (an inhibitor of PLC). The

MTT assay was used to quantify survival at specific time points.

(A) The percentage of surviving cortical cells in cultures treated with DA and forskolin was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 experiment. n=4 wells at each time point per treatment.

(B) The percentage of surviving cortical cells in cultures treated with DA and U-73122 was assessed at 1, 2, 4, 7, and 9 days. Data is derived from 1 experiment. n=4 wells at each time point per treatment.

All results are expressed as mean \pm SEM. $P < 0.05$ was considered significant compared to controls and is indicated by an asterisk.

A**B**

9.5 Discussion

9.5.1 NA and DA promote the survival of cortical cells

We presented data here which suggested that amongst the neurotransmitter substances, only the monoamines 5-HT, NA and DA increased cortical cell survival. This pointed to a specific function for these neuroactive substances during the embryonic period of cortical development. GABA is amongst the earliest of the substances to be present, while ACh shows a transient expression (reviewed in section 2.2), however, no effect was observed with these types of compound in the mediation of survival. In other studies, GABA has been shown to mediate neurite outgrowth (Redburn and Schousboe, 1987), cell proliferation (LoTurco et al., 1995; Antonopoulos et al., 1997), survival (Prasad and Barker, 1990; Meier et al., 1991), migration (Behar et al., 1996), and synaptogenesis (Belhage et al., 1997).

We also found that specific monoaminergic receptors appeared to mediate the observed survival action. For example, the D₁ dopaminergic receptor (expressed from E16 onwards), influenced cell viability whereas the D₂ dopaminergic receptor (which has a slightly later appearance at E18 in the cortical plate) did not. Actions mediated by the D₂ receptor such as neurite elongation and branching are reported at E18 (Todd, 1992). Such results suggest that survival is mediated by the *presence* and *density* of certain monoaminergic receptors. Consistent with this hypothesis are the reports of over-expression, regional or transient levels of neurotransmitter receptors during development and in the adult (Lidow et al., 1991; Whitaker-Azmitia, 1991). Actions regulated by β -adrenergic receptors have involved affecting morphological and proliferative cell parameters (Shain et al., 1987; Duncan et al., 1990; Kwon et al., 1996). Interestingly, NA has been postulated to play a neurotrophic role in the development of

the postnatal cerebral cortex, however the receptors activated were not identified (Felten et al., 1982).

9.5.2 Intracellular mechanisms involved in the survival of embryonic cortical cells

The receptors which we identified as playing important roles in mediating the survival of cells in our cultures activate various secondary pathways. As such we hypothesized that mechanisms common to both these pathways may be responsible for the observed survival responses. Such mechanisms have been proposed by other investigators where the activity of one neurotransmitter interacts with that of another (Huidobro-Toro, et al., 1996; Pedarzani and Storm, 1996; Haak et al., 1997; Obrietan and van den Pol, 1997).

We propose two possible areas of regulation downstream, namely, 1) activation of gene expression, and or 2) threshold Ca^{2+} levels. The former form of modulation by neurotransmitters is well documented (Esterle and Sanders-Bush, 1991; Hughes and Dragunow, 1995), with activation of neurotransmitter receptors such as 5-HT_{2a/c} causing localized expression of the immediate early gene c-fos in the rat cortex (Leslie et al., 1993). The 5-HT_{2a} receptor, has also been shown to increase levels of BDNF in the cortex (Vaidya et al., 1997), while the mRNA for the 5-HT_{2a} receptor can be regulated by dopaminergic agonists in striatal neurons (Laprade et al., 1996). Further control of neurotrophin mRNA levels by neurotransmitters, have been implied (Lindholm et al., 1994; Hutter et al., 1996). Our second postulation, the regulation of Ca^{2+} levels has been indicated by previous studies (Mattson et al., 1988b; Lauder et al., 1993; Surmeier et al., 1995; Watson et al., 1995). Further mechanisms which may be involved using excitatory neurotransmitters such as GLU acting in combination with growth factors have been indicated in the control of embryonic retinal survival (Meyer-Franke et al.,

1995) and cerebellar cultures (Cohen-Cory et al., 1991). Taken together, our preliminary intracellular studies favour regulation of a common element in the secondary transduction pathways activated by the 5-HT_{2a/c}, β_1 -, and D₁- receptors, however, an exact mechanism has not been established.

CHAPTER SIX

CONCLUSIONS

10.1 Neuroactive Substances Regulate Cortical Development

The environmental factors that regulate cortical cell development appear to exert multiple actions upon a particular cell phenotype. This seems to be in contrast to the rather more simple, single actions initiated within the PNS. For example, studies have shown that bFGF, BDNF, and NGF can regulate proliferation, survival, and differentiation at various stages of cortical development (reviewed in section 2.1 and Figure 2). Thus, stem cells, restricted progenitor cells for the neuronal or glia lineage or postmitotic neurons each depend at a certain time point on one or more of the above directive cues.

Recent studies have indicated that, in the cortex, uncommitted neuroepithelial cells respond to variations in the concentration of bFGF. High threshold levels of bFGF (also known as FGF2), induce oligodendroglial cell production, while low concentrations allow a “default” neuronal pathway (Qian et al., 1997). Additional factors appear to be needed to induce a glial commitment (Gross et al, 1996). Further investigations point to the required involvement of the growth factor PDGF in initiating neuronal differentiation (Williams et al., 1997). While these investigations have improved our picture of the requirements of early, cortical stem cells, other reports have suggested certain proteolipid proteins in regulating the later events of oligodendroglial survival and differentiation (Yang and Skoff, 1997), whereas endogenous BDNF and NGF jointly influence cortical dendritic growth (McAllister et al., 1997).

10.2 The Role of Serotonin and Other Neurotransmitters in Cortical Development

The investigations which we carried out in the embryonic cortex *in vitro*, have sought to increase our understanding of the actions of neurotransmitter substances and their receptors in various stages of cortical development. The main finding of this project was that the monoamine, 5-HT, increases cell viability at a defined point during corticogenesis. These effects were mediated by a specific 5-HT receptor. Specifically, stimulation of the 5-HT_{2a/c} receptor, in contrast to 5-HT_{1a}, or 5-HT₃, mimicked the action of 5-HT. The application of antagonists also provided results consistent with the observed effects. We also found that a certain population of cells within our cultures were surviving within these conditions. Taken together, our results suggest that 5-HT promotes the survival of differentiated neuronal cells, specifically, GLU expressing pyramidal neurons.

Within the neocortex it has been shown that *different* 5-HT receptors appear to be expressed by certain cell phenotypes. 5-HT₃ for example, is present in different populations of GABAergic neurons (Morales et al., 1996, 1997) along with the 5-HT_{2a/c} receptors (Morilak et al., 1993). The 5-HT_{2a} receptor is also specifically expressed by *layer V pyramidal neurons* in many areas of the rat neocortex (Hamada et al., 1996; Marek and Aghajanian, 1996). These cells, through stimulation of the 5-HT_{2a} receptor, increase BDNF mRNA (Vaidya et al., 1997). Evidence for an interesting model has subsequently been provided, whereby activation of the 5-HT_{2a} receptor mediates the release of GLU which in turn promotes excitability and BDNF production in neocortical pyramidal neurons (Figure 32A) (Vaidya et al., 1997). BDNF might then influence the

survival and development of cortical neurons. A similar mechanism has been postulated for dentate gyrus cells via the 5-HT_{2a} receptor (Figure 32B). Such models are also in agreement with our hypothesis that regulation of mRNA levels, such as those of the neurotrophins, by neurotransmitters may be responsible for the observed survival effects. We also showed that other monoaminergic neurotransmitters, such as NA and DA promoted survival. While we reproduced these actions with agonists and identified receptor subtypes that mediated these responses, further *consolidation* of these particular findings could be provided by applying the appropriate antagonists. Interestingly, DA acting through D₁- receptors on layer V-VI pyramidal neurons in the prefrontal cortex, has been reported to regulate their excitability (McCormick et al., 1993; Yang and Seamans, 1996). Additionally, the activity-dependent *survival* of cortical neurons has been shown to have a requirement for BDNF (Ghosh et al., 1994). Such evidence leads us to suggest that specific neurotransmitters regulate the survival of developing cortical neurons through activity-dependent mechanisms requiring BDNF production. Activity-dependent mechanisms have been shown to play roles in the survival, and differentiation of cells specifically regulating morphological traits (Gallo et al., 1987; McAllister et al., 1996; Marty et al., 1997).

Finally, the result that 5-HT promotes the survival of glutamatergic neurons, as opposed to dividing progenitor cells, appeared to propose the picture that as cells became further differentiated, they expressed certain receptor subtypes influential in the mediation of survival. Within the PNS, collective evidence has further strengthened this proposal with postmitotic Schwann cells displaying 5-HT responses via 5-HT_{2a} receptors in contrast to non-dividing cells labelled with BrdU (Yoder et al., 1997).

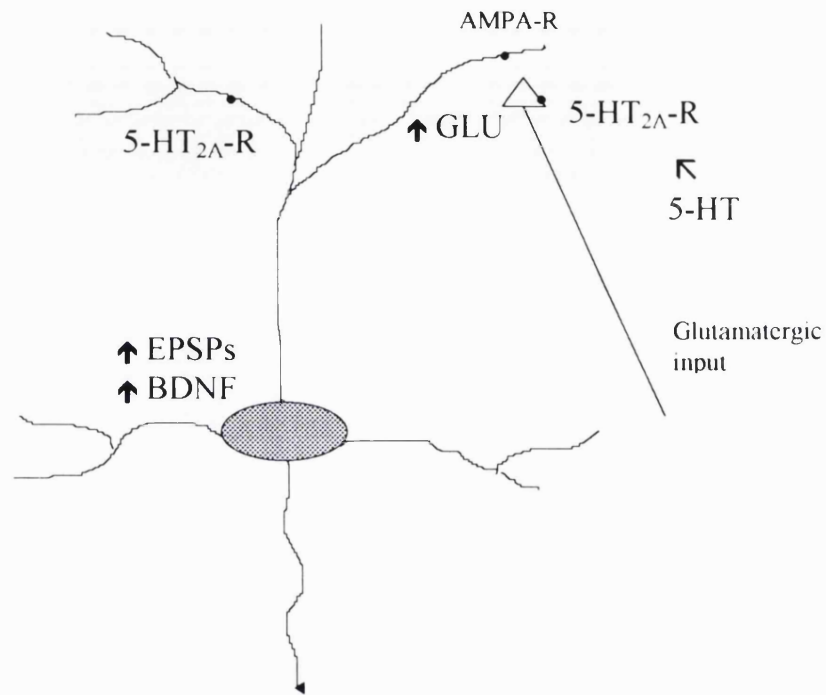
In conclusion, the actions of neurotransmitter substances should be considered to include a *broad* spectrum of effects that range from the trophic actions reviewed above, to the classic transmitter actions of mediating intercellular communication. 5-HT receptor subtypes, for example, are proposed to mediate many of the psychotropic effects in man such as depression, as well as an involvement in learning and memory processes (Elliott et al., 1994). While our present study focused on the survival promoting effects of 5-HT during development, the involvement of serotonergic systems in neurodegenerative disorders is shown by post-mortem neurochemical studies (Cross, 1988). Specifically, a degeneration of serotonergic neurones occurs in both Parkinson's disease and Alzheimer type dementia with a selective reduction of 5-HT receptors (5-HT₁ and 5-HT₂ respectively) in areas of terminal degeneration (Cross, 1988). The use of new selective serotonergic drugs during clinical studies should increase our understanding of the role of 5-HT during these processes.

Figure 32: Cellular Models for the Serotonergic Regulation of Neurotrophin Gene Expression

In the neocortex (A), 5-HT has been shown to initiate electrical activity and *increase* upregulation of mRNA for *BDNF*, in layer V pyramidal neurons through 5-HT_{2A} *receptors*. The increased electrical activity (EPSPs) is a result of increased GLU and as such indicates that 5-HT regulation of BDNF levels is mediated by neuronal activity. A similar scenario for the regulation of gene expression has been proposed in granule cells of the hippocampus (B). Here, 5-HT decreases BDNF mRNA. It is suggested that 5-HT_{2A} receptors located on GABAergic interneurons decrease electrical activity (IPSPs) and cause general inhibition of cells. This may thus cause the observed neurotrophin decrease (Adapted after Vaidya et al., 1997).

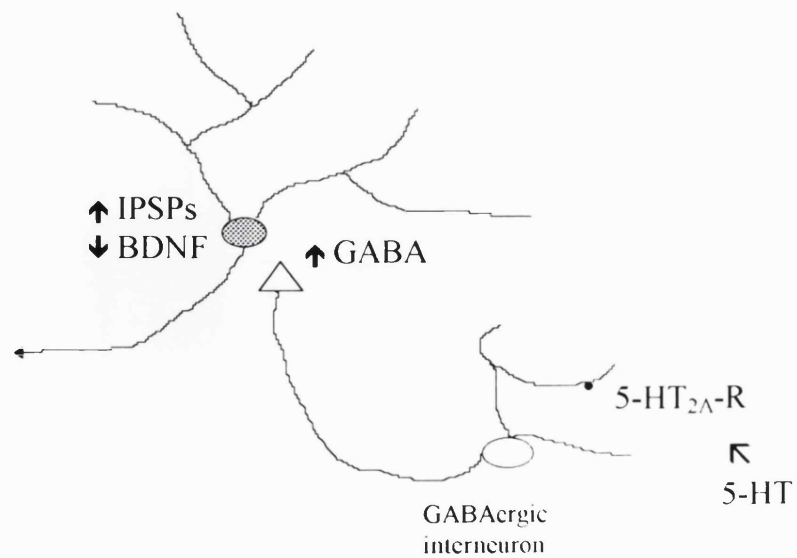
A

Neocortical Pyramidal Cell



B

Dentate Gyrus Granule Cell



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