

**THE ROLE OF MONOAMINES IN THE DEVELOPMENT OF
RAT CORTICAL NEURONAL TYPES: AN IN VITRO STUDY**

By

ALEXANDROS A. LAVDAS

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**Department of Anatomy and Developmental Biology
University College London**

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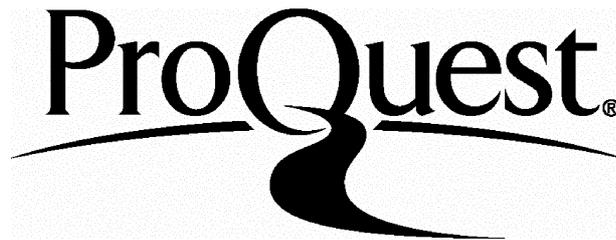
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PUBLICATIONS OF A.A. LAVDAS

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ABSTRACT

The monoamines serotonin (5-HT), noradrenaline (NE) and dopamine (DA), present in the developing brain long before they assume their neurotransmitter functions, are regarded as strong candidates for a role in the maturation of the cerebral cortex. Acetylcholine, although it appears in the cortex at more advanced developmental stages, has also been considered as a candidate. This study sought to investigate the effects of these neurotransmitters on the generation and differentiation of cortical cell types. Slice cultures, prepared from the cortices of embryonic day (E) 14, 16 and 19 rat embryos, were kept in defined medium or in defined medium plus 5-HT for 7 days. Embryonic day 16 cortices were also exposed to DA, NA or ACh for the same period. At the end of this period, the proportions of the neuronal (glutamate-, GABA-, calbindin-, calretinin-labelled), glial (GFAP) and neuroepithelial (nestin) cell types were estimated for all conditions. It was found that in E16 cultures, application of 5-HT, but not of DA, NA or ACh, significantly increased the proportion of glutamate containing neurons in a dose-dependent manner without affecting the proportions of any other cell types. A similar effect was observed in co-cultures of E16 cortex with slices through the midbrain of E19 embryos containing the raphe nuclei. The total amount of cortical glutamate, as measured with high pressure liquid chromatography, was also increased in these co-cultures. To investigate whether the effect of 5-HT was the result of changes in cell proliferation, cortical slices were exposed to bromodeoxyuridine (BrdU) every day of the culture period. Cell counts showed that the proportion of BrdU labelled cells was similar in the 5-HT

treated and control slices. These results indicate that 5-HT promotes the differentiation of cortical glutamate containing neurons without affecting neuroepithelial cell proliferation.

LIST OF ABBREVIATIONS

ABC: Avidin-biotin complex

ACh: Acetylcholine

ANOVA: Analysis of variance

BDMA: Benzydimethylamine

BrdU: 5-bromo-2-deoxyuridine

CB: Calbindin D28K

CP: Cortical plate

CR: Calretinin

DA: Dopamine

DAB: Diaminobenzidine

DDSA: Dodecynyl succinic aldehyde

DMEM: Dulbecco's modified Eagle's medium

E: Embryonic day

EDTA: Ethylene diamino tetraacetic acid

GABA: γ -aminobutyric acid

GBSS: Gays balanced salt solution

GFAP: Glial acidic fibrillary protein

Glu: Glutamate

HPLC: High pressure liquid chromatography

5-HT: Serotonin (5-hydroxytryptamin)

IZ: Intermediate zone

MZ: Marginal zone

NA: Noradrenaline

NGS: Normal goat serum

P: Postnatal day

p: Probability

PBS: Phosphate buffer saline

PPL: Primordial plexiform layer

SP: Subplate

SVZ: Subventricular zone

VZ: Ventricular zone

1. The cerebral cortex

The cerebral cortex is a sheet of cells occupying the anterior and dorsal aspect of the cerebral hemispheres. The cortex of mammals is divided into the paleocortex, the archicortex and the neocortex. The paleocortex and the archicortex (collectively referred to as the allocortex) are also found in lower vertebrate taxa. The neocortex (often referred to as “cortex” throughout this text) first appears in the reptiles (Kappers, Huber and Crosby, 1936), but it is in the mammals where it reaches its full development. Mammals with small brains have smooth cortices (lissencephalic brains) whereas large-brained animals have cortices with gyri and sulci (gyrencephalic brains). However, despite - or perhaps because of - these divergent morphological adaptations, the basic structure of the cortex is the same across different mammalian species. This type of spatial rearrangement has allowed for a vastly expanded cortical area and a more complex connectivity without the need for the modification of cortical structure or for a correspondingly larger cranium. Thus, although the human brain is 3400 times larger than that of the mouse, cortical thickness is only increased by a factor of about 3 (Kappers, Huber and Crosby, 1936), and the histological makeup of the cortex is consistent in mammals as diverse as the mouse, the rat, the cat, the macaque and the human (Rockel et al., 1980). Neurons in the cortex of the rat, as in the cortices of other mammals, are arranged in 6 layers, as visualized by Nissl stained sections. Layer I is the most superficial and contains only a small number of neurons. Layer II is made up of an outer zone of densely packed neurons and an inner zone of more widely spaced neurons. This pattern is continued throughout the thickness of layer III, so that the

border between the two layers is difficult to delineate; hence they are often collectively referred to as layers II/III. Layer IV consists of smaller, more densely packed neurons, while layer V contains loosely spaced cell bodies, mostly medium sized; large somata are also present. Two zones can be distinguished in layer VI: layer VIa, with neurons resembling those of layer IV and layer VIb, bordering the subcortical white matter, consisting of horizontally oriented neurons (Krieg, 1946a; Winkelmann et al., 1972; Schober and Winkelmann, 1975).

1.1. Cytology of the rat cerebral cortex

1.1.1 Neurons

The earliest microscopical studies of the cerebral cortex were performed during the first half of the nineteenth century, using unstained material that was unfixed or poorly fixed in alcohol. Ehrenberg and Valentin in the 1830's and Remak in the 1840's reported the existence of cortical cells, the latter stressing the continuity between the cell somata and the nerve fibers. Von Kölliker (1852) was able to classify neurons according to their shape and number of processes, initially based on observations of unstained material, and first described a subset of neurons as "pyramidal". Berlin, using carmine staining in the 1850's, reported findings in agreement with those of Kölliker, while Meynert, one decade later, first described the cytoarchitectonics of the cortex in remarkable detail (for reviews see Polyak, 1957; Jones, 1988; Jacobson, 1991). The advancement of staining techniques that allowed researchers to more accurately describe the structure of the cortex evolved

in parallel with the construction and establishment of the neural theory during the last 60 years of the 19th century (for review see Parker, 1900). The introduction of metal impregnation staining techniques by Camillo Golgi in the 1870's provided researchers with a new and powerful tool. The procedure typically stains the entire cell body and processes of only a small proportion of neurons, thus greatly facilitating the examination of neuronal morphology. Santiago Ramón y Cajal first and then his student Lorente de Nó used the Golgi technique to examine the cortex and achieved, by the turn of the century, to accurately describe its morphological organization (Ramón y Cajal, 1911; Lorente de Nó, 1922, 1949). Other studies, performed mostly in the 1970's, have further examined cortical cellular composition, organization and structure, focusing primarily on the visual cortex of various mammalian species (Lund, 1973; Szentágothai, 1973; Jones, 1975; Valverde, 1976; Parnavelas et al., 1977a; Feldman and Peters, 1978; Valverde, 1978; Peters and Regidor, 1981). There is now a general agreement that cortical neurons can be broadly classified into two major categories, the pyramidal and nonpyramidal cells.

Pyramidal neurons

Pyramidal neurons, the projection cells of the cortex, are the predominant neuronal cell type of the cortex. They are present in all layers with the exception of layer I, and comprise approximately two thirds of the neuronal population (Parnavelas et al., 1977a; Rockel et al., 1980; Winfield et al., 1980). Pyramidal cell bodies show a variety of shapes, with pyramidal or conical being the most common. They

typically have a prominent dendrite that emanates from the apex of the triangular soma and extends towards the pial surface, giving rise to numerous oblique and horizontal branches on its way and forming a terminal tuft in layers I and II. Two or more dendrites, the so-called basal dendrites, emanate from the base of the soma, travel obliquely or horizontally and, together with their branches, form the basal dendritic field. An axon typically arises from the bottom of the cell body, or occasionally from the proximal portion of a basal dendrite, and extends towards the white matter giving rise to numerous collaterals. Pyramidal cells with this typical appearance are found primarily in layer V and lower layer III. Cells in the upper part of layer II are different in that they have round or ovoid cell bodies and shorter apical dendrites or lack a prominent apical dendrite when situated near the upper border of layer II; instead, they have two or three obliquely oriented ascending dendrites that branch near the soma (Peters and Kara, 1985a). Other atypical pyramidal cells are the pyramidal-like cells of layers V and VIa, which have “apical” dendrites extending horizontally or obliquely, and the inverted pyramids of layers V and VI which have a pyramidal appearance but are inverted, so that the “apical” dendrite is oriented towards the white matter (Parnavelas et al., 1977a). However, the use of the term “pyramidal” to describe such cells is not in agreement with Marín-Padilla’s (1992) definition, according to which only those cells that retain their contact with layer I, through their apical dendrites, are true pyramids. Layer IV pyramidal cell bodies are small and oval in shape and lack a typical basal dendritic field; they give rise to primary dendrites that radiate from their somata (Peters and Kara, 1985a), a feature that prompted Lorente de Nó (1949) to name them “star pyramids”. Ultrastructural examination has shown that

pyramidal cell nuclei are electroluscent with evenly distributed heterochromatin. Their cell bodies receive exclusively Gray's type II (Gray, 1959) or symmetrical synapses on their somata, type I synapses on their dendritic spines and both types on their dendritic shafts (Parnavelas et al., 1977b). The axons of the pyramidal cells are predominantly myelinated (Peters, 1981) and their collaterals form type I (asymmetrical) synapses with dendritic spines and dendritic shafts of other cortical neurons (Parnavelas et al., 1977b; Somogyi, 1978).

Nonpyramidal neurons

Nonpyramidal neurons are the local circuit neurons of the cortex and make up 15-30% of the total neuronal population (Parnavelas et al., 1977a; Rockel et al., 1980; Winfield et al., 1980). In Golgi preparations of the rat cerebral cortex they show different soma sizes and dendritic field shapes (Parnavelas et al., 1977a; Feldman and Peters, 1978; Winkelmann et al., 1981). Feldman and Peters (1978) classified nonpyramidal neurons of the rat visual cortex according to their dendritic patterns as *multipolar*, *bitufted* or *bipolar* and, according to the abundance of their dendritic spines, as *spinous*, *sparsely spinous* and *spine free*. Spinous dendrites appear to be associated only with multipolar neurons, while sparsely spinous and spine-free dendrites can be the features of all three types.

Approximately 60% of the nonpyramidal neurons are *multipolar*; they are found in all layers, have spherical, ovoid or irregular somata and a stellate appearance, with dendrites arising from various parts of the soma and extending in different directions. Axons emanate from the superficial or deep part of the cell body or from one of the primary dendrites and form an elaborate local plexus.

Bitufted cell somata comprise approximately 20% of the Golgi stained nonpyramidal neurons and are located primarily in layers IV and V. They have vertically oriented elongated perikarya, from each pole of which one or more dendrites arise that soon branch repeatedly to form two almost symmetrical tufts. Their axons arise from the soma or from one of the primary dendrites.

Bipolar cell somata are present predominantly in layers II-IV. Most perikarya are vertically elongated, with one dendrite emanating from each of the poles, and extending for a considerable distance. Their axons usually arise from one of the dendrites and exhibit a predominantly vertical orientation.

Some additional types of nonpyramidal cells are found in layer I (Bradford et al., 1977). *Cajal-Retzius cells* are only present during the perinatal period; some of these cells subsequently undergo degeneration, while others undergo a transformation to become indistinguishable from typical layer I nonpyramidal cells (Ramón y Cajal, 1911; Marín-Padilla, 1972; Edmunds and Parnavelas, 1982). *Persisting horizontal cells* have horizontally elongated somata from which two primary dendrites emanate, one at each pole, to ramify into oblique branches. *Vertical cells* are located in the upper part of layer I; one or two short horizontal dendrites and one thick descending dendrite emanate from the cell body. Finally, a small number of *cells without axons* have been observed (Bradford et al., 1977).

A number of other types of nonpyramidal neurons, absent from the rat cortex, often appear in Golgi preparations of the cortex of higher mammals: *spinous stellate cells* with axons projecting to the white matter (Szentágothai, 1973), *basket cells* (Ramón y Cajal, 1911; Szentágothai, 1973), *midget cells* (Garey, 1971; Szentágothai, 1973) and *double bouquet cells* (Ramón y Cajal, 1911;

Szentágothai, 1973).

The combination of Golgi staining with electron microscopy has been extensively used to examine the ultrastructural features of nonpyramidal neurons (Parnavelas et al., 1977b; Peters and Fairén, 1978; Peters and Kimerer, 1981). Nonpyramidal cell nuclei appear electron dense and display a rather uneven distribution of heterochromatin; their nuclear membranes are always invaginated. Nonpyramidal cells receive both Gray's type I and type II synapses on their somata and dendrites (Parnavelas et al., 1977b; Peters and Fairén, 1978; Peters and Kara, 1985b). Their axons can be either myelinated or unmyelinated and form symmetrical synapses with both pyramidal and nonpyramidal cells in the cortex (Parnavelas et al., 1977b; Peters and Fairén, 1978; Peters, 1981).

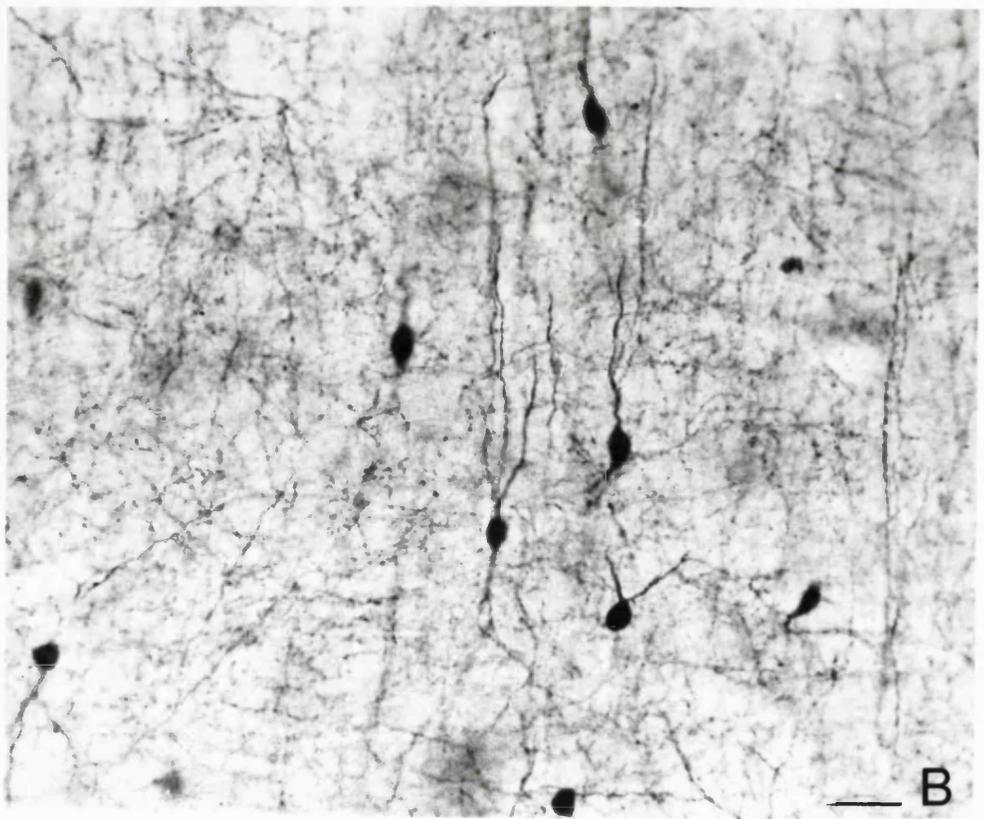
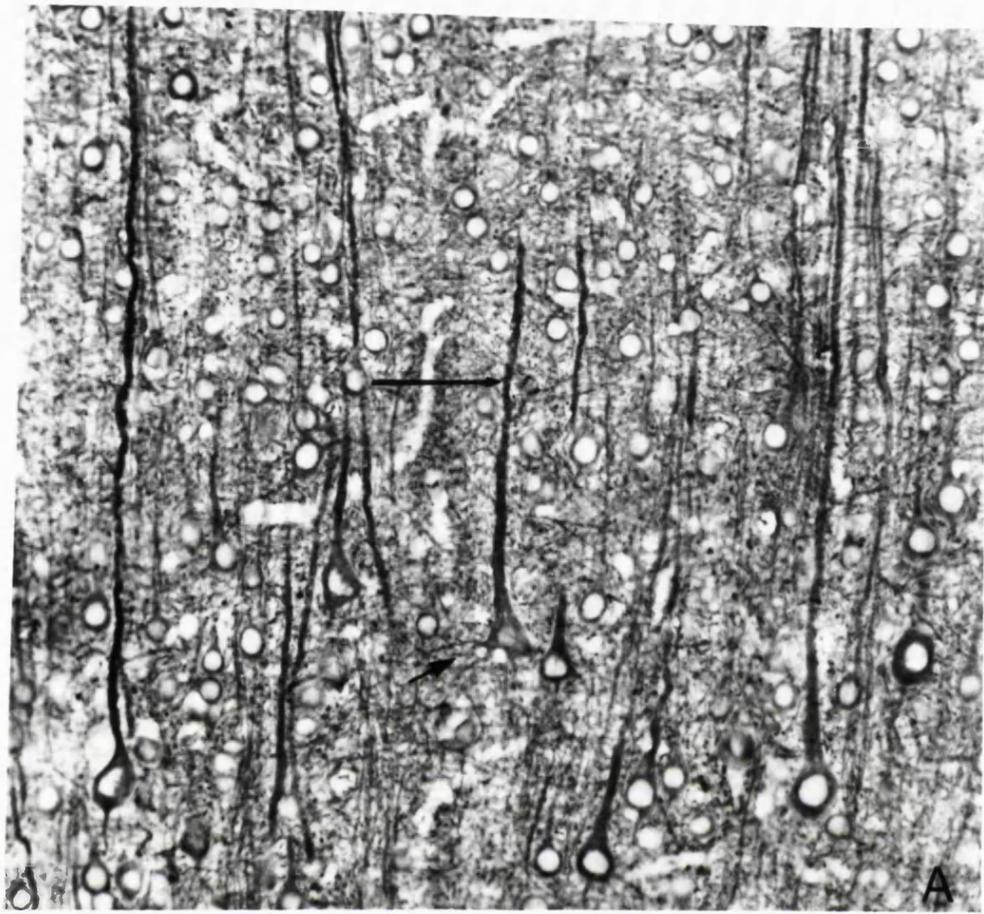
1.1.2. Glial cells

Although there were relevant publications before, it was Virchow in 1846 who first recognized the existence in the CNS of a fibrous interstitial component made up of stellate or spindle-shaped cells that could be morphologically differentiated from neurons; he named these cells neuroglia (nerve glue) (see Penfield, 1932; Privat, 1975; Sturrock, 1975; Privat and Fulckgrand, 1977). The development of selective impregnation methods in the latter part of the 19th and the early part of the 20th century allowed the recognition of distinct cells classified as: astrocytes, oligodendrocytes (together comprising the macroglia), ependymal cells (only found in the lining of the ventricles of the brain and the central canal of the spinal cord) and microglia. Macroglia and ependymal cells are of neuroectodermal origin,

Figure 1

Vibratome sections (50 μm thick) through the adult rat cortex stained with antibodies against MAP-2 (A) and the calcium-binding protein calretinin (B). Pyramidal neurons can be seen in A, with their distinctive apical dendrites (long arrow) and the shorter basal dendrites (short arrow), while a group of nonpyramidal neurons containing the calcium-binding protein CR are visualized in B.

Scale bar = 30 μm .



whereas microglia are derived from the mesoderm and invade the CNS at the time of vascularization via the pia mater and the blood vessels.

Astrocytes can be subdivided into protoplasmic (located in the grey matter) and fibrous (present in the white matter); the former extend end-feet that surround brain capillaries (Mugnaini and Waldberg, 1964) and are believed to take part in the formation of the blood-brain barrier. Astrocytes can be found at all levels in the cortex, but are more abundant close to the pial surface and in the deep layers (Parnavelas et al., 1983). In contrast to the older view that astrocytes were mere supportive elements for the neurons, there is now a long list of properties that are attributed to these cells: Buffering of extracellular ion levels so that neurons can maintain their excitability (Lux et al., 1986; Walz, 1989), non-synaptic uptake and release of neurotransmitters (Kimmelberg and Katz, 1985; Kimmelberg and Pelton, 1983), formation of scar tissue following neuronal loss (Reier, 1986), storage of glycogen (Tascopoulos and Magistretti, 1996), and provision of trophic substances for young neurons before they establish connections with their postsynaptic targets (Schwartz and Mishler, 1990). Radial glial cells, specialized cells with long processes that guide the newly generated migrating neurons during development, are transformed into protoplasmic astrocytes after completion of the process of migration (Culican et al., 1990; Hatten, 1990; Rakic, 1985).

Oligodendrocytes can be subdivided into satellite, intermediate and interfascicular (myelinating) types, which have been studied more extensively (Rain, 1982, 1984). Oligodendrocytes are present throughout the cortex, being rare in layer I and most abundant in layers V and VI. They provide the myelin sheaths around CNS axons. In contrast to their counterparts in the periphery, the Schwann

cells, oligodendrocytes can produce numerous internodes of myelin, up to 50 in the optic nerve (Rain, 1982). This, in conjunction with the slow mitotic rate and poor regenerative capacity of the oligodendrocytes, means that damage to a few of these cells can result in a large area of demyelination.

Microglial cells are in a resting state in the normal brain but are transformed into very active and mobile macrophages during disease; they also phagocytose the debris of degenerating CNS elements during normal wear and tear (for review, see Dickson et al., 1991). Three subgroups are identified: ramified microglia (extensively branched cells), perivascular microglia (less ramified, antigen-presenting cells derived from monocytes) and amoeboid microglia (the macrophage form appearing in diseased states). Microglial cells are present throughout the cortical thickness (Parnavelas et al., 1983).

1.1.3. Afferent and efferent connections

Afferent connections

Thalamic afferents: Thalamic afferents can be distinguished into two types: fibers of one type, conveying specific information, arise in sensory relay nuclei of the thalamus and form dense terminal arborescences in layer IV and deep layer III; others, non-specific afferents, arise mostly in the intralaminar nuclei, are thinner and give rise to a diffuse innervation of the entire cortical thickness (Herkenham, 1980; Parnavelas and Chatzissavidou, 1981). The former axons form type I synapses with the proximal dendrites and somata of pyramidal and nonpyramidal neurons (Peters and Feldman, 1976; Peters and Saldanha, 1976; Schober and Winkelmann, 1977).

Other subcortical afferents: A number of other subcortical systems also project, mostly ipsilaterally, to the entire cerebral cortex.: The nuclei of the basal forebrain project providing its cholinergic input (Divac, 1975; Lehman et al., 1980; Wenk et al., 1980; Henderson, 1981); the locus ceruleus in the pons sends noradrenergic fibers (Lindvall and Björklund, 1974; Jones and Moore, 1977); serotonergic fibers from the midbrain dorsal and median raphe nuclei (Fuxe, 1965; Bobilier et al., 1976; Azmitia and Segal, 1978; Moore et al., 1978; Lidov et al., 1980; Parent et al., 1981; Waterhouse et al., 1986) and dopamine-containing fibers from the midbrain ventral tegmental area (Lindvall and Björklund, 1974; Törk and Turner, 1981) (See also section on neurotransmitters).

Corticocortical afferents: The organization of cortical callosal connectivity has been studied using degeneration, autoradiography and horseradish peroxidase (HRP) tracing techniques (Nauta and Bucher, 1954; Jacobson, 1970; Ribak, 1977; Miller and Vogt, 1984a; Olavarria and Sluyter, 1984). The neurons that give rise to these projections as well as those that receive connections from the contralateral hemisphere are found in layers II-VI but predominantly in layers III and V, suggesting a homotopic connectivity in terms of cortical layer; the connectivity is also primarily homotopic in terms of cortical areas. Callosal afferents form type I synapses with somata and dendrites of cortical pyramidal and nonpyramidal neurons (Lund and Lund, 1970). In addition to callosal connections there exist corticocortical connections reciprocally connecting different cortical areas ipsilaterally (Ribak, 1977; Olavarria and Montero, 1981; Miller and Vogt, 1984b).

Cortical efferents

Pyramidal neurons in the cerebral cortex of the rat project to a number of subcortical structures. These include: the spinal cord, thalamus, superior colliculus, pons and the striatum. (Webster, 1961; Lund, 1966; Gosavi and Dubey, 1972; Ribak, 1977; Mihailoff et al., 1981)

1.2. Neurotransmitters

1.2.1. Amino acids

Glutamate and Aspartate

The neurotransmitter roles for L glutamate (Glu) and L-aspartate (Asp) in the cortex have been supported by a number of studies since the early 1960's (for reviews, see Curtis and Johnston, 1974; Fagg and Foster, 1983). Up until the 1980's, however, a direct demonstration of the neurons utilising these substances remained elusive, as both amino acids are key metabolites and it was not possible to discern the metabolic from their neurotransmitter pools. Indirect methods were used for that purpose; lesions of various cortical efferent systems (corticopontine, corticocollicular, corticothalamic, corticostriatal) resulted in a drop in endogenous levels, release and high affinity uptake of Glu and Asp in the corresponding subcortical targets (McGeer et al., 1977; Lund-Karler and Fonnum, 1978; Thangnipon et al., 1983; Fosse et al., 1984b; Fosse and Fonnum, 1987). Retrograde labelling by [³H]-Glu and [³H]-Asp was also performed at subcortical and callosal target sites of cortical projections (Baughman and Gilbert, 1981; Rustioni et al., 1983; Matute and Streit, 1985; Barbaresi et al., 1987).

Immunohistochemical localization of Glu and Asp was achieved following the production of antibodies directed against the amino acids (Storm-Mathisen et al., 1983). It is now widely accepted that Glu is the neurotransmitter utilized by cortical pyramidal neurons, and Glu-containing cells are found in cortical layers II-VI; Asp is also localized in the same layers, but approximately 10% of Asp-containing cells have been found to be nonpyramidal neurons (Dori et al., 1989). The proportions of Glu- to Asp-containing pyramidal cells in the cortical projection systems vary according to the target (Dori et al., 1992). In the cortex, Glu- and Asp- containing axon terminals have been estimated to form asymmetrical synapses, primarily with dendritic spines, both in the cat (DeFelipe et al., 1988; Conti et al., 1989) and in the rat (Dori et al., 1992).

Neurotransmitter Glu is primarily synthesized from glutamine by glutaminase, though other routes also exist: from α -ketoglutarate by transamination (the metabolic pathway that leads to GABA) or by inversion of glutamic acid dehydrogenase action, or from ornithine. Oxaloacetate, Glu, glutamine and asparagine are all considered to be Asp precursors. Different pools of both Glu and Asp have been demonstrated, but no association between different pools and anatomical features has been established (Fonnum, 1984).

Both Glu and Asp strongly excite cortical neurons when applied iontophoretically (Krnjevic and Phillis, 1963b; Krnjevic, 1964). Effects of Glu are instant and rapidly terminated; its application is followed by a marked drop in membrane resistance and Na^+ permeability. Asp action is similar to that of Glu, although not as potent.

Four types of excitatory amino acid (EAA) receptors are recognized, each

having a distinct pattern of distribution: N-methyl-D-aspartate (NMDA), quisqualate (QA) or α -amino-3-hydroxy-5-methyl-4-isoxazole (AMPA), kainate (KA) and L-aminophosphonobutyric acid (AP4) (Fagg and Foster, 1986; Cotman et al., 1987). Molecular studies have shown the existence of numerous subtypes (see Doble, 1995 for review). The NMDA is the best characterized receptor class; its function differs from that of other EAA receptors in that it is blocked in a voltage-dependent fashion by physiological concentrations of Mg^{+2} and becomes operative when the membrane is depolarized by Na^{+} influx, presumably as a result of excitation of other Glu receptors. Excessive quantities of EAA can cause neuronal overexcitation resulting in damage (excitotoxicity). This neurotoxicity is mediated by NMDA, QA and KA receptors. Degeneration of cells in the retina and hypothalamus can occur after systemic high dose EAA administration in immature animals or after intraventricular administration in mature ones, whereas microinjection of EAA in various brain areas can produce focal degeneration (McGeer et al., 1978; Lodge, 1987; McGeer et al., 1987). Such toxicity can also be produced by a number of other amino acids that act as analogues of Glu or Asp (cysteinsulfinic, ibotenic, quinolinic, homocysteic, and folic acid). Excessive activity of endogenous EAAs has been implicated in a number of pathological processes, including ischaemic/hypoxic brain damage (Rothman and Olney, 1986), Huntington's disease (McGeer and McGeer, 1976), epilepsy (Meldrum, 1985), and olivopontocerebellar atrophy (Plaitakis et al., 1982).

There has been one report of Glu-positive cell presence in the developing rat cerebral cortex as early as embryonic day (E) 19 (Götz and Bolz, 1994). However, in a detailed study of EAA development in the rat visual cortex, Dori

and Parnavelas (1996) reported that individual Glu- and Asp- positive cells are first visualized in the deep layers of the cortex around postnatal day (P) 3, with only diffuse staining seen at E20 and P0. The cells gradually mature in morphology and increase in number and, by the end of the second postnatal week, they are distributed uniformly across layers II-VI; the adult distribution is reached by the beginning of the fourth week (Dori and Parnavelas, 1996).

γ -aminobutyric acid

Gamma-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the cerebral cortex. The presence of GABA in the brain was first discovered independently by Roberts and Awapara in 1950, and its inhibitory properties demonstrated by Florey in 1953 (for review, see Curtis and Johnson, 1974; McGeer and McGeer, 1989). The presence of GABA-containing neurons in the cortex was first traced by autoradiography using [³H]-GABA, while a number of immunocytochemical studies subsequently localized GABAergic neurons in the cortex, with antibodies either against glutamic acid decarboxylase (GAD, the enzyme that synthesizes GABA) (Ribak, 1978; Chromwall and Wolff, 1978; Emson and Lindvall, 1979; Hendry and Jones, 1981) or against GABA itself (Storm-Mathisen et al., 1983; Somogyi et al., 1985; Meinecke and Peters, 1987).

GABAergic cells in the rat cerebral cortex appear as multipolar, bipolar or bitufted nonpyramidal cell types (Ribak, 1978; Lin et al., 1986; Meinecke and Peters, 1987; Parnavelas et al., 1989). They make up approximately 20% of the total neuronal population in the rat cortex, and the proportion is more or less the

same across different areas and different species (Hendry et al., 1987; Meinecke and Peters, 1987). Lin (1986) observed a band with an increased density of GAD-positive cells in layer IV, whereas Meinecke and Peters (1987) reported that the distribution of GABA-positive neurons was homogeneous across the thickness of the cortex. Ultrastructural studies have shown that GABAergic axons form symmetrical synapses with somata and dendrites of cortical cells as well as with axon initial segments of pyramidal neurons (for review, see Houser et al., 1988). There is evidence that some acetylcholine (ACh) containing neurons in the basal forebrain that project to the cortex also contain GABA (Fisher et al., 1988, 1989); GABAergic cells in the posterior hypothalamus and the zona incerta have also been reported to project to the cortex (Vincent et al., 1983; Kohler et al., 1985; Lin et al., 1990).

GABA is synthesized by glutamate which in turn comes from α -ketoglutarate, an intermediate in the Krebs cycle; the decarboxylation of glutamate is catalyzed by GAD. GABA is transaminated by GABA-oxoglutarate-transaminase (GABA-T) to succinic semialdehyde which is then rapidly oxidized to succinic acid. The transamination of GABA to succinic semialdehyde can only take place, however, if α -ketoglutarate is the acceptor of the amine group, resulting in glutamate synthesis. Thus, a GABA molecule can only be destroyed when a precursor molecule is synthesized. Alternative precursors for GABA have been proposed, such as putrescine and γ -hydroxybutyrate, but they have not been shown to play a significant role in the brain (for review see McGeer and McGeer, 1989).

The inhibitory action of GABA on cortical cells has been demonstrated by iontophoretic application (Krnjevic and Phillis, 1963a; Krnjevic and Schwartz,

1967; Krnjevic, 1974). There are two major classes of GABA receptors: GABA_A and GABA_B. The GABA_A receptors are ligand-gated ion channels and have a heterooligomeric structure comprising a number of different subunits. Their properties depend upon the combination of subunits from which they are assembled. It has been demonstrated by molecular cloning that the number of these subunits is at least 16 (α_{1-6} , β_{1-4} , γ_{1-3} , δ and ρ_{1-2}), suggesting a potentially large number of GABA_A receptor subtypes. GABA_A sites are mostly postsynaptic and are responsible for the classic postsynaptic inhibitory action of GABA (for reviews see Sieghart, 1995; Stephenson, 1995; Yeh and Grigorenko, 1995). Until recently, little was known about the GABA_B receptor, but that has now changed with the recent report of its cloning by Kaupman et al. (1997). It belongs to the a G-protein coupled type, acts by reducing the flow of other transmitters and its sites are both on presynaptic and postsynaptic neuronal elements (Hill et al., 1981; Wojcik and Holopainen, 1992; Bowery and Brown, 1997).

It has been demonstrated by combined [³H]-thymidine autoradiography and GAD or GABA immunohistochemistry, that GABAergic cells in the rat neocortex are generated throughout the period of cortical neurogenesis (E14-E21) with a peak at E17 (Wolff et al., 1984; Miller 1986) and follow the same inside-out pattern of development as pyramidal cells (Miller, 1986b). GABAergic neurons in the rat first appear in the primordial plexiform layer at E14, subsequently innervating the intermediate zone (E15) and the rest of the cortical primordium by the end of E16. From E19 onwards more cells appear in the cortical plate and fewer in the intermediate and subventricular zones (Lauder et al., 1986; Van Eden et al., 1989). GABAergic neurons in the marginal zone and the subplate are also transient

(Luskin and Shatz, 1985b; Valverde and Facal-Valverde, 1988); the cells in the marginal zone are the Cajal-Retzius cells (Marin-Padilla, 1988).

1.2.2. Monoamines

Serotonin

5-hydroxytryptamine (serotonin; 5-HT)-containing neurons in the brainstem raphe nuclei were initially visualized in the 1960's with the Falck-Hillarp method of formaldehyde-induced histofluorescence (Dahlstrom and Fuxe, 1964; Fuxe 1965). However, the instability of the fluorophore produced meant that this method could not provide information on the serotonergic innervation of the cortex at the same level of sensitivity as it did for the catecholaminergic innervation (see below). Alternative methods were subsequently used to localize 5-HT in the cortex: autoradiography either at the light microscope (Conrad et al., 1974; Bobillier et al., 1976, 1979; Azmitia and Segal, 1978; Moore et al., 1978) or at the electron microscope level (Aghajanian and Bloom, 1967; Descarries et al., 1975); lesion studies combined with biochemistry (Geyer et al., 1976) or histochemistry (Ungerstent, 1971); or, more recently, the preferred method of immunohistochemistry with antibodies against 5-HT for demonstrating the full extend of the cortical serotonergic innervation (Steinbusch et al., 1978; Lidov et al., 1980; Lidov and Moliver, 1982a).

All the 5-HT present in the cortex is derived from the serotonergic afferents arising in cell bodies situated in the dorsal and median raphe nuclei of the midbrain. The dorsal raphe nucleus provides innervation to the motor,

somatosensory and visual cortex, and the median raphe nucleus projects to the motor portion of the frontal cortex (Moore et al., 1978; Waterhouse et al., 1986). In the adult cortex, 5-HT axons are oriented tangentially (parallel to the pial surface) in layers I and VI, radially in layers II and III, with shorter curved or obliquely oriented axonal segments typically found in layers IV and V (Papadopoulos et al., 1987b). In addition to this laminar heterogeneity, there seems to be a morphological heterogeneity in the fibers themselves, with the axons originating from the dorsal raphe nucleus being finer with smaller varicosities and the ones coming from the median raphe nucleus being thicker with large varicosities (Lidov et al., 1980; Kosofsky and Molliver, 1987; Mulligan and Törk, 1988). Serotonergic axons have been shown to form both type I and II synapses in the visual cortex, usually on dendritic shafts, spines and apical dendrites of pyramidal neurons (Papadopoulos et al., 1987a,b).

Serotonergic neurons synthesize 5-HT using dietary tryptophane, which is converted to 5-hydroxy-tryptophane by tryptophane 5-hydroxylase; this is the rate limiting step in the biosynthesis of 5-HT. 5-hydroxy-tryptophane is, in turn, converted to 5-HT by aromatic-L-aminoacid decarboxylase. The production rate has been proposed to be adjusted by impulse-coupled regulation of tryptophan hydroxylase activity (for review see Cooper et al., 1986). Serotonin itself is metabolized primarily into 5-hydroxy-indole-acetaldehyde by monoamine oxidase (MAO), the enzyme that oxidatively deaminates all monoamines to their corresponding aldehydes; further oxidation by aldehyde dehydrogenase leads to 5-hydroxy-indole acetic acid (see Osborne, 1982).

Serotonin is released by stimulation of the serotonergic cell bodies in the

raphe nuclei. These cells contain 5-HT autoreceptors, suggesting that 5-HT regulates its own release. However, other neurotransmitters and modulators (dopamine, noradrenaline, acetylcholine) have also been implicated in this regulation. Drugs and experimental compounds like reserpine, π -chloroamphetamine (PCA), and methylene-dioxy-methamphetamine (MDMA/"Ecstasy") can also cause release of 5-HT (for review, see Green et al., 1995). The action of 5-HT upon postsynaptic receptors, as studied by iontophoretic application, has been demonstrated to be, in most cases, inhibitory (Reader, 1978), although it can also have (or facilitate) excitatory activity (Nedegaard et al., 1987). This action is terminated by reuptake into the presynaptic terminal; the uptake system has a high specificity and does not interact with other monoamines.

Serotonin receptors were originally classified as D and M types in the 1950's and later as 5-HT₁, 5-HT₂, 5-HT₃ and, more recently, 5-HT₄. The advent of molecular cloning techniques brought about an explosive increase in the number of distinct 5-HT receptor subtypes that can be identified reaching the current number of 14. 5-HT₃ is a ligand-gated ion channel, while the rest belong to G-protein coupled types (Teitler and Herrick-Davis, 1994; Leonard, 1996).

Serotonergic cell bodies appear very early (E12) in the embryonic development of the rat, when they form a cluster on either side of the floor plate of the rhombencephalon, accompanied by short, non-varicose fibers (Aitken and Törk, 1988). This cluster gives rise to the ascending fibers, while a second one, appearing caudally at E14, gives rise to the descending ones. The dorsal and the median raphe nuclei, which innervate the cortex, both arise from the first cluster; the serotonergic fibers first arrive at the telencephalic region at about E17, forming

two layers on either side of the cortical plate (Lidov and Molliver, 1982a; Wallace and Lauder, 1983). The innervation of the cortex is continued during the prenatal and well into the postnatal period; during the latter, the fibers that were prenatally extending without branching, form extensive ramifications and cover the entire cortex (Wallace and Lauder, 1983). A transient serotonergic innervation localized predominantly in layer IV of primary sensory areas of the rat cortex has been observed during the first two weeks of postnatal life. In the somatosensory cortex this transient innervation is coextensive with the cortical barrels, aggregates of cortical cells that receive thalamic input from individual vibrissae (D'Amato et al., 1987; Dori et al., 1996). However, recent findings in the mouse suggest that the transient appearance of 5-HT in layer IV of the somatosensory cortex coincides with the thalamocortical projection, whose axons and terminals take up 5-HT through high affinity uptake sites (Lebrand et al., 1996).

Dopamine

The existence of a dopaminergic innervation of the cerebral cortex was first suggested by biochemical studies (Thierry et al., 1973a,b, 1974) and subsequently confirmed by anatomical work, utilizing glyoxylic acid-induced histofluorescence (Lindvall et al., 1974; Lindvall and Björklund, 1974), histofluorescence in combination with tract-tracing (Törk and Turner, 1981), autoradiography (Descarries et al., 1987), immunohistochemistry against the synthesizing enzyme tyrosine hydroxylase (TH) in combination with destruction of the NA system (Berger et al., 1985) and, finally, immunohistochemistry with antibodies against dopamine (DA) itself, ending the problems of distinction between DA and NA that

had hampered earlier work (Papadopoulos et al., 1989b)

The dopaminergic innervation of the cerebral cortex arises in the midbrain ventral tegmental area from cell bodies that lie medial to the substantia nigra and innervate the cortex through the medial forebrain bundle. This, however, is not the main dopaminergic pathway in the brain, as nearly 80% of all of the brain's DA is found in the caudate nucleus and putamen of the corpus striatum, which is densely innervated by the zona compacta of the substantia nigra via the nigrostriatal pathway. The innervation of the cortex is widespread (Berger et al., 1985; Papadopoulos et al., 1989b) and exhibits varying density in different cytoarchitectonically distinct areas (Levitt et al., 1984). Dopaminergic axons in the rat visual cortex have been demonstrated, by electron microscope immunocytochemistry, to form predominantly (but not exclusively) type I synapses on dendritic shafts and spines (Papadopoulos et al., 1989b)

Dopamine is synthesized from tyrosine, which is first converted into dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase, the rate-limiting enzyme in the biosynthetic pathway of DA; DOPA is, in turn, decarboxylated to DA by DOPA decarboxylase. Dopamine synthesis is modulated by end product inhibition of tyrosine hydroxylase (TH) (negative feedback); in addition, depolarization of dopaminergic cells results in TH activation, thus maintaining the balance between DA synthesis and release (Joh et al., 1978; Masserano et al., 1988). Like the other monoamines, DA is catabolized by MAO.

In studies using iontophoretic application, DA has been demonstrated to inhibit cortical neurons in the cat (Krnjevic and Phillis, 1963a; Reader, 1978) and rat (Bunney and Aghajanian, 1976; Reader et al., 1979a) but, like 5-HT, it has

also been shown to have excitatory action on some neurons. The action of DA is terminated by reuptake into the presynaptic terminals by a DA-specific reuptake carrier (Coyle et al., 1969).

Dopamine receptors were originally classified as type D_1 and D_2 in the 1970's but, since then, more have emerged with the cloning of types D_3 , D_4 , D_5 and their isoforms; they belong to G-protein coupled types (for review see Wolfarth and Ossowska, 1995).

The first presence of DA is detectable in the substantia nigra at E14 (Specht et al., 1981). It has been demonstrated that dopaminergic fibres first arrive at the developing rat cerebral cortex at E15, entering the intermediate zone, while the cortical plate is reached at the time of birth. Prior to the time of birth, the innervation consists of thick, straight fibres darkly stained with anti-DA, whereas a morphological change occurs after that period and, between P2 and P4, all the thick straight fibres are turned into thin, often twisted fibres with irregularly spaced varicosities (Kalsbeek et al., 1988). D_2 receptors have been found in the rat cortex at E18 (Noisin and Thomas, 1988) and the presence of D_1 receptors has been demonstrated as early as E16 (Reinoso et al., 1996).

Noradrenaline

The noradrenergic innervation of the cerebral cortex was first demonstrated by the Falck-Hillarp method of formaldehyde-induced histofluorescence in the 1960's (Fuxe et al., 1968), and subsequently supported by numerous studies using glyoxylic acid - induced histofluorescence (Lindvall and Björklund, 1974), autoradiography (Jones and Moore, 1977), immunohistochemistry using antibodies

against dopamine- β -hydroxylase (Morrison et al., 1978), and finally immunohistochemistry against NA itself (Papadopoulos et al., 1989a).

The noradrenergic innervation of the cerebral cortex originates in the locus ceruleus (LC), a nucleus located at the isthmus, on the lateral aspect of the fourth ventricle and the noradrenaline (NA)-containing axons enter the cortex rostrally via the medial forebrain bundle (Lindvall and Björklund, 1974; Jones and Moore, 1977; Moore and Card, 1984). The same nucleus also innervates the thalamus and the cerebellum, while other noradrenergic cells, located in the lateral tegmental area innervate the basal forebrain, hypothalamus, brain stem, and spinal cord (Moore and Bloom, 1979). A dense network of thin and varicose NA-containing fibers is present in all layers of the rat cerebral cortex. The distribution of fibers resembles that of the serotonergic system: tangential axons predominate in layers I and VI, while straight radial fibers are found in layers II and III; these axons progress in a meandrous fashion within the boundaries of the two layers. Layers IV and V are characterized by short twisted axons often oriented obliquely (Levitt and Moore, 1978; Morrison et al., 1978; Papadopoulos et al., 1989a). As demonstrated by high pressure liquid chromatography (HPLC), the level of NA is highest in layer I and lowest in layer V (Parnavelas et al., 1985). Immunocytochemistry at the ultrastructural level has demonstrated that NA containing axons form mainly asymmetrical (type I) axodendritic and axosomatic synapses in the visual cortex (Papadopoulos et al., 1987a, 1989a).

Noradrenaline is synthesized by hydroxylation of DA by dopamine- β -hydroxylase. Thus the hydroxylation of tyrosine is, as in the case of DA, the rate-limiting step in NA biosynthesis. Its synthesis is, also like that of DA, modulated

by end-product inhibition and depolarization activation of TH (Joh et al., 1978; Masserano et al., 1988).

Studies employing iontophoretic application of NA have demonstrated that it can either increase or decrease the activity of cortical cells (Reader et al., 1978). The action of NA released at the synapse is terminated by reuptake into presynaptic nerve terminals (Axelrod, 1971). The uptake mechanism is selective for NA and does not interact with dopamine (Coyle et al., 1969). Noradrenergic receptors are classified as α and β subtypes since the late 1940's (Ahlquist, 1948), while distinct subtypes are now recognized; they belong to the G-protein coupled type (for review see Weiner and Molinoff, 1989).

Noradrenaline is first detected in the LC at E14 (Olson and Seiger, 1972), and NA-containing axons have been demonstrated to enter the cerebral cortex rostrally at E17, both in layer I and the intermediate zone (Levitt and Moore, 1979; Verney et al., 1984). They subsequently enter the cortical plate at E18, extend rostrocaudally and innervate the entire cortex by the time of birth. The levels of NA increase transiently during the first 3 days and then decrease again and reach the adult level in the second month. They attain the adult pattern of innervation by the end of the first week of life (Levitt and Moore, 1979)

1.2.3 Acetylcholine

The cholinergic innervation of the rat cerebral cortex was first demonstrated in the 1960's (Shute and Lewis, 1967) with acetylcholinesterase (AChE) histochemistry, and subsequently confirmed by numerous studies, the more recent of which utilized

immunohistochemistry for choline acetyltransferase (ChAT; Mesulam et al., 1983a,b; Eckenstein et al., 1988).

The cholinergic innervation of the rat cortex consists of two components. The extrinsic component is made up, primarily, of fibres originating in distinct areas of the basal forebrain, namely the horizontal and the vertical limbs of the diagonal band of Broca, the nucleus basalis of Meynert and the substantia inominata; these have been suggested to project to distinct cortical areas (for reviews see Eckenstein and Baughmann, 1984; Fibiger,1982). The extrinsic component also includes a projection from the midbrain tegmental area to the medial frontal cortex (Vincent et al., 1983). There exist laminar differences in the density of innervation, with a higher density in layers I-III and V, lower density in layer IV and varying densities an layer VI, but this pattern varies according to cortical area (Eckenstein et al., 1988). There is, also, an intrinsic component of cortical ACh innervation (Eckenstein and Thoenen, 1983; Houser et al., 1983, 1985; Eckenstein et al., 1988); it consists of mainly bipolar and some multipolar nonpyramidal neurons found in layers II-VI and predominantly in layers II and III (Parnavelas et al., 1986). ChAT-positive terminals have been found to form symmetric synapses with small diameter dendritic shafts (Houser et al., 1983a).

Acetylcholine (ACh) is synthesized from its two immediate precursors, choline and acetyl-coenzyme A in a reaction catalyzed by ChAT. Acetyl-CoA comes from pyruvate that is formed from glucose, while choline, which cannot be synthesized in the neurons, is supplied by breakdown of phosphatidylcholine as well as from recycling of released Ach, which is broken down by acetylcholinesterase (AChE).

Both excitatory and inhibitory effects of ACh on cortical neurons have been documented (Krnjevic and Phillis, 1963b,c; Stone, 1972; Sillito and Kemp, 1983; Kelly and Rogawski, 1985). It has been suggested that the excitatory and the inhibitory action of ACh might be related to the extrinsic and intrinsic cholinergic components of the cortex, respectively (Parnavelas et al., 1986). Acetylcholine action is terminated by its breakdown by AChE.

Cholinergic receptors are divided in the nicotinic (ligand-gated ion channels) and the muscarinic (G-protein coupled) types, which are further divided into a number of subtypes (Maelicke, 1986; Perolta et al., 1988).

The cholinergic innervation of the cortex appears relatively late in development (Coyle and Yamamura, 1976; Kvale et al., 1983; Mc Donald et al., 1987); ChAT-positive perikarya are first observed at E17 in the basal forebrain (Armstrong et al., 1987; Dinopoulos et al., 1989) as well as the cortex (Dori and Parnavelas, 1989), but the cortical cells are only transiently positive, and the staining disappears after birth. The cortex then remains devoid of ChAT-positive elements until the middle of the second postnatal week, when weakly stained fibers and neurons appear; their number and staining intensity increases up to the fifth week, at which stage the density and distribution of cholinergic neurons and axons assumes an adult appearance (Dinopoulos et al., 1989; Dori and Parnavelas, 1989).

1.3. Neuropeptides

Several independent lines of investigation have led to the recognition of the involvement of peptides in CNS function. The concept of the “peptidergic neuron”

(Schamer, 1978) was initially related to the neurosecretory cells of the hypothalamus that release oxytocin and vasopressin into the blood stream from terminals in the posterior pituitary. However, the investigation of releasing factors and the characterization of thyrotropin-releasing factor broadened this concept (Fink, 1976; Guillemin, 1978). The isolation and characterization of substance P (Leeman and Mroz, 1974), a substance that had been detected by von Euler and Gaddum in 1931, was also pointing to the direction of peptides acting as neuromodulators and/or transmitters. A number of peptides have been demonstrated to be present in the mammalian cerebral cortex (for reviews see Parnavelas and McDonald, 1983; Jones and Hendry, 1986).

Somatostatin (somatotropin release inhibitory factor, SRIF) has been localized in the neocortex in many studies (Bennett-Clarke et al., 1980; Finley et al., 1981; Laemle et al., 1981; Morrison et al., 1981). SRIF-containing neurons are distributed throughout layers II-VI, primarily in layers II/III, V and VI of the rat neocortex (McDonald et al., 1982a; Lin et al., 1986; Meinecke and Peters, 1986). These are mostly multipolar or bitufted cell forms. Iontophoretic application has been demonstrated to cause both excitation (Ioffe et al., 1978; Olpe et al., 1980) and inhibition (Reneud et al., 1975) of cortical neurons. SRIF-containing neurons are first detectable on E17 (Eadie et al., 1987).

Vasoactive intestinal polypeptide (VIP) is present in 3% of the total cortical neuronal population, throughout layers II-VI, and primarily in layer II/III. These cells are vertical bipolar or multipolar (Emson et al., 1979; Lorén et al., 1979; Besson et al., 1982; McDonald et al., 1982b; McGregor et al., 1982). Iontophoretic application of VIP results in excitation of cortical neurons (Phillis et al., 1978;

Phillis and Kirkpatrick, 1980; Kelly, 1982). VIP-containing cells are first detected in the rat neocortex on the fourth postnatal day (McDonald et al., 1982b).

Cholecystokinin (CCK) is present in approximately 1% of the cortical neurons, primarily in layer II/III, but also in all other cortical layers (Emson, 1979; McDonald et al., 1982c; Hendry et al., 1983; Peters et al., 1983; Emson and Hunt, 1984). Iontophoretic application of CCK results in excitation of cortical neurons (Phillis and Kirkpatrick, 1980; Kelly, 1982; Chiodo and Bunney, 1983). CCK-positive neurons first appear in the rat neocortex at the time of birth.

Neuropeptide Y (NPY) has been demonstrated in the cortex by numerous studies (Lorén et al., 1979; McDonald et al., 1982d; Hendry et al., 1984; Wahle et al., 1986; Van Reeth et al., 1987). NPY-containing cells comprise 1-2% of the total neuronal population, are found in layers II-VI but mostly in II/III (McDonald et al., 1982d) and are first detectable at birth (Eadie et al., 1990).

A number of other peptides have also been reported to be present in the neocortex: *opioid peptides* (Finley et al., 1981; McGinty et al., 1984), *neurotensin* (Kobayashi, 1977; Emson et al., 1982), *substance P* (Brownstein et al., 1976; Nicoll et al., 1980), *motilin* (Chey et al., 1980), *secretin* (O'Donohue et al., 1981), *bombesin* (Brown et al., 1978), *α -melanocyte stimulating hormone* (O'Donohue et al., 1979), and *γ_3 -melanocyte stimulating hormone* (Shibasaki et al., 1981).

1.4. Calcium-binding proteins

There is a variety of proteins that are able to bind calcium ions (for reviews see Baimbridge et al., 1992; Andressen et al., 1993). These proteins are classified into

different families according to their molecular structure. CNS cells contain calcium-binding proteins (CaBPs) belonging to the EF-hand family, defined by the presence of a consensus amino acid sequence with a characteristic three-dimensional configuration; this is the site of high affinity Ca^{2+} binding, which is able to bind Ca^{2+} at physiological concentrations (Persechini et al., 1989). EF-hand family proteins can be further subdivided into “trigger” and “buffer” CaBPs; the former (calmodulin and troponin C) change configuration after binding Ca^{2+} , and can then modulate the activity of enzymes and ion channels, while the latter are believed to play a more passive role, buffering intracellular Ca^{2+} concentration. It is not clear, however, whether they have any additional roles (Baimbridge et al., 1992; Andressen et al., 1993). Three EF-hand buffer proteins occur in distinct neuronal subpopulations in the cortex, almost exclusively in nonpyramidal neurons.

Calbindin-D28K (CB) is found in bitufted, bipolar and double-bouquet cells of all layers, making up 5% of the total neuronal population. The strongest immunostaining is present in layer I, but there are weakly stained cells (which are, however, positive for CB mRNA) in other layers, including a small number of pyramidal cells (Celio, 1990; De Felipe et al., 1989a; Sequier et al., 1990).

Calretinin (CR) containing cells are bipolar, bitufted or vertically oriented multipolar forms. They are found in all layers being more abundant in layers II/III, and comprise less than 5% of the neuronal population (Jacobowitz and Winsky, 1991; Resibois and Rogers, 1992).

Parvalbumin (PV) is found in multipolar, bitufted or basket cells. Cells containing PV comprise 10% of the total neuronal population and are found in all layers except layer I, being most abundant in layers III and IV (Blümcke et al.,

1990; Celio, 1990; De Felipe et al., 1989b; Lewis and Lund, 1990).

However, not all of the cells belonging to these types express the respective CaBPs (Lewis and Lund, 1990; Rogers, 1992). Thus, immunolabelling for CaBPs cannot be used to estimate total neuronal populations; however, these proteins are excellent markers for qualitative analysis of different neuronal types as, by virtue of their high solubility, they are present throughout the cytosol, even in the thinnest processes, giving a Golgi-like appearance to immunolabelled neurons.

There is no colocalization of CB and CR, and only very limited colocalization between PV and CB or CR (Rogers and Resibois, 1992; De Felipe, 1993). There is generally no colocalization of CaBPs with neuropeptides; however, CR is colocalized with VIP in bipolar cells of the infragranular layers, and there is a limited colocalization of CB with SRIF (Rogers, 1992). There is also a small proportion of CaBP-containing neurons that are not GABA-positive (De Felipe and Jones, 1992; Rogers, 1992). It is interesting to note that, as in the case of peptides, CaBP content reveals neurochemical heterogeneity within populations considered as homogeneous by morphological criteria (Andressen, 1993).

The specification of CaBP phenotype is not dictated by lineage and may be correlated to neuronal connectivity (Mione et al., 1994). Calbindin and CR appear early in embryonic life (Sánchez et al. 1992, Fonseca et al, 1995), while PV appears only between postnatal days 4-8 in the rat cortex (Solbach and Celio, 1991). The appearance of the CaBPs is followed by a long period of maturation, during which transient expression has been observed. It has been postulated that these transient patterns of expression may be related to cell division, process elongation and cell motility during development, as Ca^{2+} ions play an important

role in these processes. In any case, the evolutionary conservation of the CaBPs aminoacid sequences (particularly those of CB and CR) argues for an important role in cell function, either during development or in maturity (Andressen, 1993).

1.5. Development of the cerebral cortex

1.5.1. Laminar organization

The diverse cell population that makes up the adult cerebral cortex has its origins in the proliferative zones that line the telencephalic ventricles during embryonic development (Boulder Committee, 1970). The *ventricular zone* (VZ) is a layer that contains pseudostriated epithelial cells (Sauer, 1935). These cells divide continuously and their nuclei move to and fro the ventricular surface during each cell cycle (interkinetic nuclear migration) (Sauer and Walker, 1959; Hinds and Ruffet, 1971; Seymour and Berry, 1975). S phase begins as the nuclei situated at the outer portion of the VZ start descending towards the ventricular surface; while descending, nuclei go through G₂, and divide (M phase) near the ventricular surface, after which they start ascending again as they go through the G₁ phase (Takahashi et al., 1993). The second zone to appear after the VZ is the *marginal zone* (MZ) (E14 in the rat). This is situated directly beneath the pial surface, consists of tangential fibers and a number of the earliest generated neurons of the cortex, primarily the Cajal-Retzius cells (Ramón y Cajal, 1911), and later becomes layer I of the mature cortex. Cajal-Retzius cells degenerate for the most part (Derer and Derer, 1990), but some persist as nonpyramidal neurons of layer I (Parnavelas and Edmunds, 1983). At this stage the MZ coincides with the structure described as

the *primordial plexiform layer* (PPL) by Marín-Padilla (1992). In contrast to older views, Marín-Padilla demonstrated this structure to be divided by the formation of the cortical plate (see below), its upper portion remaining as the MZ, while its lower part becomes the *subplate* or *layer VIIb* (or “*layer VII*”) (at E17-18 in the rat). The subplate, together with the MZ, contains the earliest generated cortical neurons (neurons generated before the splitting of the PPL in two) as was first observed by Cajal many decades before the introduction of the concept of PPL, and demonstrated with H³-autoradiography more recently (König et al., 1977; Raedler and Raedler, 1978; Luskin and Shatz, 1985b; Chun et al., 1987). The early synaptic (König et al., 1975; Kristt and Molliver, 1976; Raedler and Sievers, 1976; König and Marty, 1981) and neurochemical (Antonini and Shatz, 1990) maturation of these layers, as well as their early innervation by monoaminergic (Lidov and Molliver, 1982a; Wallace and Lauder, 1983; Verney et al., 1984) and thalamic fibers (Shatz and Luskin, 1986) suggest a possible early functional and/or organizational role in the developing cortex. In the subplate, some of the early neurons eventually die (Luskin and Shatz, 1985a; Valverde and Facal-Valverde, 1987), while others differentiate into interstitial neurons of the white matter in the adult (Kostovic and Rakic, 1980; Valverde and Facal-Valverde, 1988).

The next zone to appear after the MZ is the *intermediate zone* (IZ; E16 in the rat); it is situated between the VZ and the MZ and is made up of young neurons migrating away from the ventricular surface (Gadisseux et al., 1990). These neurons form the *cortical plate* at the intersection of the MZ and the IZ, splitting the plexiform primordium in two, as already mentioned. The cortical plate will accumulate all the neurons born from this point on, will gradually thicken and give

rise to cortical layers II through VI. Tritiated thymidine autoradiography experiments have demonstrated the existence of an “inside-out” pattern of neurogenesis: neurons destined to occupy the deeper layers are generated first, while the ones destined for the more superficial layers are generated later and migrate past the earlier cells to reach their final position (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1974; Raedler et al., 1980; Luskin and Shatz, 1985a). As a part of a more general reassessment of the established views on cortical structure and development, Marín-Padilla (1992) has suggested that the need of newly generated neurons to establish contact with layer I is behind the inside-out pattern of corticogenesis. Furthermore, pyramidal cells are redefined as those neurons that, while being displaced into deeper layers by newly arriving neurons, retain this contact (through their apical dendrites), in contrast to the nonpyramidal neurons that lose it. In this context, neurons that possess a morphology resembling that of pyramidal cells but do not retain contact with layer I (e.g. inverted pyramids) cannot be considered as true pyramids (Marín-Padilla, 1992).

At the base of the IZ appears the *subventricular zone* (SVZ), at E16 in the rat. Cells of this zone are small and undergo mitosis but not interkinetic nuclear migration (Bayer and Altman, 1991). The SVZ gives rise to glial cells during development and in adulthood and has also been demonstrated to give rise to some neurons destined for the olfactory bulb (Smart, 1961; Luskin, 1993; Lois and Alvarez-Buylla, 1993).

1.5.2. Neuronal migration

Various hypotheses have been put forth in the attempt to interpret the phenomenon of neuronal migration. Berry and Rogers (1965), observing Golgi-stained material at the light microscope level, described what they thought were binucleate cells with processes reaching both the ventricular and the pial surface of the developing cortex of the rat; these, they hypothesized, were cells that had had a nuclear (but not cytoplasmic) division at the level of the VZ. The new nucleus was supposed to be translocated to the cortical plate travelling through the apical process; after this would be completed, cytoplasmic division would take place. Morest (1970), using the same techniques to study the opossum embryo, also suggested that the cells' processes reach both surfaces but argued that, after terminal division, the nucleus is translocated through the apical process while the ventricular process is retracted.

However, it is the mechanism proposed by Rakic (1971b) that is widely accepted today: according to this interpretation, which was based on studies of the monkey cortex, neurons divide in the VZ and start migrating using radially oriented glial fibers to guide them to the cortical plate. Serial section electron microscopy has shown that the entire length of the migrating neuron maintains an intimate contact with radial glial fibers; it is this intimate contact that makes the two cells appear as one at the light microscope level (Rakic 1972, 1974). Similar observations have been made in the neocortex of the rat (Peters and Feldman, 1972) and mouse (Pinto Lord et al., 1982), as well as in the cerebellum (Rakic, 1971a) and the hippocampus (Nowakowski and Rakic, 1981) of the monkey. This

interpretation has led to the construction of the *protomap model* of development of the neocortex (Rakic, 1988). According to this model, the VZ is regarded as a mosaic consisting of proliferative units, each containing many clones of dividing cells. The cells generated in each unit move along the same glial fibre fascicles, thus projecting the spatial relationships of the VZ cells to the cortical plate and forming “ontogenetic columns”.

The recognition of radial glial cells by young neurons might be depending on cell adhesion molecules (Edelman, 1983) while the actual propulsion along the glial fibre is presumed to be achieved by mechanisms of differential cell adhesion. This can involve the local exo-endocytotic recycling of neuron membrane components with specific adhesion properties, that would result in the bound cytoplasmic process extending towards the direction of highest affinity to the glial fibre (Rakic, 1985). The release of proteolytic enzymes such as plasminogen and plasminogen activator (Moonen et al., 1982) at the tip of the growing process could enable cells to migrate through processes and between other cells, an issue becoming more important as cell packing density increases (Rakic, 1985).

Neuron-neuron and neuron-extracellular matrix (ECM) interactions may also be of importance, particularly in the termination of migration, as it has been suggested by D’Arcangelo et al. (1995) in their paper reporting the cloning of the *reelin* gene. *Reeler* is an autosomal recessive mutation of the mouse that results in tremor, impaired motor coordination and ataxia (Falconer, 1951). The pathological condition results from an abnormal pattern of migration: neurons migrate normally towards the cortical plate but stop upon reaching it, instead of migrating through it; this results in an inverse (outside-in) cortical lamination (Caviness and Sidman,

1973). The sequence encoded by the reelin gene (reelin protein) has an absence of transmembrane domains, indicating that it is a secreted extracellular protein showing homology to F-spondin, a protein secreted by the floor plate of the developing spinal cord that is considered to regulate growth and guidance of axons on the spinal cord and the peripheral nervous system and to tenascin (D'Arcangelo et al., 1995). D'Arcangelo et al. (1995) demonstrated by in situ hybridization that the reelin gene is expressed in the MZ of the normal mouse, presumably by Cajal-Retzius cells. It has also been shown that the MZ of the reeler mouse appears to contain less ECM than that of the normal animal (Goffinet et al., 1984). These findings led the authors to postulate that reelin is secreted by Cajal-Retzius cells to form an ECM environment appropriate for the normal termination of neural migration.

It must be added that that the radial migration hypothesis, although generally applicable, does not seem to explain the migratory behaviour of all cortical neurons. Horizontally oriented cells have been found in the SVZ and IZ (Lauder et al., 1986; Parnavelas, 1992; De Diego et al., 1994). Tangential migration has been demonstrated in the IZ (O'Rourke et al., 1992, 1995), while lateral dispersion of progenitor cells within the VZ has also been shown in cortical explants (Fishell et al., 1993). Walsh and Cepko (1992), using a cocktail of genetically engineered retroviral vectors, have demonstrated that daughters of a single progenitor cell can be located in widely spaced cortical areas.

Figure 2

Toluidin blue stained semithin sections through the E16 (A) and E19 (B) rat cortex.

ppl= primordial plexiform layer

svz= subventricular zone

iz= intermediate zone

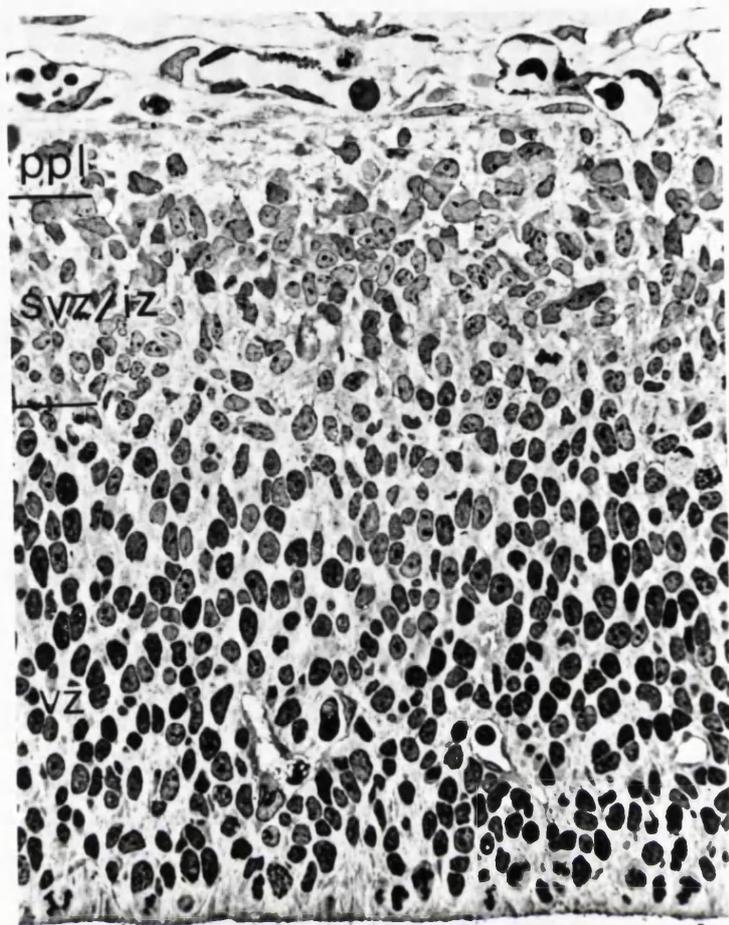
vz= ventricular zone

I= layer I

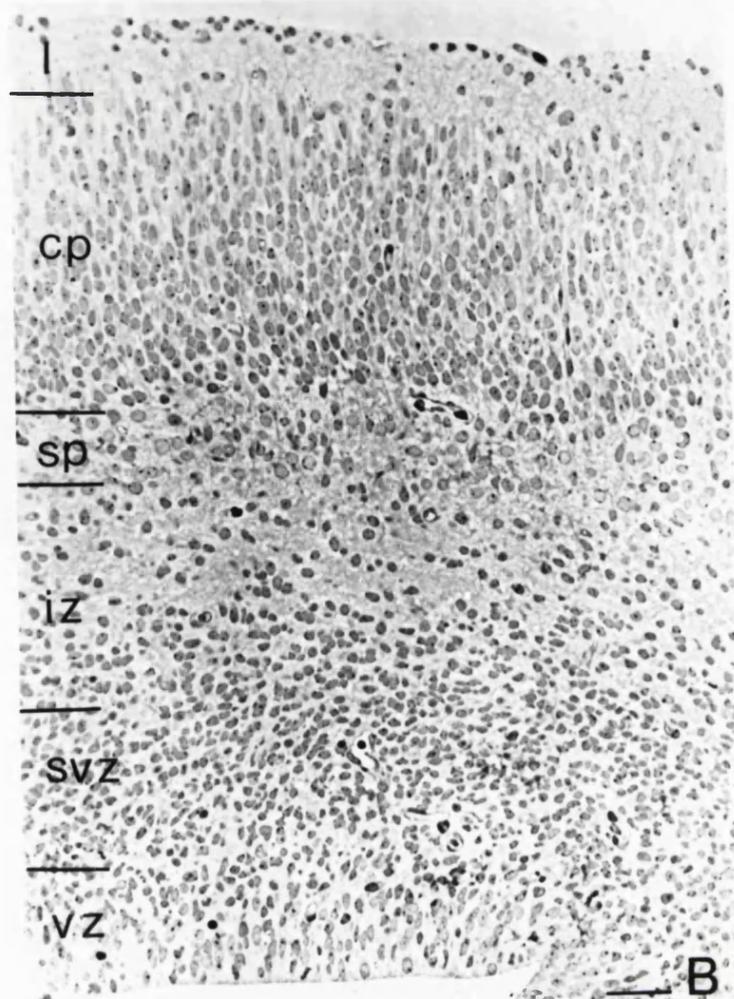
cp= cortical plate

sp= subplate

Scale bars: A= 20 μm , B = 40 μm



— A



— B

1.5.3. Differentiation

The elucidation of the processes involved in the development of different cell types with specialized structure and function from their less specialized progenitors, all the way back to the zygote, is the main goal of developmental biology. In the cortex, this issue is particularly related to the properties of the VZ. Numerous studies in the recent years (for reviews, see McConnell, 1988, 1991, 1995) have focused on different aspects of the same fundamental question: are VZ cells committed to the production of a specific type of cortical cells, or are they pluripotential, each being able to give rise to different cell types? Are the two scenarios mutually exclusive? Predating the current discussion by a century (for review see Jacobson, 1991), the issue of how the VZ is able to produce both neurons and glia has been argued since 1889, when His suggested that two major cell types coexist in the “germinal matrix” (VZ), the “germinal cells” (neuronal precursors) and the “spongioblasts” (glial precursors), which he described as forming cellular syncytia. Eight years later, Schaper proposed that what His had observed were in fact cells of the same type at different phases, something later confirmed by Sauer (1935). Schaper concluded that the same cells give rise to both neurons and glia. The study of Fujita (1963), which employed autoradiography, also supported the homogeneity of the VZ. Fujita’s interpretation of the production of the two cell types was that, after neurogenesis ceases, the same dividing population generates glia. In a study of the developing monkey cortex utilizing immunocytochemistry for GFAP and electron microscopy, Levitt et al. (1981) demonstrated the existence of glial elements in the VZ at the time of neurogenesis, proving the concept (though not the details) of His’s interpretation to be correct.

Using a recombinant retrovirus as a lineage marker, studies have shown that neuronal and glial cell lines have been shown to have diverged as early as E12-E14 in the mouse (Luskin et al., 1988; Sanes, 1989) and E15-E16 in the rat (Luskin et al., 1993). In an important step towards identifying the mechanisms behind the generation of the two cell types, it has been demonstrated that the presence or absence of a recently identified gene, the glial cell missing (gcm) gene, can determine the choice of neuronal or glial fate (Hosoya et al., 1995; Jones et al., 1995; Anderson, 1995). One step further in the same line of investigation is the question whether neuronal progenitors are subdivided into pyramidal and nonpyramidal neuron progenitors. The questions associated with neuronal differentiation are not in fact any more recent than the debate on neuron-glia divergence: in 1896, von Kölliker wrote that "...all nerve cells at first possess the same function and...their differentiation depends solely and entirely on the various external influences or excitations which affect them..." (translation from Jacobson, 1991). As in the neuron/glia conundrum, the issue of how different cell types are produced is inextricably linked with the question when the cell lines that produce them diverge. In the rat, retroviral studies have shown that neuronal clones generated from progenitors labelled as early as E14 are exclusively pyramidal or nonpyramidal (Luskin et al., 1993; Mione et al., 1994), supporting the existence of separate pyramidal and nonpyramidal progenitors in the cortex. The possibility that some of the visualized clusters could be parts of bigger clones cannot be dismissed, in view of the evidence for widespread dispersion of clonally related cells (Walsh and Cepko, 1992). However, the fact that the cells that do stay in the same cluster are of the same phenotype may in itself provide clues relating the

cells' phenotype and their migratory behaviour. Cortical area specification has also been shown to occur early, as expression of the limbic system associated protein (a marker of limbic cortex cells) has been detected in young postmitotic neurons destined for the limbic cortex as early as E14 (Levitt et al., 1984; Barbe and Levitt, 1991). Laminar specification is not lineage-related, as cells in a clone can span several layers (Luskin et al, 1988,1993; Mione et al., 1994). McConnell and Kaznowski (1991) have demonstrated that commitment to a laminar position occurs in the VZ at the time of mitosis; cells transplanted late during the cell cycle from younger to older animals migrate to a low layer position, as they would do in the donor, whereas cells taken at earlier stages of the cycle assume the same position as cells of the host generated during the same period.

1.5.4. Neurotransmitters as developmental signals

Aminoacids

Different types of *EAA* receptors have distinct patterns of distribution, and periods of peak receptor expression coincide with critical periods in synaptogenesis and plasticity (for reviews see Constantine-Paton et al., 1990; McDonald et al., 1990). Receptors appear before the initiation of synaptic transmission (de Barry et al., 1980; Slevin and Coyle, 1981; Voukelatou et al., 1986) and, during early stages of differentiation, neurons express supersensitive Glu receptors (Garthwaite and Balazs, 1978; Baudry et al., 1983). As the elimination of neurons is a fundamental process in CNS development (Cowan et al., 1984; Clarke, 1985), it is possible that exposure to slightly higher concentrations of EAAs during development or the

presence of more sensitive receptors is a developmental mechanism that uses excitotoxicity as a means of shaping the cortex (Meier et al., 1994). This is also supported by the finding that the vulnerability of neurons to EAA changes during development depending on the neuron and receptor type (Garthwaite and Garthwaite, 1986; Hajos et al., 1986; Frandsen and Schousboe, 1990). However, trophic effects have also been demonstrated, as Balasz et al. (1988a,b) reported that stimulation of NMDA receptors on cerebellar granule cells in culture prevented the cell loss normally observed in defined medium. NMDA has also been reported to enhance the differentiation of these neurons (Moran and Patel, 1989), while depolarization by high $[K^+]$ produces similar effects (Scott and Fisher, 1970; Gallo et al., 1987; Collins and Lile, 1989), suggesting that the effects of NMDA are a result of the depolarization it induces. Aruffo et al (1987) reported that low doses of Glu promote neuronal growth and differentiation in vitro, while LoTurco et al. (1995) demonstrated that Glu depolarizes cells in the VZ of the developing cortex, increases $[Ca^{+2}]$ (in part through activation of voltage-sensitive Ca^{+2} channels), and decreases the number of cells synthesizing DNA. This effect, which is mediated by AMPA and KA receptors, could be replicated by elevated $[K^+]$, indicating, in agreement with previous studies, that EAA can exert a developmental role, at least in part, by acting as depolarizing agents. The same effect was also observed as a result of *GABA* application. This effect was shown to be mediated by the $GABA_A$ receptor, whose presence in the VZ had been demonstrated a few years previously (Herb et al., 1992). $GABA_A$ receptor subunits are expressed prenatally in a region- and age-specific manner (Araki et al., 1992; Poulter et al., 1992). Other *in vitro* studies have also suggested a possible role for GABA as trophic or

regulatory factor in development (Spoerri et al., 1988; Eins et al., 1983; Hansen et al., 1984). Transient GABA-containing cells in the developing cortex have a mature appearance at a time when other cortical neurons are still immature. This, together with the early neurotransmitter expression, synaptogenesis and projections of these cells suggests that they are part of a transient network with a role in development (Van Eden et al., 1989; Lauder, 1993).

Monoamines and acetylcholine

Serotonin is regarded as a strong candidate for a role as a developmental signal (Lauder et al., 1990, 1993). It appears very early (E12) in the embryonic rat brain (Aitken and Törk) and is present in the cortex from as early as E16 (Lidov and Molliver, 1982a; Wallace and Lauder, 1983). The presence of 5-HT receptors in the embryonic cortex (Whitaker-Azmitia et al., 1987; Roth et al., 1991; Hellendal et al., 1993; Lidow and Rakic, 1995) and in neuroepithelial cells (Johnson and Heinemann, 1995) has been documented, and the demonstration of the “functional” status of embryonic 5-HT receptors (Whitaker-Azmitia et al., 1987) further support the notion of 5-HT acting as a developmental signal *in utero*. A number of studies have indicated that 5-HT could affect various aspects of neuronal development and differentiation, before assuming its neurotransmitter function in the adult brain. In *Helisoma* neurons *in vitro*, 5-HT has been demonstrated to inhibit neurite outgrowth (McCobb et al., 1988a,b), while stimulation of 5-HT_{1A} receptors has the same effect on cultured rat cortical neurons (Sikich et al., 1990). The addition of 5-HT to the culture medium has been reported to stimulate

synaptogenesis and morphological differentiation, glial proliferation and myelination as well as to accelerate the appearance of spontaneous activity in neocortical (Chubakov et al., 1986) and hippocampal (Gromova et al., 1983; Chubakov et al., 1993) slices, while it promotes glial influences in cultured 5-HT- and DA-containing neurons (Liu and Lauder, 1992). Depletion of 5-HT during embryonic development has been shown to retard neuronal differentiation (as determined by the termination of mitotic activity) in regions containing 5-HT target cells (Lauder and Krebs, 1976, 1978). Serotonin has also been shown to be involved in postnatal cortical development, influencing plasticity in the kitten cortex (Gu and Singer, 1995).

The early appearance of *dopamine* in the brain (Specht et al., 1981), the early arrival of dopaminergic fibers (Kalsbeck et al., 1988) as well as the presence of DA receptors in the embryonic cortex of the rat (Reinoso et al., 1996; Diaz et al., 1997) and the macaque monkey (Lidow and Rakic, 1995) suggest the possibility that this monoamine is involved in cortical development. Exposure of cultured *Helisoma* neurons to DA results, as in the case of 5-HT, in a decrease in neurite outgrowth (McCobb et al., 1988a,b). A similar effect, mediated by the D₁ receptors, has been observed in cultured chick retinal (Lankford et al., 1987, 1988) and rat cortical neurons (Reinoso et al., 1996), while stimulation of D₂ receptors results in enhanced neurite growth in cultured cortical neurons (Todd, 1992; Reinoso, 1996). It has also been demonstrated that DA can regulate the size and the expression of cytoskeletal components of cortical neurons *in vitro* (Reinoso et al., 1996). Studies utilizing lesions of the ventral tegmental area have suggested a trophic role for DA in the early postnatal period (Kalsbeck et al., 1987, 1989).

Noradrenaline is, like the other monoamines, present from early on in the brain (Olson and Seiger, 1972) and reaches the cortex during the period of rat cortical neurogenesis (Levitt and Moore, 1979; Verney et al., 1984). Adrenergic receptors (of the β_1 type) have been demonstrated in the cortical plate of the mouse at E14 (Goffinet et al., 1986). Noradrenaline has been proposed to play a role in synaptogenesis in the immature rat visual cortex (Blue and Parnavelas, 1982; Parnavelas and Blue, 1982), as well as to be involved in cellular maturation (Morris and Slotkin, 1985) and to have a neurotrophic role (Felten et al., 1982). In addition, it has been implicated in the regulation of plasticity in the rat (Kolb et al., 1992) and kitten (Pettigrew and Kasamatsu, 1978; Kasamatsu et al., 1979, 1985) visual cortex.

Until recently, little attention had been given to the possibility that *acetylcholine* might have a developmental role in the cortex, as the cortical cholinergic innervation is established relatively late (Dori and Parnavelas, 1989). However, a number of studies have suggested such a role for ACh. *In vitro*, it has been shown to stimulate glial cell proliferation (Ashkenazi et al., 1989) and prevent the inhibitory action of 5-HT on *Helisoma* neurite outgrowth (McCobb et al., 1988a), while blockade of ACh action with antagonists enhances process outgrowth from rat retinal ganglion cells (Lipton et al., 1988). Acetylcholine has also been suggested to influence plasticity (Bear and Singer, 1986; Gu and Singer, 1993; Liu et al., 1994), the maturation of cellular morphology (Höhmann et al., 1991) and cytoarchitecture (Höhmann et al., 1988) in the developing cortex.

2. Materials and methods

2.1 Materials

Neurotransmitters: Serotonin, NA, DA and ACh (Sigma).

Culture media and supplements: DMEM/F12, glutamine, bovine serum albumin, insulin, progesterone, putrescine, selenium, thyroxine, transferrin triiodothyronine (Sigma), bovine holo-transferrin (Gibco).

Antibodies: Rabbit anti-glutamate, rabbit anti-GABA, (Sigma), rabbit anti-calbindin-D_{28KD} (SWant, Switzerland), rabbit anti-calretinin (SWant, Switzerland), rabbit anti-nestin (gift of Dr. R. McKay, NIH), rabbit anti-fibrillary acidic protein (GFAP; Dakopatts), rabbit anti-5-HT (Incstar), mouse anti- BrdU (Sigma), mouse anti-TUJ1 (gift of Dr A. Frankfurter), biotinylated goat anti-rabbit and biotinylated goat anti-mouse (Vector).

Materials used for high pressure liquid chromatography: sodium phosphate dibasic (Fluka, Microselect), sodium phosphate monobasic (J.T. Baker, Ultrapure), sodium tetraborate, β -mercaptoethanol, methanol (Baxter, HPLC grade), perchloric acid (69%), phosphoric acid (85%), o-phthalaldehyde (Eastman-Kodak). All amino acids were purchased from Sigma. Chromatography columns: C18 reverse phase column (Spherisob ODS2, Hichrom Ltd, Reading, UK) and HR 80 reverse phase column (ESA).

Other materials: avidin and biotin (Vector), foetal calf serum (FCS; Gibco), normal goat serum (NGS; Seralab), Gays balanced salt solution (GBSS; Gibco), Hanks balanced salt solution (HBSS; ICN), phosphate buffer saline (PBS) tablets

(Unipath), boric acid (Aldrich), 30mm culture plate inserts (Millipore), 24-well plates (Greiner), penicillin/streptomycin (P/S; ICN), diaminobenzidine (DAB) tablet sets, ethylene-diamino-tetraacetic acid (EDTA), poly-l-lysine (PLL), 5-bromo-2-deoxyuridine (BrdU), glucose, trypsin, agar and low gelling temperature agarose (Sigma), halothane (ICI pharmaceuticals). Resin ingredients: Araldite CY212, dodecynyl succinic anhydrite (DDSA), plastisizer dibutyl phthalate and accelerator benzyldimethylamine (BDMA), all from Agar Scientific.

Preparations were observed under a Leica microscope and photographed using Ilford PAN-F (ISO 50) black and white negative film, and the plates were printed on Ilford resin-coated multigrade IV paper. Ultrathin sections were obtained with a Reichert-Jung Ultracut E ultramicrotome and observed under a Zeiss 910 electron microscope.

2.2. Experimental procedures

2.2.1. Slice culture preparation

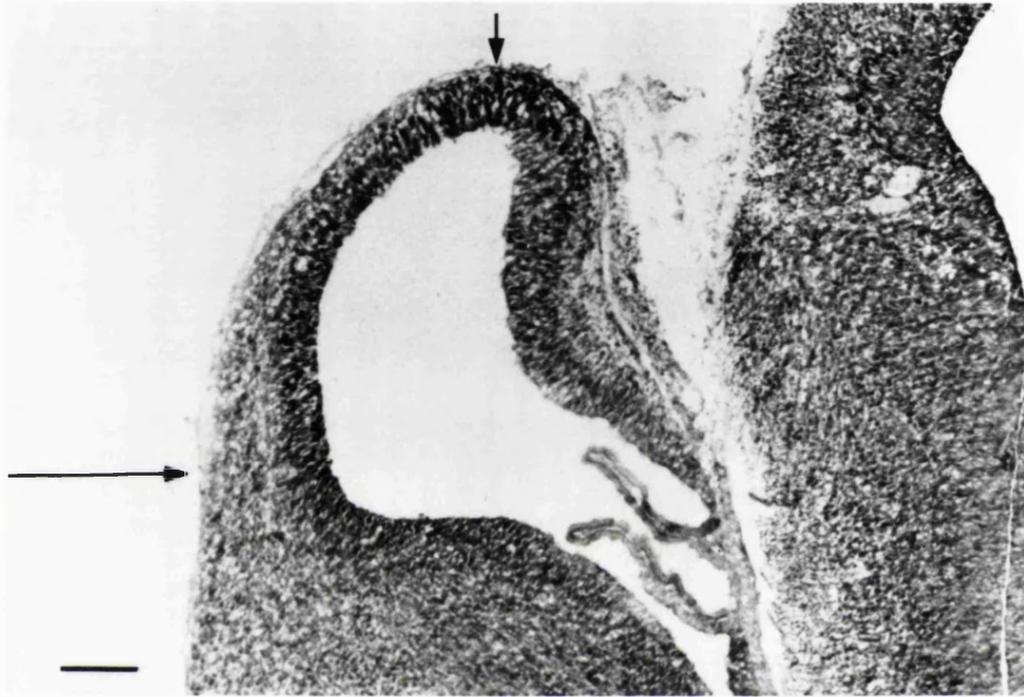
Pregnant Sprague-Dawley rats were sacrificed by cervical dislocation on the 14th (N=20), 16th (N=85), and 19th (N=36) day of gestation. The embryos were rapidly removed and placed in 4°C GBSS supplemented with glucose (6.5 mg/ml). Newborn rats (N=14) were anaesthetized with halothane. The following procedures were all performed under sterile conditions. The brains were removed and placed in a 3% solution of agar in 0.1 M pH 7.2 phosphate buffer saline (PBS) at 40°C. The agar was subsequently hardened on ice, and the brains were cut

with a Vibroslice (Campden Instruments) at 400 μm . The slices were kept for 50 minutes in 4°C GBSS/glucose to allow for deterioration of enzymatic activity released by damaged cells. In each experiment, slices taken from the cerebral wall of all embryos of a pregnant rat were dissected out and transferred onto millicell CM membranes in 30 mm petri dishes (according to Stoppini et al., 1991) containing 1 ml of either defined medium (DM) or DM+ neurotransmitter (plus 5% FCS for the first day *in vitro*). Excess medium, carried over when transferring the slices, was removed by aspiration after the slices had been positioned. Defined medium consisted of DMEM/F12 mixture supplemented with 6.5 mg/ml glucose, 0.1 mM glutamine, 100 mg/ml Penicillin/Streptomycin, 100 mg/ml bovine serum albumin, 5 mg/ml insulin, 20nM progesterone, 16 mg/ml putrescine, 30 nM selenium, 0.4 ng/ml thyroxine, 100 mg/ml transferrin and 0.3 ng/ml triiodothyronine. Serotonin (200 μM), NA (100 μM), DA (50 μM) or ACh (100 μM) was added to the medium of E16 slices for the duration of the culture period, normally 7 days; E14 and E19 cultures were exposed to 5-HT only. In 4 experiments, E16 cultures were exposed to 5-HT for the first 4 or the last 3 days in culture only. In 3 experiments, after an effect of 5-HT had been established, cortical slices were also exposed to different concentrations of 5-HT (20, 40, 80, 120 and 160 μM), so as to produce a dose-response curve. In 7 experiments co-cultures were prepared, in which slices containing the raphe nuclei of E19 rat embryos (according to Altman and Bayer, 1995) were placed onto the membranes, adjacent to E16 cortical slices; these cultures were grown in defined medium only. A number of E16 slices were kept for 10 days *in vitro* (DIV) in defined medium

Figure 3

Coronal (slightly oblique in the rostrocaudal direction) 15 μm thick cryostat section of an E16 rat brain stained with toluidin blue. For slice culture preparation, the cortical primordium was excised at the points indicated, between the prospective hippocampal structures (medial to the short arrow) and the basal ganglia and piriform cortex (medial and ventral to the long arrow).

Scale bar = 160 μm .



only. In all cultures, the medium was exchanged every second day. Culture survival was assessed by the change in the colour of the medium.

2.2.2. Immunohistochemistry

At the end of the culture period, ten slice cultures were fixed with 4% paraformaldehyde in 0.1 PBS and processed as wholemounts for immunohistochemistry for Glu or GABA in order to qualitatively assess their neurochemical composition. Slices taken from three experiments (control and 5-HT treated cultures) were also fixed, sectioned with a cryostat, collected onto poly-l-lysine coated slides, and stained with an antibody against TUJ1, a marker of immature neurons (1:500; Lee et al., 1990). All other cultures were dissociated, embedded in agarose, and immunostained as described by Vaccarino et al. (1995). According to this method, the medium was washed away with 0.1 M PBS and slices were dissociated with 0.25% trypsin containing 0.003% EDTA. In the case of the co-cultures, the cortical slice was separated from the slice of midbrain, using a razor blade, and transferred into a different petri dish prior to dissociation. Thirty minutes later, the cell suspension was centrifuged at 200 g for 3 minutes. The supernatant was then removed and the cells resuspended and fixed with 4% paraformaldehyde in PBS for 1 hour. They were subsequently washed in PBS and resuspended in a solution of 1.8% low gelling point agarose in 0.1M PBS at 45° C. The agarose solution containing the cells was then poured between two glass slides separated by two No1 coverslips, and was left to set in the refrigerator for 10 minutes. Pieces of the agarose films containing the cells were subsequently

incubated with one of the following cell-specific markers: anti-Glu (1:500), anti-GABA (1:500), anti-CB or anti-CR (1:1000; subpopulations of nonpyramidal cells), anti-nestin (1:1000; neuroepithelial cells; Lendahl et al., 1990) and anti-GFAP (1:500; astrocytes). The bound immunoglobulins were visualized with the avidin-biotin-peroxidase (ABC) method (Bayer and Wilchek, 1980; Hsu et al., 1981) using DAB as the substrate. Embryonic day 16 cultures that were kept for 10 days in vitro (DIV) were only stained for Glu and GABA.

The stained agarose films were mounted on slides (in PBS/glycerol), and fields of labelled cells were observed under the microscope. The total number of cells and the number of immunostained cells per field were counted in randomly selected fields with the use of a 250 μm square graticule under a x20 objective lens. A minimum of 10 fields (approximately 500 cells) were counted in each of 4 separate experiments for each culture condition, and the proportion of immunostained cells in the whole cell population was calculated for each experiment. Analysis of variance (ANOVA) was used to compare the mean percentages of cells stained with each antibody in the control group to those in the cortices exposed to monoamines and in the co-cultures. Once an effect of 5-HT on E16 slices had been established with the concentration used, additional concentrations were tested in order to produce a dose-response curve. In 3 experiments, cortical cells from animals of all 4 ages were not put in culture but were acutely dissociated, immunostained and quantified as above.

To examine whether the axons of 5-HT-containing neurons in the raphe nuclei innervated the cortical slices during the culture period, raphe-cortex co-cultures were fixed with 4% paraformaldehyde in PBS for 1 hour and removed

from the membranes en bloc. They were then stained as wholemounts with anti-5-HT antibody (1:500 in PBS containing 0.5% Triton-X 100 and 10% NGS overnight at room temperature) using the ABC method and DAB as substrate.

The immunohistochemical procedures were as follows: agarose slices or culture wholemounts were placed in 24-well plates and incubated in 0.1M PBS + 0.5% Triton-X-100 + 10% NGS, in order to mask non-specific binding sites. Cryostat sections were ringed with a DAKO pen and the solutions were applied on the slides, which were kept in a moist chamber. After 1 hour of incubation, the serum solution was removed and the specimens were incubated in the primary antibody, diluted in 0.1M PBS + 0.5% Triton-X-100 + 10% NGS overnight in 4°C. Specimens were then rinsed 3 times in PBS and incubated for 3 hours in room temperature (RT) on a shaker in biotinylated goat anti-rabbit or goat anti-mouse antibody (for detection of bound polyclonal and monoclonal primary antibodies, respectively) diluted 1:200 in PBS. During this incubation, the avidin-biotin complex was prepared by mixing avidin (1:100) with biotin conjugated with horseradish peroxidase (1:100) in PBS and leaving the solution on a rotator for >30 minutes. After the end of the incubation period, the specimens were again rinsed in PBS and then incubated in the avidin-biotin complex for 2 hours on a shaker. They were then washed in PBS and incubated in 0.03% DAB + 0.01% H₂O₂ in 0.1 M Tris buffer. The development of the DAB was monitored under a stereomicroscope and the reaction was stopped when necessary, by removing the DAB solution and washing with PBS. To increase section adhesion, microscope slides used for the collection of cryostat sections had been coated with poly-L-lysine (Huang et al., 1983) according to the following procedure: Slides were

placed in a metal rack and immersed in chromic acid for at least 3 hours. They were then washed in running tap water for 30 minutes, washed twice in distilled water and immersed for 5 minutes in a poly-L-lysine solution at RT. They were finally dried overnight in a 37⁰C oven.

Six cortical slices that had been co-cultured with raphe slices were processed for electron microscopy following immunohistochemical staining for 5-HT: they were osmicated (1% OsO₄ for 30 min), washed with 0.1 M sodium acetate, stained with uranyl acetate (1 % for 30min) and dehydrated through a series of graded alcohols: 25% (2x5 min), 50% (2x5 min), 70% (2x5 min), 95% (2x20 min), 100% (2x30 min). The resin mixture was prepared as follows: 53 g Araldite, 47 g DDSA and 1.5 ml plasticizer were mixed thoroughly, then 1 ml BDMA was added and the solution was once again mixed thoroughly. The slices were kept in this mixture overnight in room temperature, and the following morning they were placed in a fresh mixture and flat embedded in Araldite on large glass slides, sandwiched between two acetate sheets. The resin was cured for 48 hours at 70⁰ C. After that period, the slices were removed from the slides using a razor blade under a stereomicroscope and were mounted on Araldite stubs for ultrathin sectioning. Ultrathin sections (silver-gold interference colours) were collected on carbon-coated nickel grids. Sections were then stained with uranyl acetate (3% for 30 min), washed in distilled water, stained with Reynol lead citrate (30 min) , washed and dried before observation.

2.2.3. High pressure liquid chromatography

High pressure liquid chromatography (HPLC) was used to measure the concentration of 5-HT in the medium of cortical cultures at various time points after the addition of the monoamine, as well as to quantitate the Glu content in the cortical slices in raphe-cortex co-cultures. Measurements of 5-HT were performed using HPLC with coulometric detection. 200 μ l samples were taken from the culture medium of 3 cultures every 6 hours. The first sample was taken at 18 hours and the last at 36 hours *in vitro*. The samples were first frozen and stored at -70° C, and were subsequently diluted 1:10 with 0.1 M perchloric acid containing 0.4 mM sodium bisulphite before use. After low speed centrifugation the supernatants were analyzed using the method of Reinhard et al. (1980). Separation was achieved with a C18 reverse phase column using a mobile phase of 0.1 M sodium acetate buffer (pH 4.7) containing 0.1 mM EDTA and 12% (v/v) methanol at a flow rate of 2.0 ml/min. Quantitation was achieved with a coulometric detector with the following settings: guard cell: +0.35V; detector 1: +0.33 V; detector 2: -0.30V.

For measurement of the Glu content, 8 groups each of 3 control slices and 9 groups of the same number of slices that had been co-cultured with raphe nuclei (taken from 4 pregnant rats) were frozen and stored at -70° C until protein extraction. To extract protein, frozen cultures were gently removed from the membrane on which they had been grown, using ice-cold PBS, and their weights determined. Ten volumes per weight of 0.2 N perchloric acid were added, and the tissue was sonicated on ice twice for 30 seconds. The contents were then centrifuged at 8000 g for 1 minute. The supernatants were collected, filtered through Millipore filters and frozen. These samples were stored at -20° C until the

amino acid analysis was performed. A modified HPLC method for isocratic separation and determination of amino acids was used to measure levels of Glu (Donzati and Yamamoto, 1988). The method used provided conditions which optimized the sensitivity, resolution and stability of precolumn derivatization of amino acids using o-phthaldehyde (OPA) and b-mercaptoethanol (BME). Briefly, precolumn derivatization was performed by a Gilson 231 XL sampling injector with a 20 ml titanium loop (autosampler). A diluted OPA/BME stock solution was mixed with the sample solution in a proportion of 2:1. To correct for injection variability, an internal standard (homoserine) was used in the sample preparation. After a 2 minute incubation time, the samples were injected on a reverse phase HR-80 column (ESA). Amino acids were detected with an ESA coulochem II multi-electrode detector and the data was collected by a SP4400 integrator (Thermo Separation Products). Glutamate content, expressed as mmoles/l, was determined by comparing the area under the peak for each amino acid versus a standard peak for that amino acid. Three measurements were taken from each culture group, and a mean was calculated. Eight such means from the controls and 9 from the co-cultures were used in total, and the two conditions were compared using an ANOVA test.

2.2.4. BrdU incorporation experiments

5-bromo-2-deoxyuridine incorporation was used to assess cell proliferation (Miller and Nowakowski, 1988). In 4 experiments, slice cultures were exposed to BrdU (10^{-5} M) for 16 hours and then fixed with 4% paraformaldehyde in 0.1 M PBS for 1 hour; different slices were exposed to BrdU at each of the 7 DIV. They were

subsequently cryoprotected in 20% sucrose in PBS and sectioned at 10 μm with a cryostat. The sections were collected onto microscope slides coated with poly-L-lysine and treated with 2N HCl for 1 hour. They were then incubated in 0.1 M borate buffer, pH 8.4 (2x15 min), rinsed with PBS, and stained with anti-BrdU antibody (1:500 in PBS containing 0.25% Triton-X 100 and 5% NGS overnight at 4° C) using the ABC method and DAB as substrate. The calculation of the mean percentage of labelled cells in the proliferative zones for each day and culture condition was performed as above.

3. RESULTS

The results of this study can be divided into two parts. In the first part, a description is given of the morphology and the neurochemical content of cell populations present in slice cultures prepared at four stages of the development of the rat neocortex and kept for 7 days *in vitro*. In the second part, the effects of exposing the cultured slices to a number of neurotransmitters (5-HT, DA, NA, ACh) are described. The study focuses on the effects of the neurotransmitters in the neurochemical markers expressed by the cortical cells, and these effects are assessed by quantitating the changes in proportions of the cells in the overall cell population. Of the neurotransmitters tested, 5-HT was found to significantly affect the proportions of the Glu-containing cells. The effect of the addition of 5-HT was further verified by utilizing a co-culture system, in which the cortical slices were innervated by 5-HT-containing neuronal processes emanating from adjacently placed slices of the raphe nuclei, thus replicating *in vitro* the phenomenon of the early invasion of the cortex by serotonergic afferents.

3.1 Cell phenotypes in the developing cortex prior to culturing

Cortices from rats at the four developmental stages used in this study, namely E14, E16, E19 and P0, were acutely dissociated, embedded in agarose and immunostained for cell-specific markers so as to obtain information on the

neurochemical content of the cortices before culturing (fig. 18). *Glutamate* immunoreactivity was not detected in the embryonic cortex at any of the three stages studied, whereas a discernible but not quantifiable background staining first appeared in the newborn. *GABA* immunoreactivity at E14 resembled that of Glu at P0, but was readily quantifiable at more advanced stages. Thus, cells labelled with immunohistochemistry against GABA comprised 6.96 % of the total cell population at E16, 8.76 % at E19 and 29.1 % at P0. *Calbindin*-positive cells increased from 5.4 % at E14 to 10.5 % at E16, and later decreased both in proportion and intensity of immunoreactivity at E19 (3.2%) and P0 (5.1%). *Calretinin*-positive cells showed the highest proportion at E14 (7.02%), while at E16 they represented 5.4% and at E19 4.1 % of the cell population, starting to increase again at P0 (6.0%). Nestin-positive cells, a population comprising the undifferentiated neuroepithelial cells of the developing cortex as well as radial glial cells, constituted the majority of the cell population at E14 (73.6%), appearing in decreasing proportions in E16 (43.9%), E19 (26.7%) and P0 (7.4%). Glial fibrillary acidic protein-containing cells, the astrocytic element of the cortex, were absent from the embryonic preparations at all three stages, whereas a small proportion of cells (<1%) were found to be reactive for GFAP in the cortex at birth.

3.2 Control slice cultures

3.2.1. E14

Cell phenotypes

Wholemound immunostained slices revealed the presence of both Glu- and GABA-containing neurons. Glutamate - containing cells had an immature appearance with a round or ovoid cell body shape (fig.4A). GABA-containing neurons (fig.4B), as well as the neurons reactive for CB (fig. 5A) and CR (fig. 5B), seemed to be in a somewhat more advanced stage of morphological differentiation, showing discernible processes. They, none the less, also looked immature, with small ovoid cell somata and unbranched processes. Some CR-containing cells, however, were bigger with longer processes (fig. 5B), and were identified as Cajal-Retzius cells (Del Rio et al., 1995). Nestin staining revealed monopolar cells and long linear processes throughout the slice (fig. 6A), whereas GFAP- positive cells were few in number, weakly stained and with only a few small processes (fig. 6B).

Cell counts

In agarose films, cells labelled with cell-specific markers (Glu, GABA, CB, CR, nestin, GFAP) appeared as round and darkly stained, often in clusters of 3 or more cells (figs. 16, 17). Unlabelled cells showed light background staining, and their identification was confirmed at times with phase contrast microscopy. Counts of labelled and unlabelled cells were made, and the proportion of each cell population was determined . In slices prepared from E14 animals, Glu-labelled neurons were

19.2% of the total cell population, GABA containing neurons represented 29.9%, nestin immunoreactive cells were 11.4%, and CB-, CR- and GFAP-labelled cells comprised 2.8%, 3.5% and 3.2% of the overall cell population respectively (fig. 19A)

3.2.2. E16

Cell phenotypes

Wholemout immunostained slices revealed the presence of both Glu- and GABA-containing neurons throughout the cortical thickness. Most of these cells had more intensely stained somata than those found in E14 slices, and a number of short and sparsely branched processes. (fig. 7) Overall, the GABA-containing neurons were once more in a more advanced stage of differentiation as compared to the Glu-containing cells, and they also showed more elaborate and longer processes (fig. 7B) as compared to GABA- containing cells in E14 cultures. Cells immunoreactive for CB (fig. 8A) or CR (fig. 8B) were generally of similar morphology as those stained for GABA, with CR- containing cells often exhibiting longer stained processes. Labelling for nestin again revealed a radial network of fibers, although this time denser and more intensely stained (fig. 9A). Cells immunopositive for GFAP were still sparse, although the staining was more intense than in E14 cultures, and the morphologies of the cells were somewhat more developed, with longer and more prominent processes (fig. 9B).

Cell counts

The proportions of most cell types in slices prepared at E16 were generally similar to those calculated in E14 cultures. Glutamate-containing cells comprised 22.7 % of the total cell population, CB-positive cells were 3.2%, CR-positive cells were 2.7%, nestin was detected in 13.9% of the cells, while cells containing GFAP comprised 4.6 % of the total population. The only significant change was found in the population of the GABA-containing cells, which increased to 41.3% ($p=0.008$) (fig. 19B).

3.2.3. E19

Cell phenotypes

The appearance of Glu- (fig. 10A) and GABA- (fig. 10B) containing cells in cultures prepared at E19 was not markedly different to that found in E16 cultures, although a substantial shift in the proportion of the Glu-containing cells was evident before quantitation. Cells immunoreactive for the calcium-binding proteins CB (fig. 11A) and CR (fig. 11B) had a similar morphology as those stained for GABA. The major morphological changes observed in E19 cultures were related to nestin- and GFAP-labelling. Immunohistochemistry for nestin revealed, as previously, a network of fibers with more or less radial general orientation. However, this time the trajectory of the fibers was undulated rather than straight, and a small number of multipolar cells suggestive of early astrocytes were observed (arrow; fig. 12A). GFAP-immunoreactive cells were evidently much more numerous than in previous stages and appeared to have become more intensely stained with numerous and

elaborate processes, covering the entire cortical slice (fig. 12B).

Cell counts

A different picture emerged in cultures prepared from E19 animals (fig. 20A). In these cultures, when maintained in control conditions, the proportion of Glu neurons (41.1%) was markedly higher than in control slices prepared from E14 ($p=0.02$) or E16 ($p=0.03$) animals. The proportion of GABA-containing neurons in control cultures was reduced to 23.7% as compared to 41.3% calculated for E16 slices ($p=0.0006$), whereas GFAP labelled cells increased to 17.3% compared to 2.7% observed in E16 cultures ($p=0.0002$). Cells containing CB and CR were also present in proportions higher than those found in E16 slices (CB: 6.1%, $p=0.05$; CR: 5.7%, $p=0.004$). Nestin-positive cells (8.2%) were present in a proportion similar to that found in slices prepared from E16 animals.

To examine whether slices prepared from E16 embryos and cultured for an additional 3 days could acquire the same proportion of Glu-containing cells as cultures prepared at E19, a number of these cultures were kept for a total of 10 DIV. When these slices were dissociated and immunostained, it was found that the proportion of Glu containing neurons was significantly ($p=0.05$) lower than those observed in E19 cultures and not markedly different from that estimated in E16 slices kept for 7 DIV (fig. 21). This suggested that the proportion of Glu-labelled neurons was related to the age of the animal used to prepare the culture, and that the prolonged cultivation of slices explanted at earlier developmental stages was not sufficient for the cell population to acquire the neurochemical phenotype seen in

cultures prepared at more advanced stages.

3.2.4. P0

Cell phenotypes

Glutamate-containing cells appeared to be in a more advanced stage of morphological differentiation in these slices than in slices prepared at earlier stages, showing a conical soma shape and longer processes (fig 13A). GABA-containing cells (fig. 13B) also showed a more mature morphology as compared to the previous stages, some of them exhibiting multipolar morphologies. Cells labelled for CB (fig. 14A) and CR (fig. 14B) were not markedly different from those visualized in E19 slices, although a dense network of immunoreactive fibers could be visualized.. Nestin staining was remarkably different from what had been found in earlier stages, revealing a rather disorganized and non-linear arrangement of a smaller number of immunoreactive fibers. As in E19 cultures, a number of cells exhibited multipolar morphologies (arrow; fig. 15A). GFAP-containing cells were more numerous and even more intensely stained than those in E19 slices and formed a more elaborate network of processes (fig. 15B).

Cell counts

The proportion of Glu-containing cells in cultures prepared from newborn animals (52.5%) was higher than the one found in E19 slices, continuing the trend towards higher proportions in more advanced stages. The proportions of the other cell types were similar to those found in E19 cultures. GABA positive cells comprised 23.2%

of the population, GFAP positive cells were 21% and CB-, CR- and nestin-containing cells consisted 8.3%, 7.3% and 10.6% of the total cell population respectively. (fig. 20B).

3.3. Slice cultures exposed to 5-HT: cell counts

3.3.1. E14

When slices prepared from E14 rat cortices were exposed to 200 mM 5-HT, the proportions of the different cell types labelled with cell-specific markers were not significantly different from those found in the control group of the same age. Glutamate-containing cells comprised 22.3 % of the total cell population, GABA-containing cells were 25.2%, the CaBPs CB and CR were present in 3.4 % and 4.6 %, respectively, nestin labelled 8.8% of the cells and GFAP was detected in 2.7% (fig . 19A).

3.3.2. E16

In contrast to the finding in E14 slices, exposing E16 cultures to 5-HT resulted in a change in the neurochemical makeup of the cell population. In these cultures, the proportion of Glu-positive cells was found to be significantly ($p=0.001$) higher in the 5-HT group (38.1%) as compared to the control slice cultures (22.8%), whereas no significant differences were found for the other cell types (fig. 19B). Counts of cells labelled for TUJ1 (a marker of immature neurons) were performed in the 5-HT treated and control cultures, in order to examine whether the effect of 5-HT was a result of a

change in the proportion of the newly produced neuronal cells. However, no difference between the two groups was found, with these cells comprising a little more than 70% of the overall cell population under both conditions.

In order to decipher at which stage of the culture period the effect of 5-HT is exerted, a group of slices was exposed to the indolamine during the first 4 DIV, and then kept in DM for the remaining 3 days, while another group was kept in DM for the first 4 days and then exposed to 5-HT for the remainder of the culture period. The proportion of Glu positive cells in E16 slices exposed to 5-HT only during the first 4 days of the 7 day culture period was significantly higher than in the control slices ($p=0.0002$), showing no significant difference from the slices exposed to the neurotransmitter for the whole period (fig. 22). In contrast, cultures exposed to 5-HT only during the last 3 days of the culture period did not show an increase in the proportion of Glu neurons, indicating that the action of 5-HT on the developing Glu-containing cell population is completed by the fourth day *in vitro*.

After an effect of 200 μM 5-HT was established in E16 cultures, 20, 40, 80, 120 and 160 μM were also applied, so as to produce a dose-response curve. These different concentrations were maintained for the entire culture period. Application of 20 or 40 μM 5-HT resulted in no detectable effect (with proportions of Glu-containing cells at 19.7 % and 22.3 %, respectively), whereas 120 μM and 160 μM (Glu-containing cells: 39.1 % and 42.7 %, respectively) were as effective as 200 μM . The presence of 80 μM of 5-HT in the medium resulted in a proportion of Glu-containing cells (31.8%) that was significantly higher than the controls ($p=0.05$), and at the

same time lower than what was obtained with 200 μM 5-HT, the difference being just below the limit of statistical significance ($p=0.06$). Concentration of 5-HT over 250 μM resulted in cytotoxicity, as assessed by the change of the colour of the culture medium and the appearance of the cells under phase contrast microscopy.

3.3.3. E19

When cultures prepared from E19 cortices were exposed to 200 μM 5-HT, no significant difference was found in the proportions of the different cells types between the control and the 5-HT treated groups. Glutamate-containing cells comprised 45.1 % of the cell population, GABA-containing cells were 23.0 %, CB- and CR- containing cells were 6.4 % and 5.2%, respectively, nestin labelled 10.6 % of the cells while GFAP was present in 18.3 % of the total cell population (fig. 20A).

3.4. HPLC quantitation of medium 5-HT content over time

To examine whether the added 5-HT was stable in the culture medium, high pressure liquid chromatography was used to quantitate the concentration of the monoamine in the medium over time. Samples of 200 ml of medium were collected in 6-hour intervals starting at 18 hours *in vitro*. After 18 hours the concentration of 5-HT had dropped from 200 to 153.6 μM on average, but remained more or less stable up to 36 hours *in vitro*.

3.5. Co-cultures

3.5.1. Morphology

To confirm the observed increase in the proportion of Glu positive cells in cortical slices cultured in the presence of 5-HT in an alternative system, cortical slices were exposed to their natural source of 5-HT, namely the serotonergic cells of the raphe nuclei in the midbrain. Co-cultures of E16 cortical slices and slices taken from E19 raphe nuclei were grown side by side for 7 days. Co-cultures were then fixed and stained with anti-5-HT antibody that revealed a number of large immunoreactive cell somata in the raphe nuclei giving rise to numerous 5-HT containing processes (fig. 25A). These processes were seen to invade the whole cortical slice (fig. 26). In agreement with earlier descriptions of the serotonergic innervation of the cortex *in vivo* (Papadopoulos et al., 1987b), they showed predominantly an orientation parallel to the pial surface in layer I, and vertical or oblique orientations in the remaining cortical thickness. These serotonergic processes were relatively thick, sparsely branched, and showed a typical beaded appearance. Growth cones were frequently seen at the leading ends of the serotonergic axons in the cortical slices. They were intensely stained and in some cases extended filopodia were visualized, indicating the active state of the growth cone at the time of fixation (arrow, fig. 25B). In order to assess whether any incoming processes had formed synaptic contacts with cortical cellular elements, wholemount immunostained cortical slices that had been co-cultured with raphe slices were processed for electron microscopy, and ultrathin sections were cut and examined under the electron microscope. The examination did not reveal the presence of synaptic contacts between the

immunostained processes and other cellular elements in the cortical slice.

3.5.2. Cell counts

A number of cortical slices from these co-cultures were dissociated as described above, immunostained, and the mean proportion of Glu positive cells was assessed. In these slices, Glu neurons made up 32% of the cell population as compared to 22.8% in the control group (fig. 27A), a difference that was statistically significant ($p=0.002$), indicating that the effect of 5-HT on the developing Glu-positive cell population could be exerted through the raphe afferents.

3.5.3. HPLC quantitation of Glu content

High pressure liquid chromatography was used as a further means of determining changes in Glu content in cortical slices brought about by co-culturing with slices containing the raphe nuclei. The mean concentration of Glu in the slices of cortex co-cultured with raphe nuclei was 4.123 mmol/L as compared to 1.711 mmol/L in the control cultures, showing a significant increase ($p=0.028$) of 141% on average (fig. 27B).

3.6. Assessment of cell proliferation in slice cultures using BrdU

In order to reveal possible changes in proliferation brought about by culturing E16 slices in 5-HT containing medium, different cultures were exposed to BrdU for 16 hours at each of the 7 DIV, and then fixed. Cells that had incorporated BrdU were

immunohistochemically identified (arrow, fig. 24A). The mean proportion of BrdU labelled cells in the proliferative zones ranged from about 50% on the first DIV to about 10% on the last day in culture, both in control conditions and in the presence of 5-HT (fig. 25B). The mean proportions, in the control and the 5-HT group respectively, were: 49.7 % and 56.2 % (1 DIV), 47.5 % and 51.8 % (2 DIV), 36.3 % and 46.0 % (3 DIV), 35.8 % and 27.5 % (4 DIV), 18.2 % and 15.8 % (5 DIV), 12.1 % and 10.6 % (6 DIV), and 10.6 % and 13.4 % (7 DIV). Although the mean values were higher in the 5-HT group for the first 3 days, statistical analysis of these preparations showed that the differences did not reach the level of statistical significance, with the p value reaching a minimum of 0.065 on the third day, when the difference between the mean proportions of BrdU+ cells in the two groups was more pronounced. These results indicate that 5-HT does not alter the rate of cell proliferation in the developing cortex. Even if the observed increases were indeed indications of some existing effect, its scale would be too small to be related to the significant increase of the Glu-containing cell population observed in cultures exposed to 5-HT.

3.7. Effects of other neurotransmitters

In order to test whether the neurotransmitters DA, NA or ACh might also exert an influence on the neurochemical makeup of the developing cortical slice cultures, cortices from E16 rat embryos were exposed to these neurotransmitters, initially at a concentration of 10 μ M. No effects were observed, and subsequently concentrations of 50 μ M (DA) and 100 μ M (NA and ACh) were used. These

concentrations also did not significantly alter the proportion of any cell types in the cultures (fig. 19B). Concentrations over 50 μM DA or 100 μM NA were found to have cytotoxic effects, as assessed by the colour of the medium and the appearance of the cells under phase contrast microscopy. No toxic effects of ACh were observed.

Figure 4

A. Immunostaining of E14 cultures with an antibody against Glu revealed a number of round or ovoid cell somata with some giving rise to short processes .

B. Staining for GABA revealed a cell population that appeared to be in a more advanced state of morphological differentiation than Glu-containing cells, but still immature none the less. Cells were mono- or bi-polar, with easily identifiable processes and an ovoid or sometimes round cell body.

Scale bar = 20 μm .

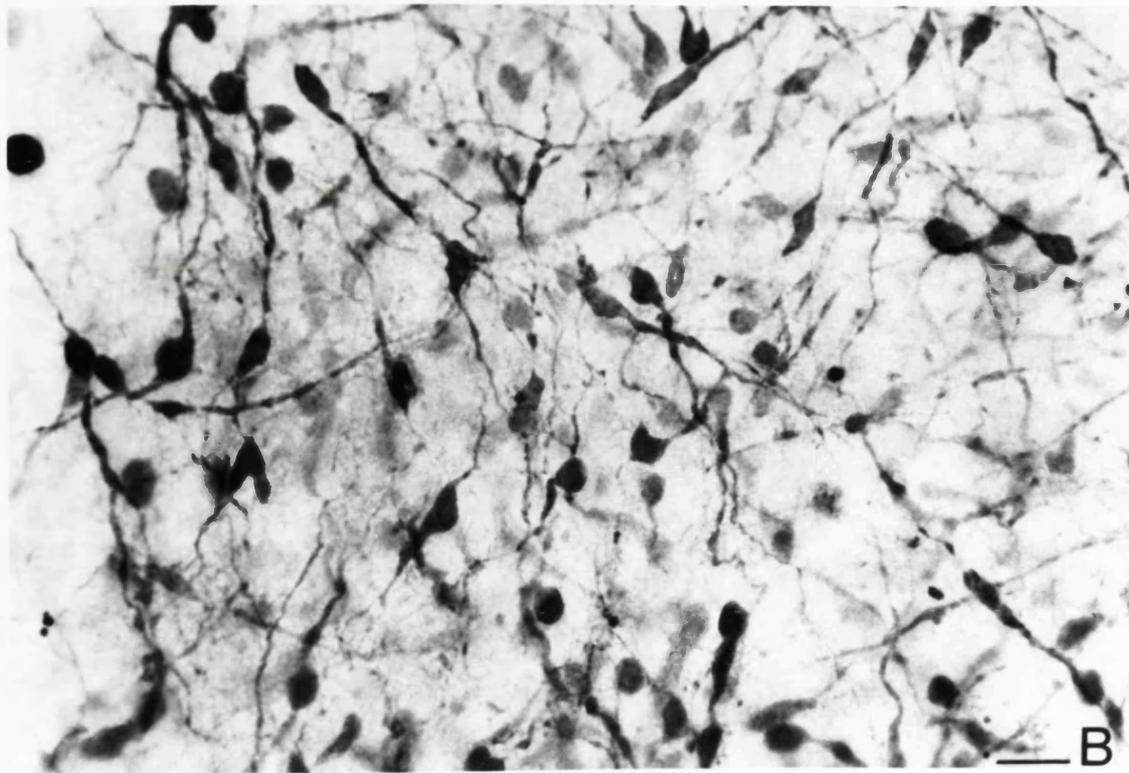
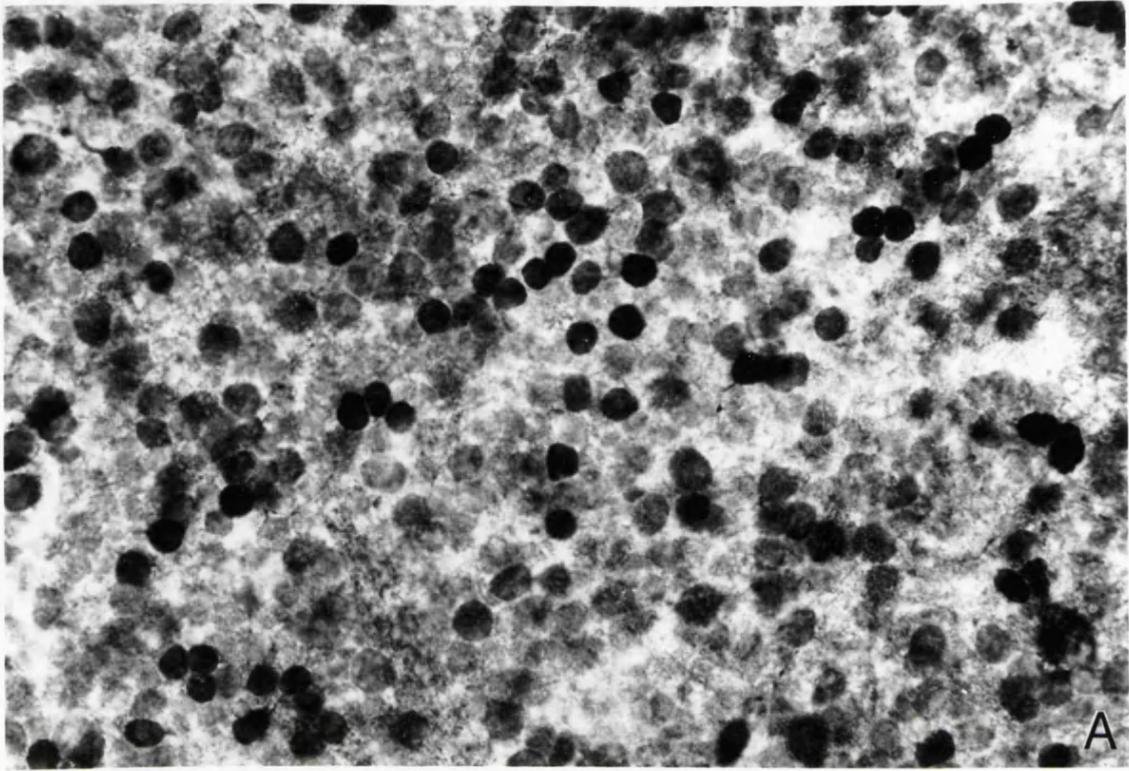


Figure 5

Staining for CB (A) and CR (B) in E14 cortical slices at 7 DIV. The large CR-labelled cells are probably Cajal-Retzius cells.

Scale bar = 20 μm

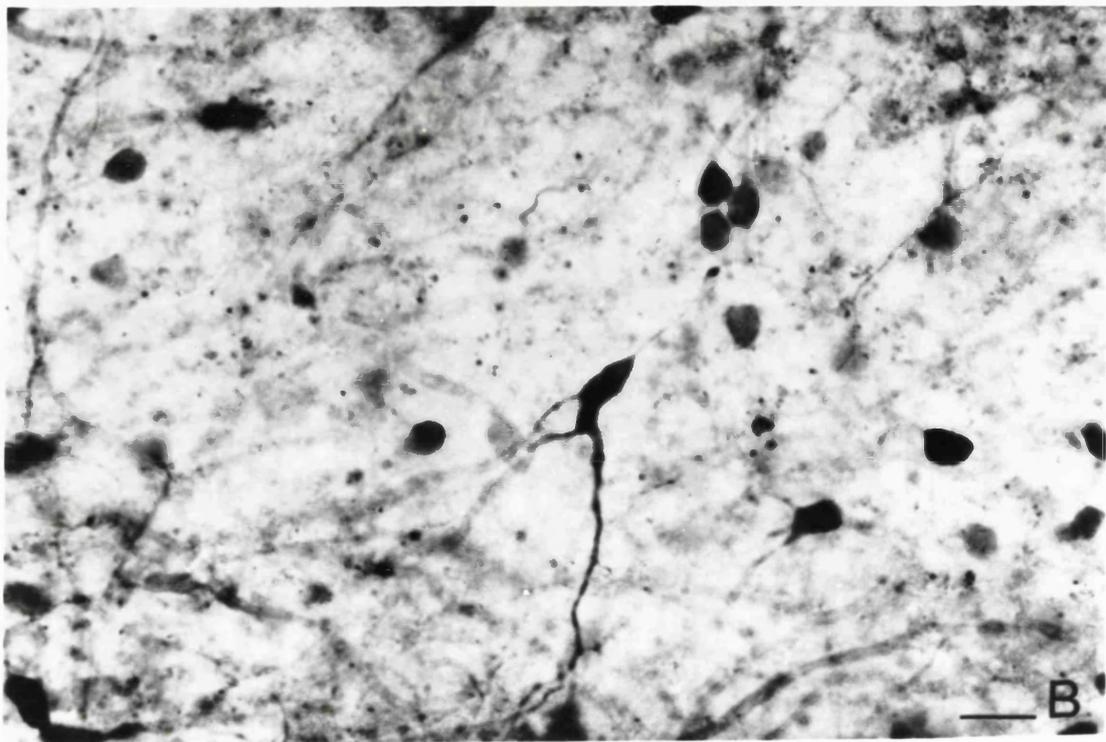
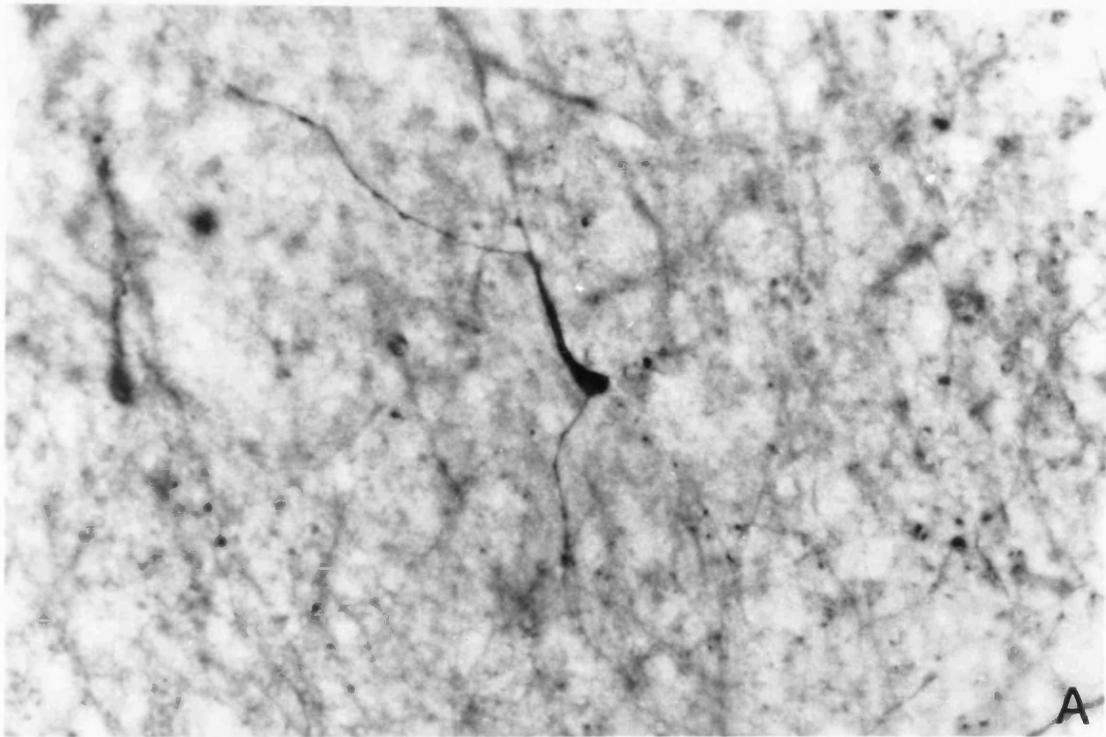


Figure 6

A. Nestin staining of E14 slices revealed a number of cells as well as long radial glial processes spanning the thickness of the slice.

B. Only a small number of cells were immunostained with a GFAP antibody in E14 slices. They showed cell bodies of small size with short processes.

Scale bar = 20 μm

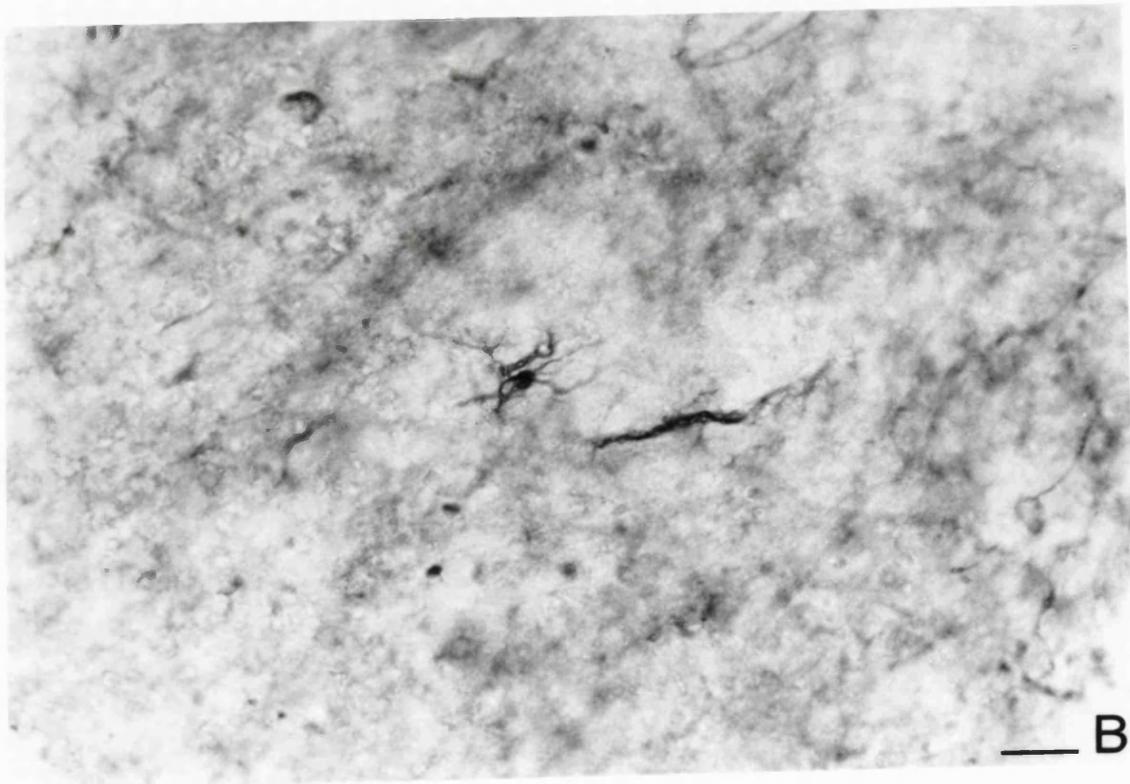
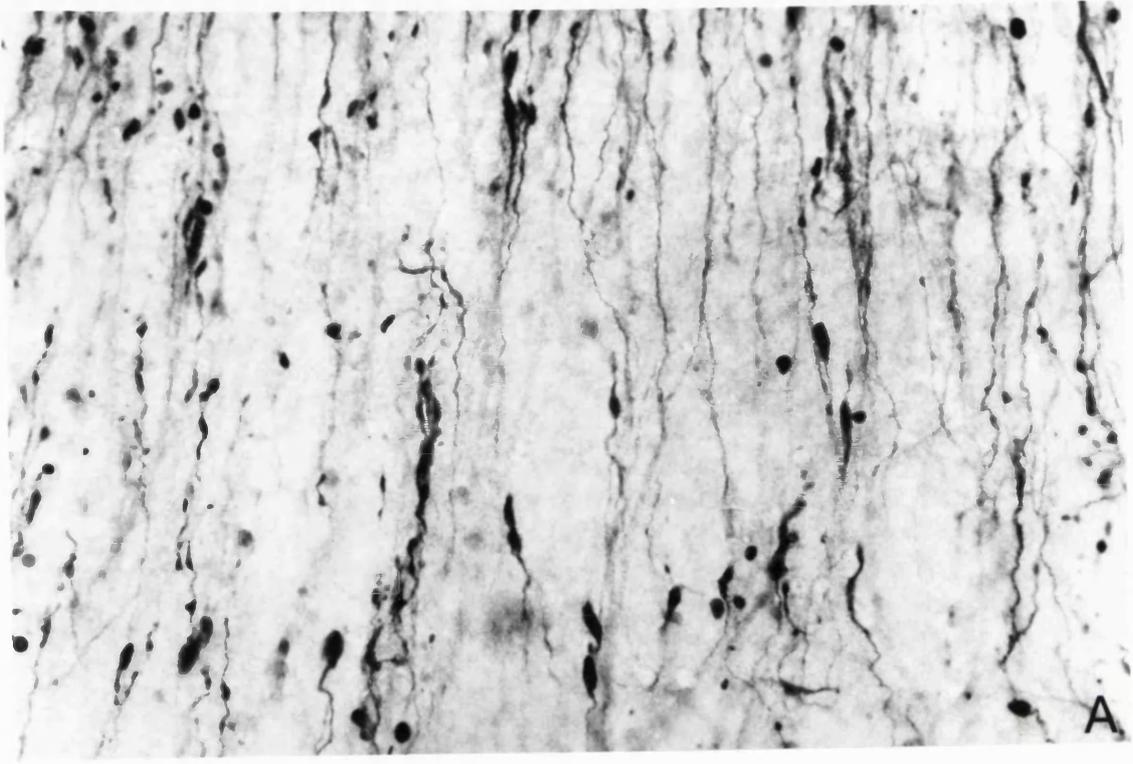


Figure 7

A. Glutamate-containing cells in E16 slices appeared to be morphologically more differentiated than those in E14 cultures (fig. 5A), with well stained processes, some of them possessing a small number of varicosities.

B. GABA-containing neurons in E16 slices were at a more advanced stage of morphological differentiation than their E14 counterparts (fig 5B). They appeared more mature than the Glu-containing neurons of the same stage, with long and more elaborate processes and numerous varicosities.

Scale bar = 20 μ m.

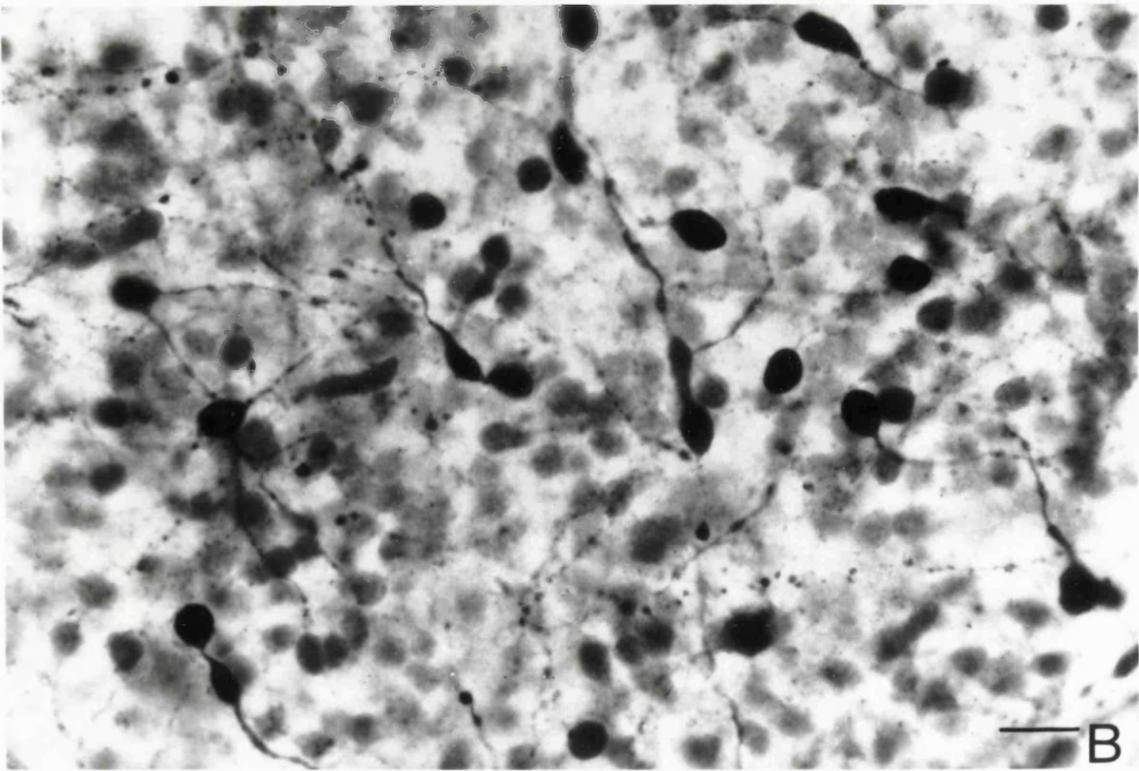
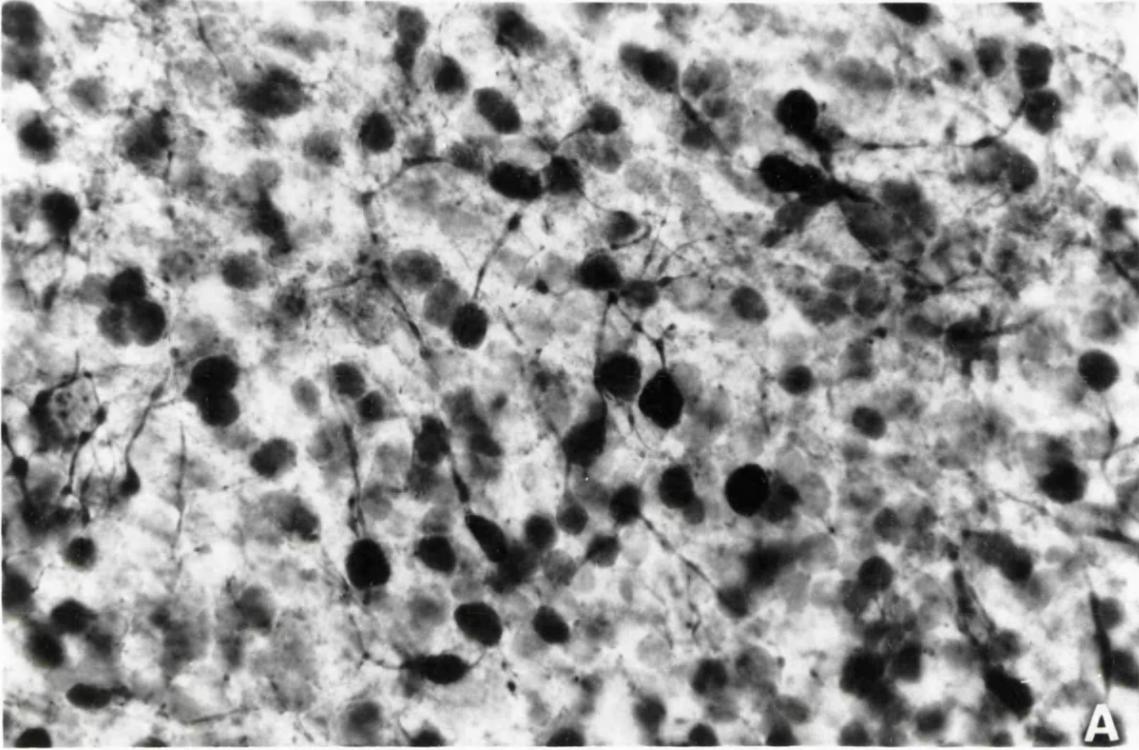


Figure 8

Cells containing CB (A) and CR (B) in E16 slice cultures. The cells appeared bigger and morphologically more mature than those found in E14 slices. Longer processes were usually associated with CR-containing cells.

Scale bar = 20 μ m.

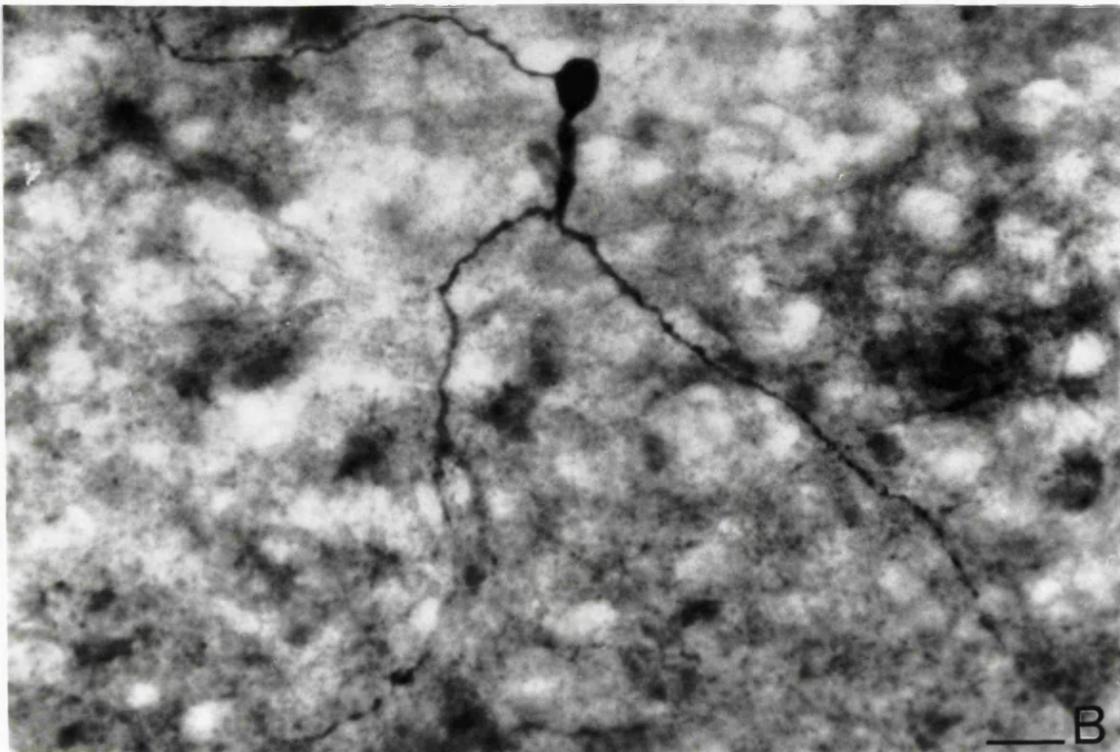
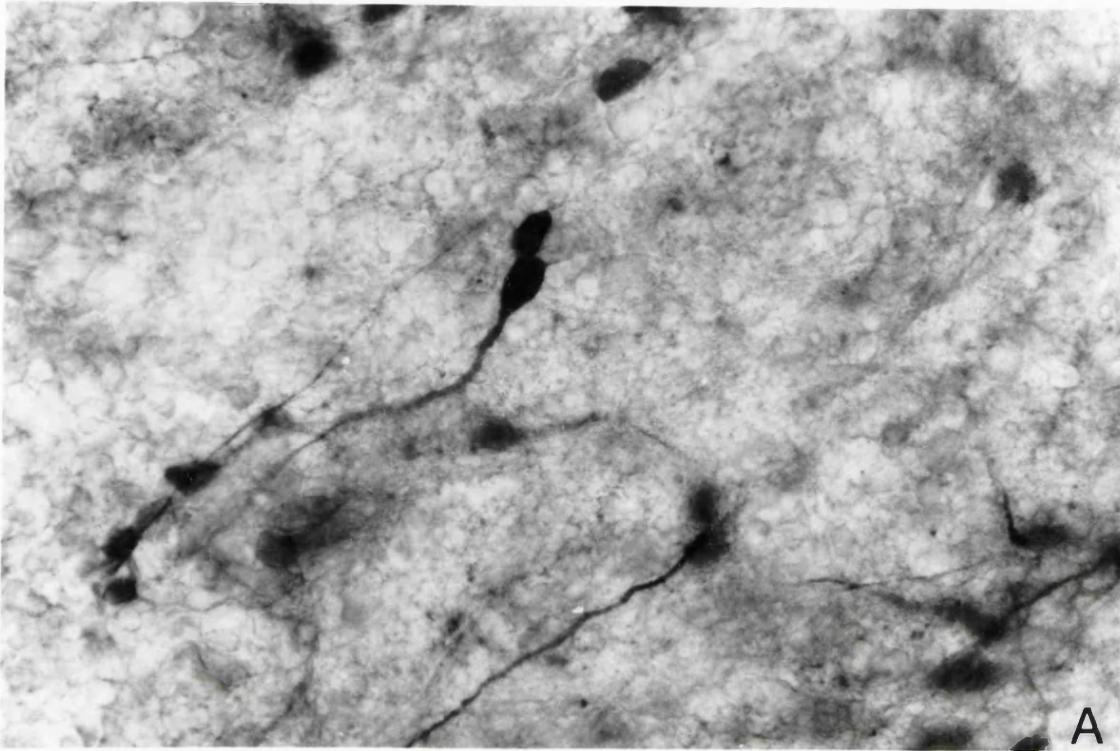


Figure 9

A. Immunostaining for nestin revealed a number of monopolar cells as well as the dense scaffolding of radial glial fibers in E16 slices.

B. Still only a few GFAP-positive cells were found in E16 slices, but their cell body sizes were bigger and the processes longer than in E14 cultures (fig 7B).

Scale bar = 20 μ m.

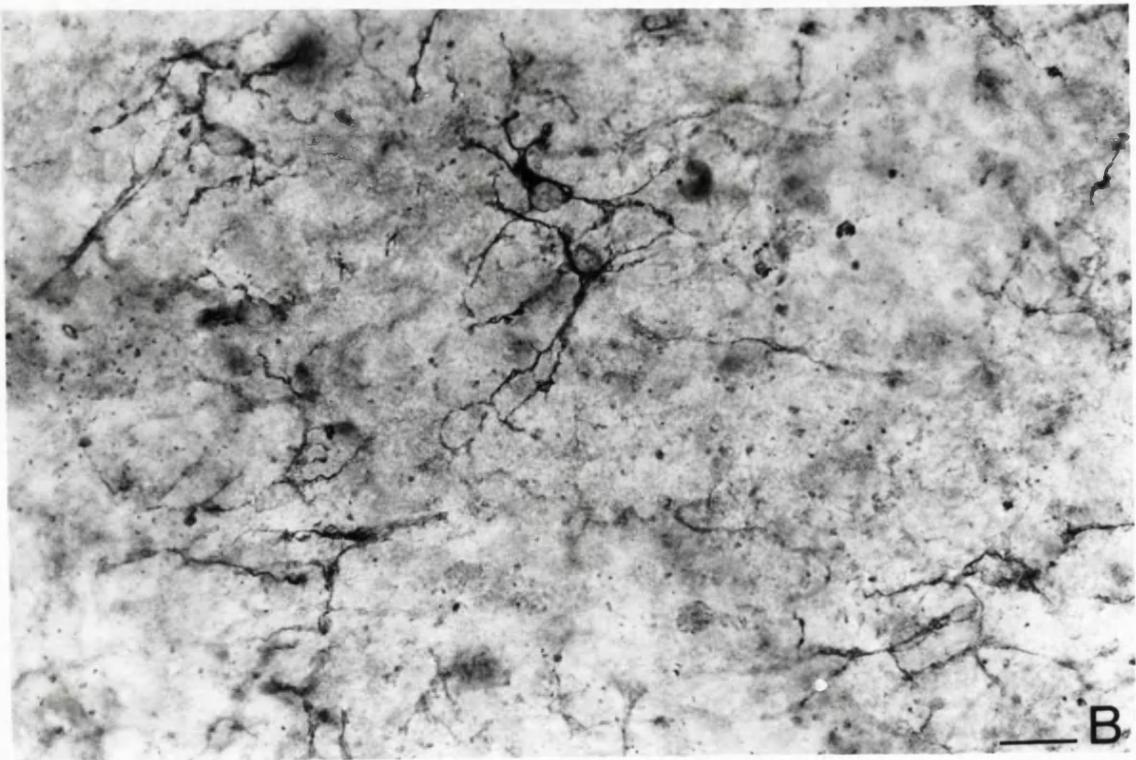
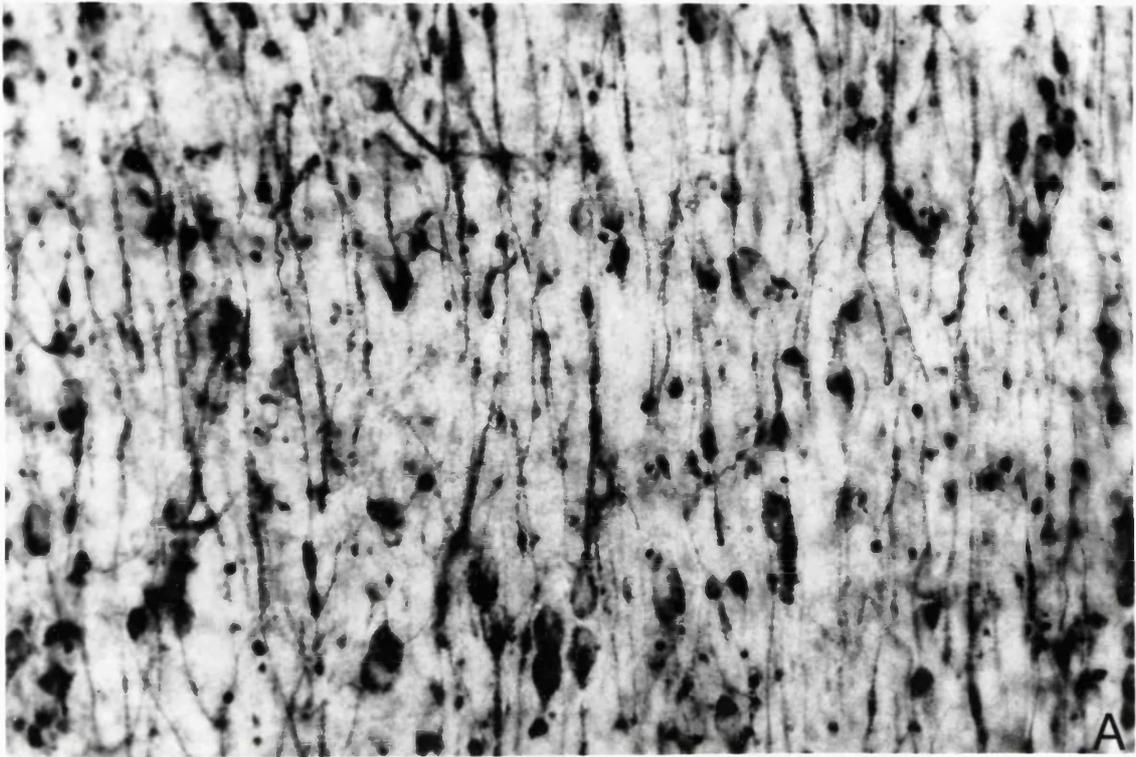


Figure 10

Glutamate-(A) and GABA-(B) containing cells in E19 slices showed no marked morphological differences as compared with those in E16 slices (fig. 8).

Scale bar = 20 μ m.

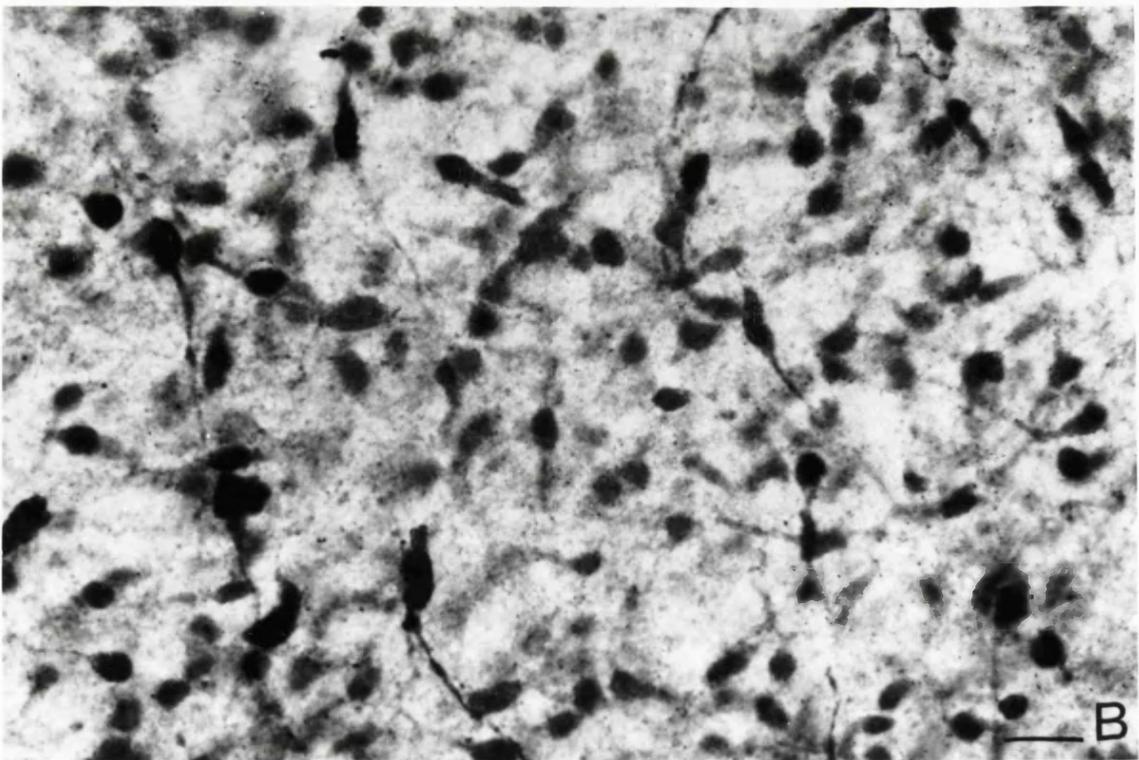
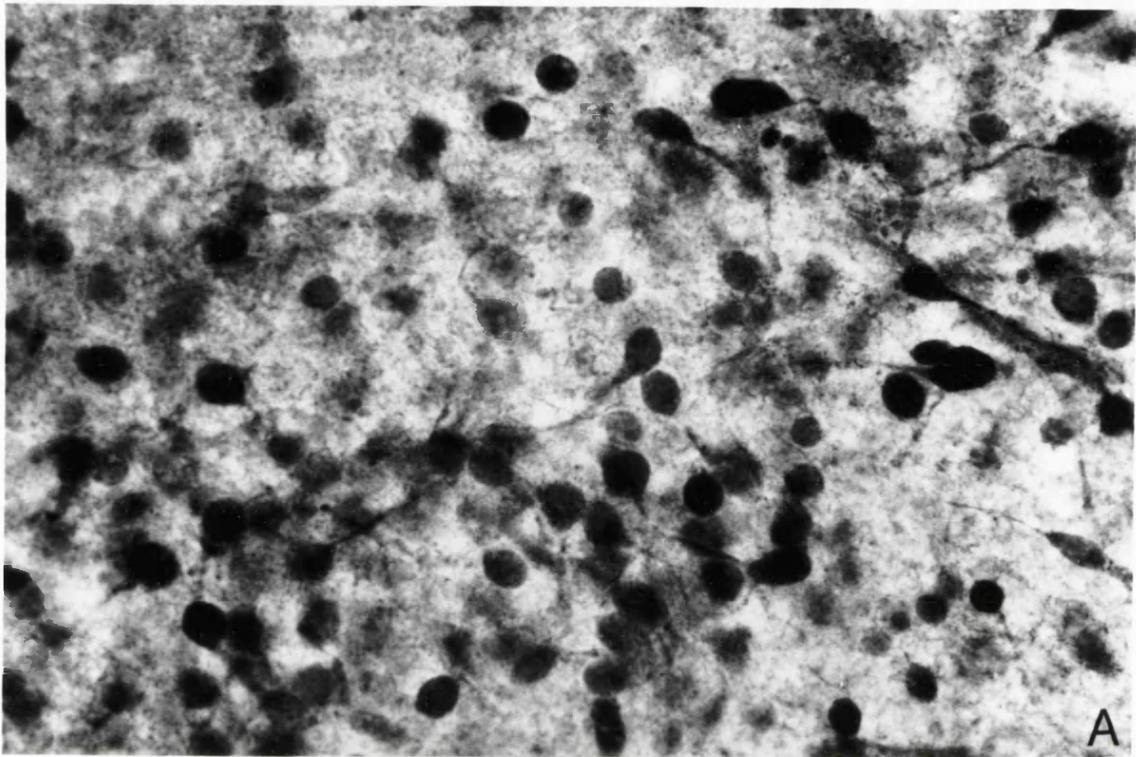


Figure 11

Calbindin-(A) and CR-(B) containing cells in E19 slices. No marked morphological differences are observed between CaBP-containing cells in these cultures and those prepared from E16 brains (fig.9)

Scale bar = 20 μ m.

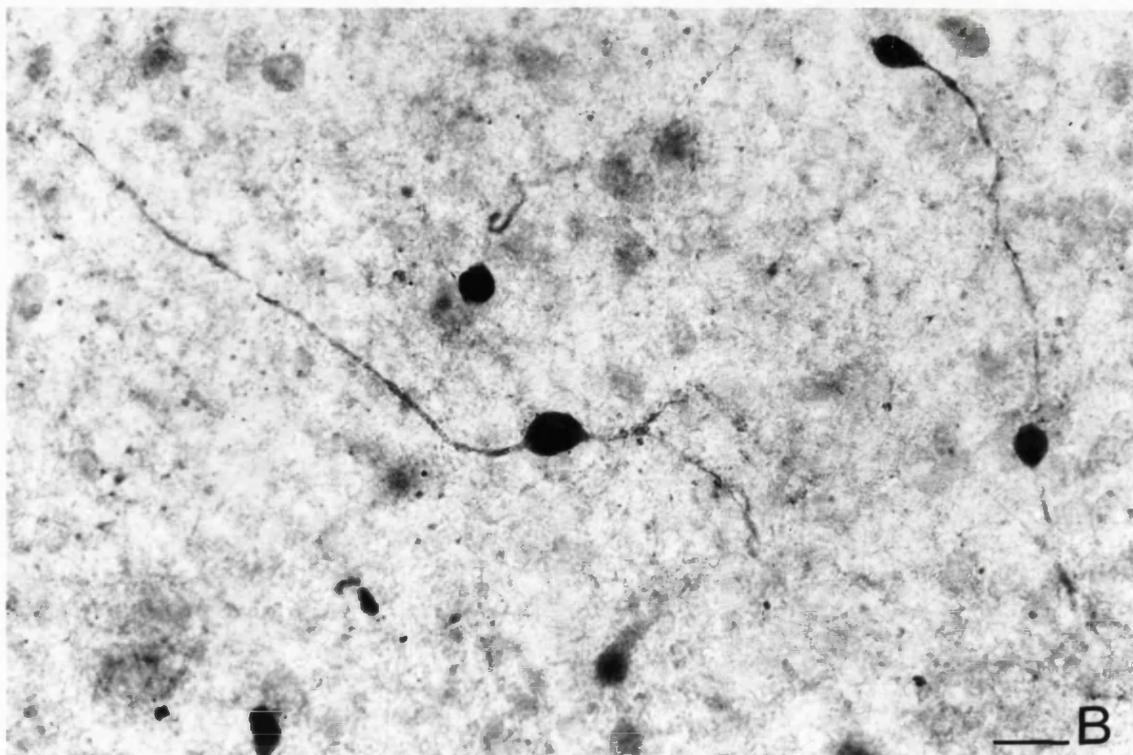
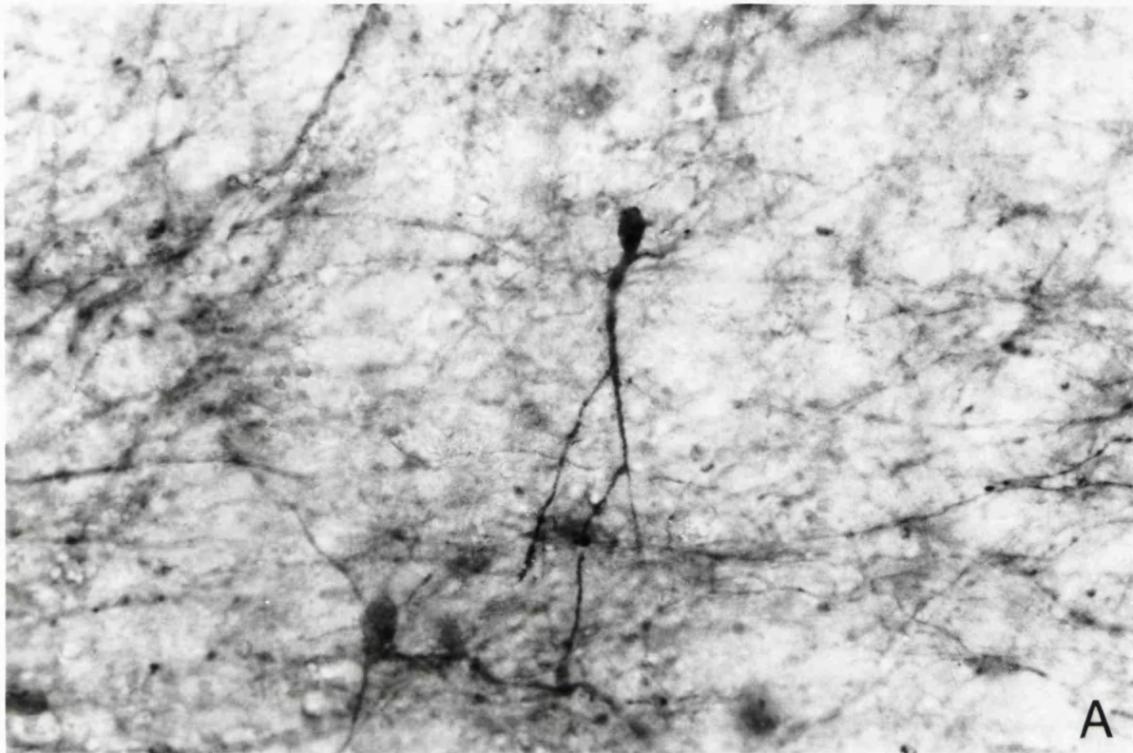


Figure 12

A. Immunostaining of E19 slices for nestin revealed, as in previous stages, a number of cells and a dense network of fibers. However, while the fibers were still radial in orientation, they traversed the thickness of the cortical slice in a somewhat undulated fashion; a number of multipolar cells (arrow) were visualized for the first time.

B. An increased number of GFAP-positive cells was revealed at E19 as compared to E16 slices (fig. 10B); they showed longer processes that form a loose network in the cortical slice.

Scale bar = 20 μ m.

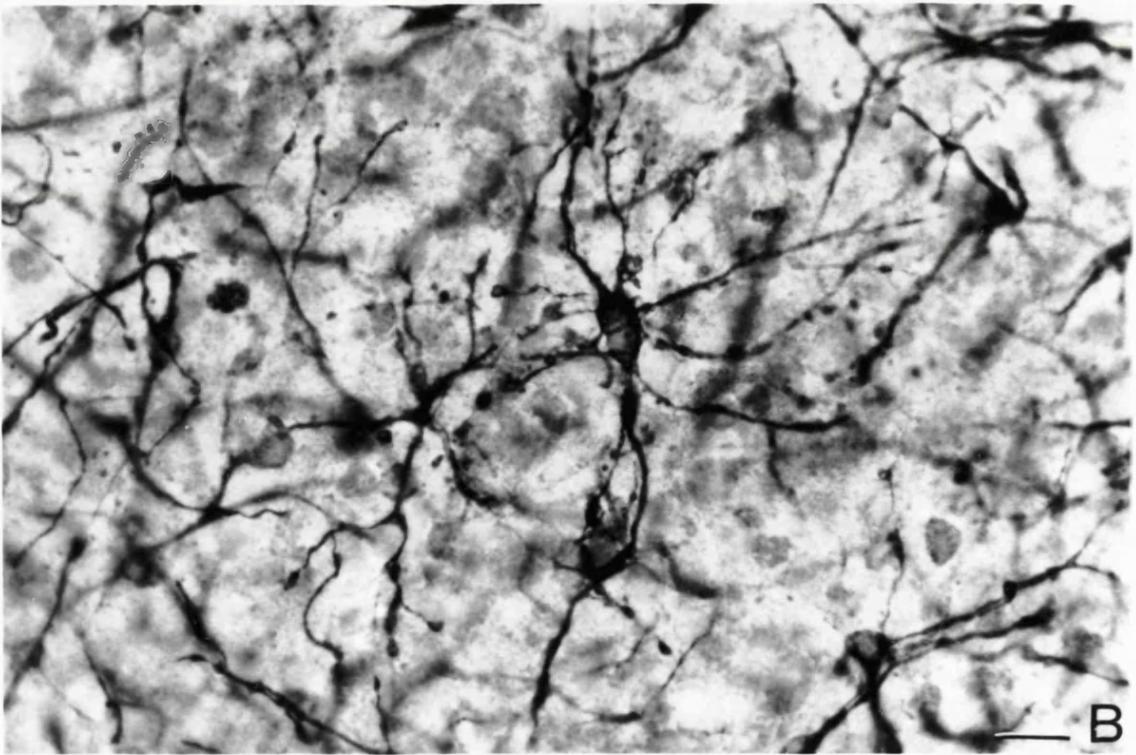
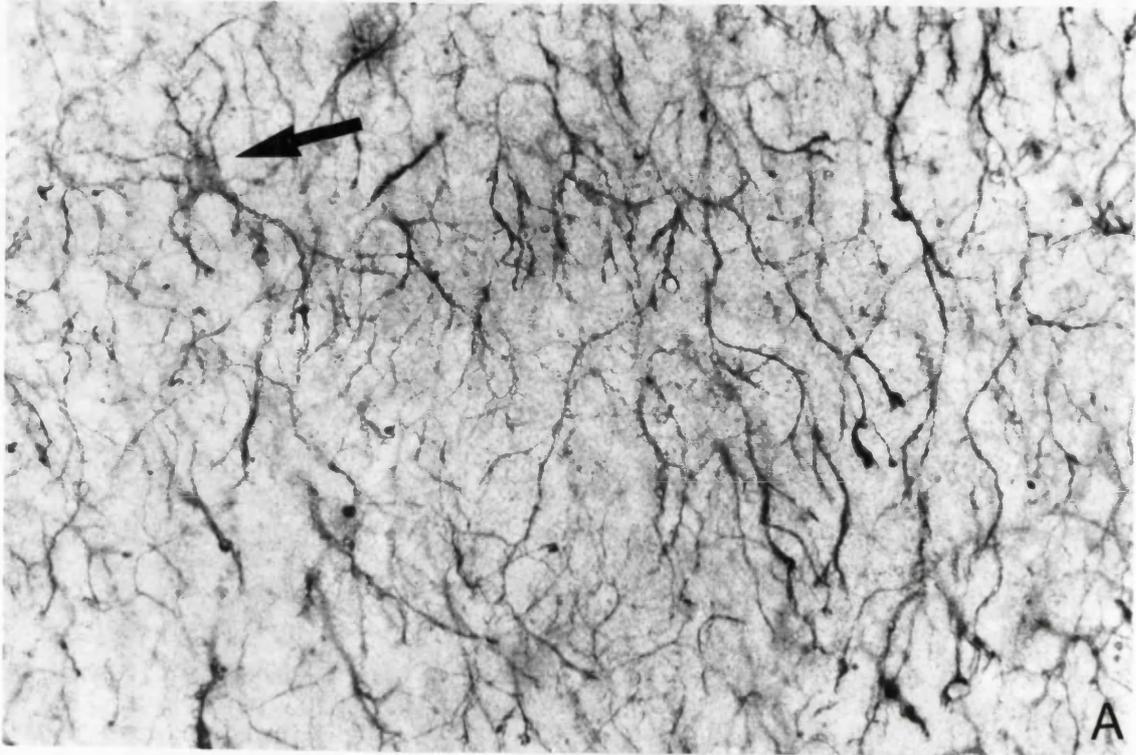


Figure 13

After staining with antibodies to Glu (A) and GABA (B), slices prepared from newborn animals showed morphologically more mature cells, as compared to previous stages. Bigger cell bodies and longer processes can be seen in both cases, with a substantial number of multipolar GABA-containing cells (B).

Scale bar = 20 μ m.

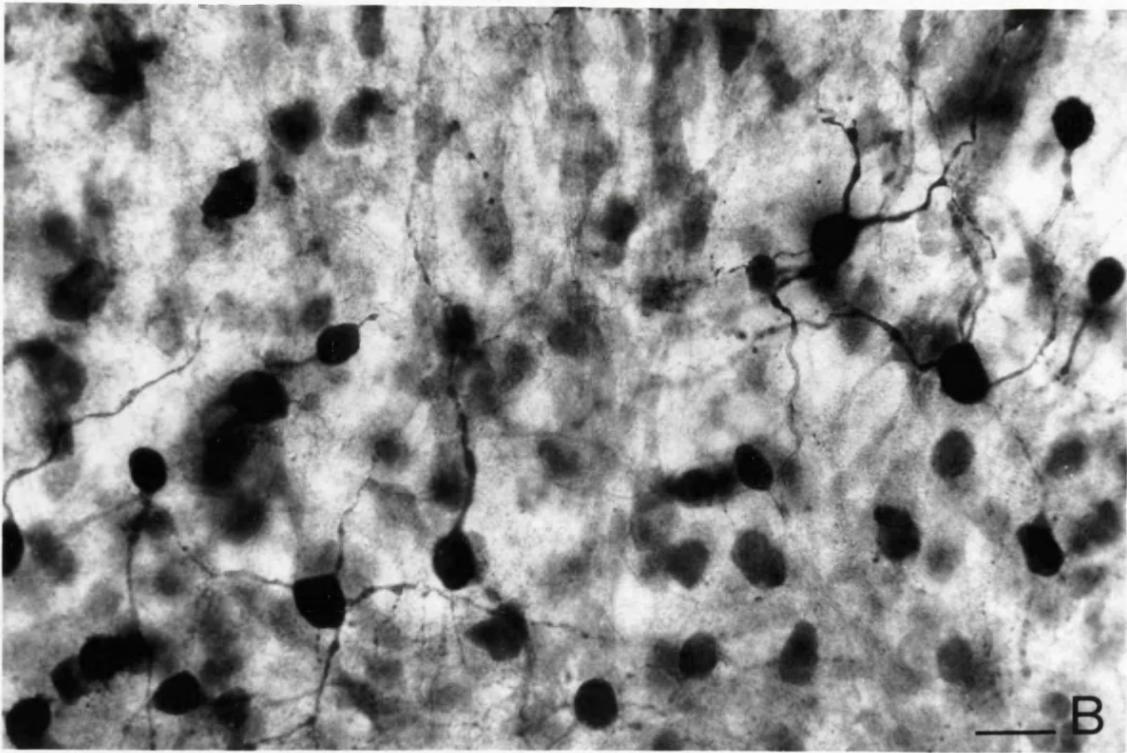
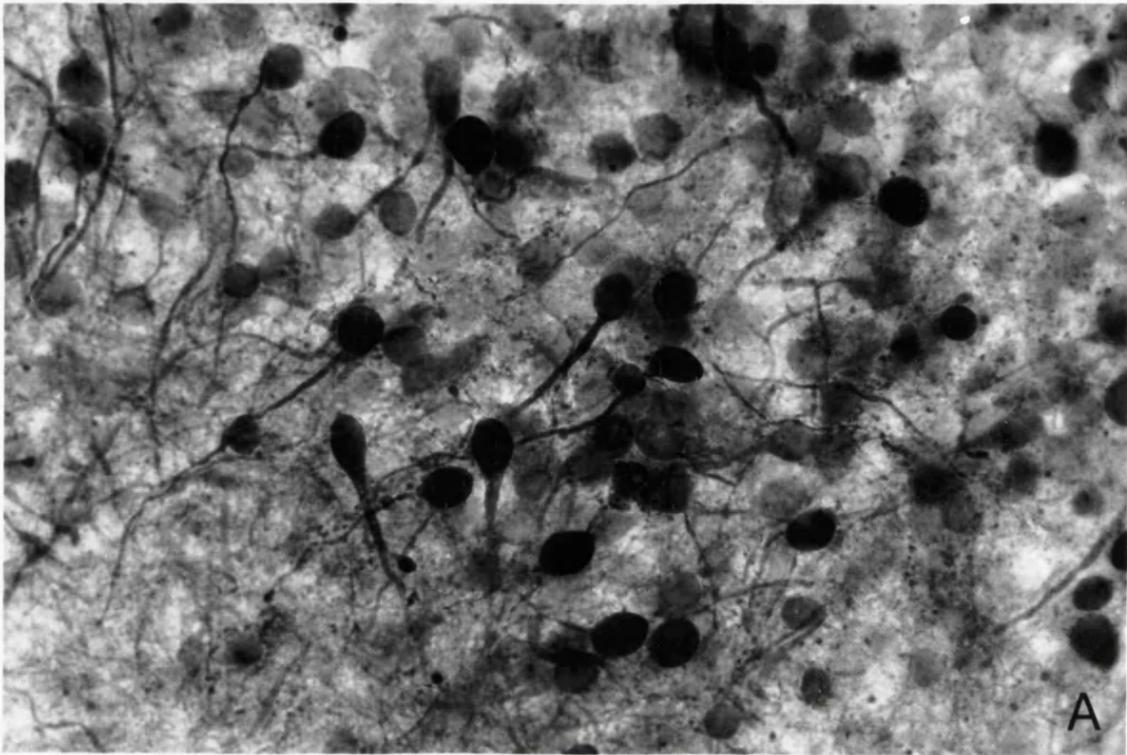
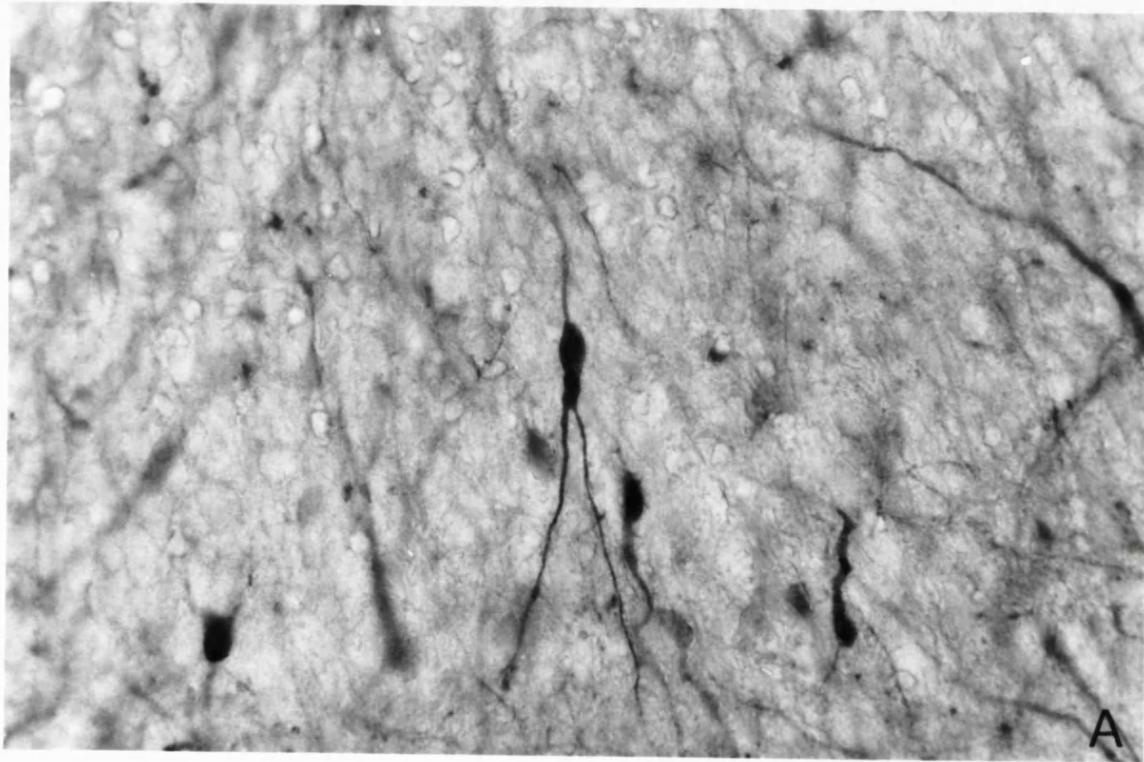


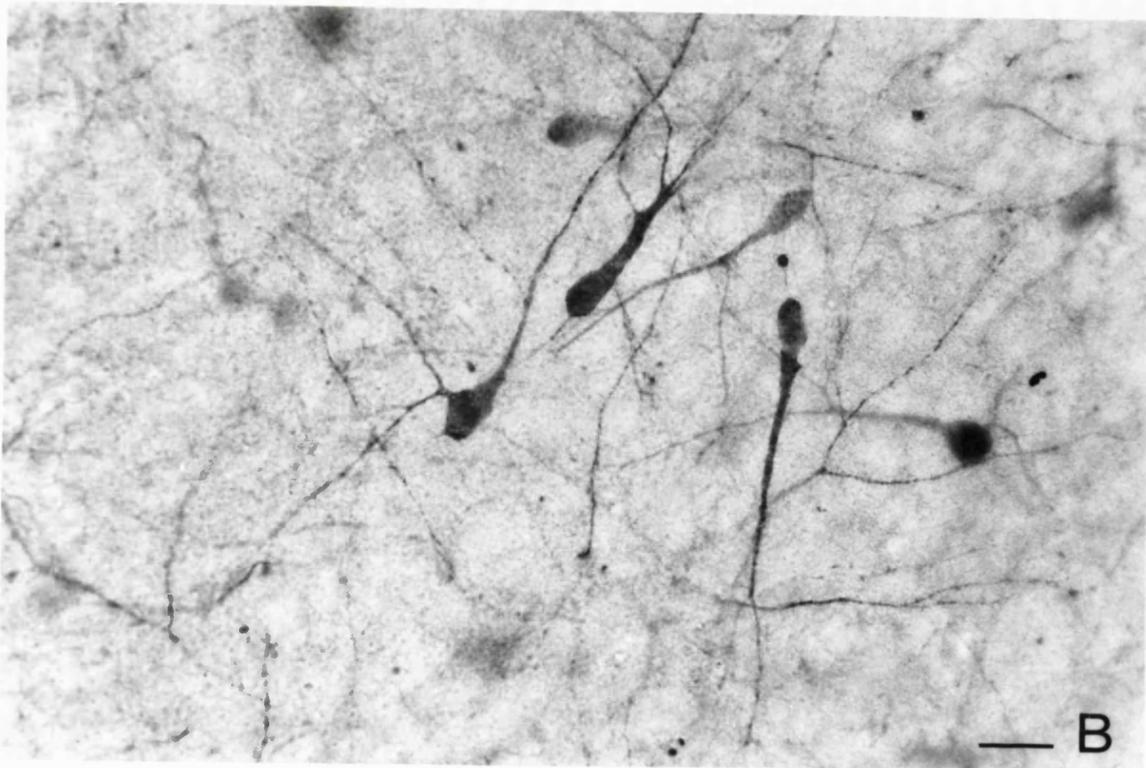
Figure 14

Calbindin-(A) and CR-(B) containing cells were not markedly different in their morphology in P0 slices from those in E19 slices. However, a somewhat denser network of stained processes could be visualized at this stage (fig. 12).

Scale bar = 20 μ m.



A



— B

Figure 15

A. The appearance of P0 nestin-immunostained slices was markedly different to that found at all previous stages. The network of fibers seemed now to have totally lost its earlier radial arrangement, and the overall number of fibers was smaller. As in E19 slices, multipolar cellular forms could be identified (arrow).

B. Immunostaining with GFAP was strikingly increased in P0 cultures, revealing a dense network of GFAP-containing fibers that covered the whole cortical slice.

Scale bar = 20 μ m.

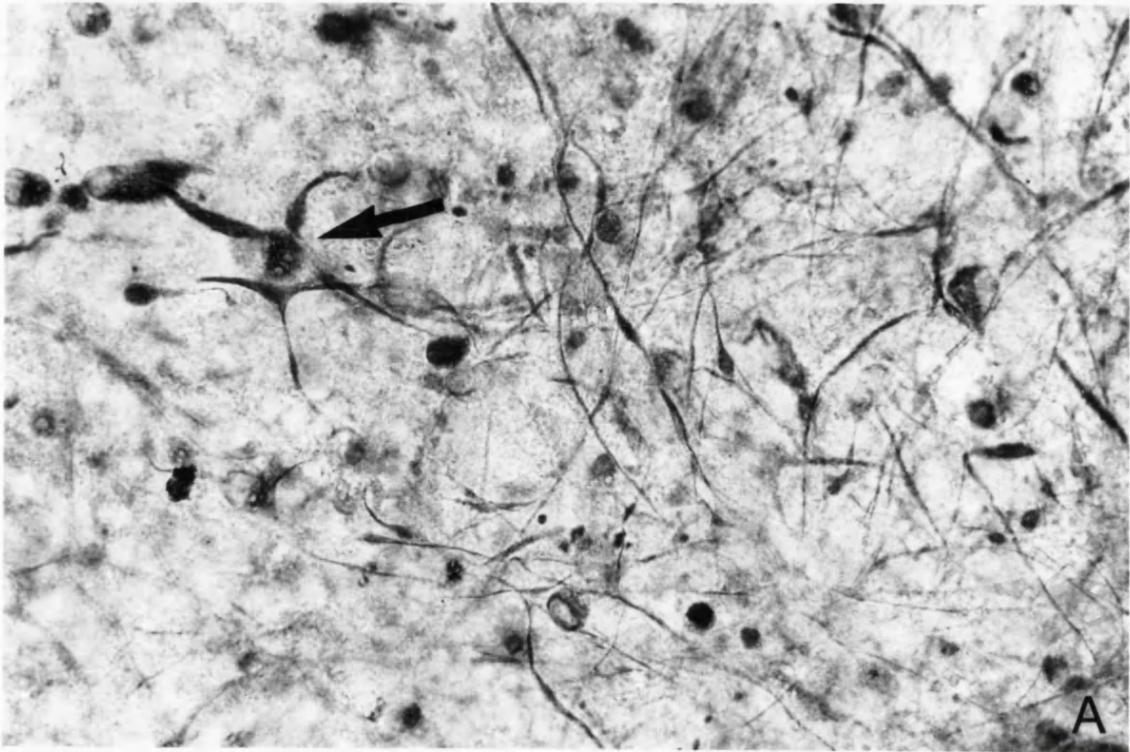
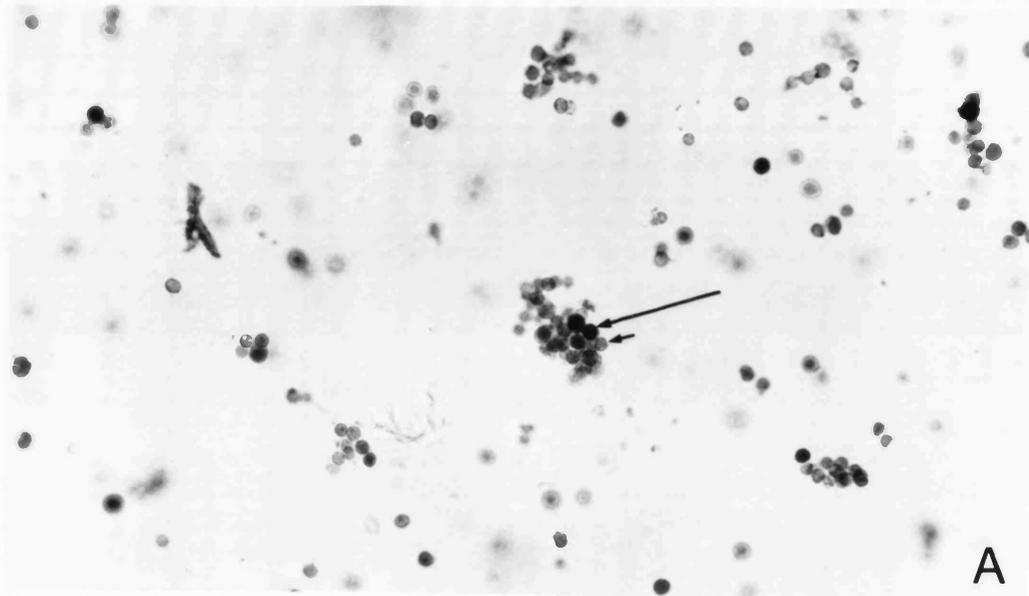


Figure 16

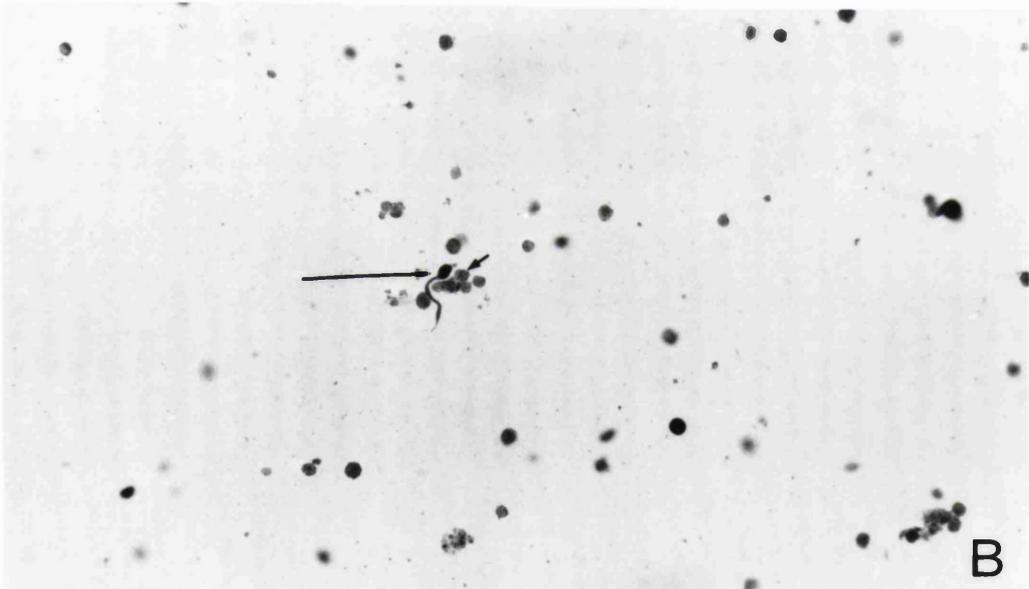
At the end of the culture period the cultures were dissociated, and the cells were embedded in agarose and immunostained for a number of cell-specific markers .

A: Glu, B: GABA, C: GFAP. Long arrows indicate immunopositive and short arrows immunonegative cells.

Scale bar = 40 μ m.



A



B



C

Figure 17

Dissociated slice culture cells stained for CB (A), CR (B) and nestin (C).

Long arrows indicate immunopositive and short arrows immunonegative cells.

Scale bar = 40 μ m

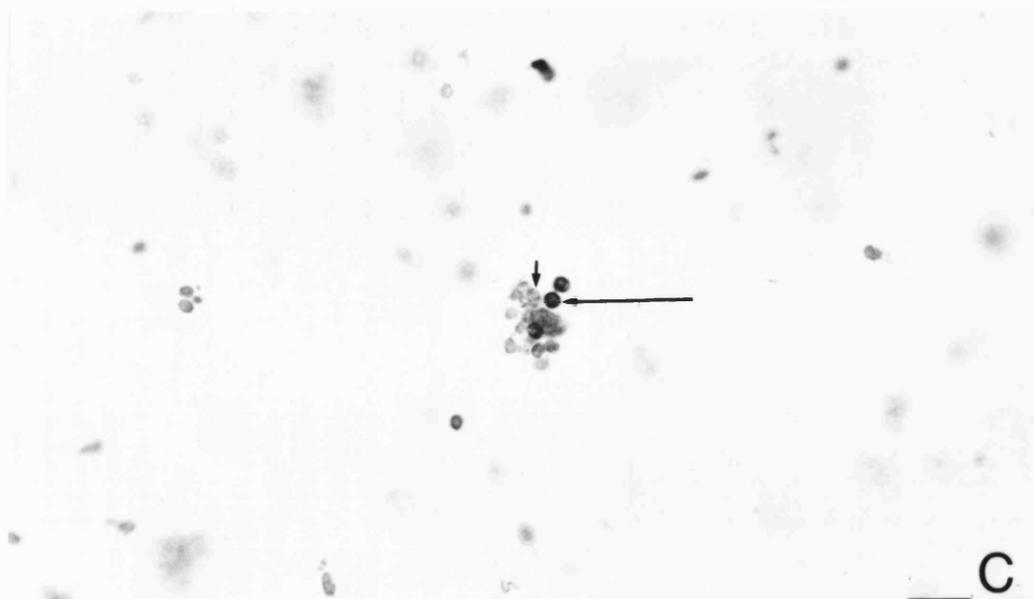
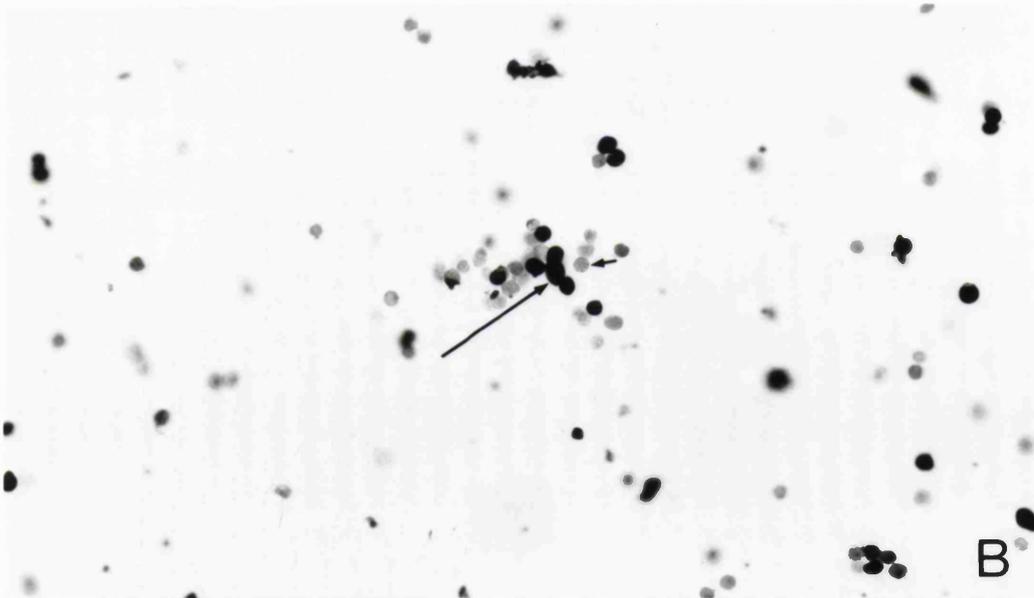
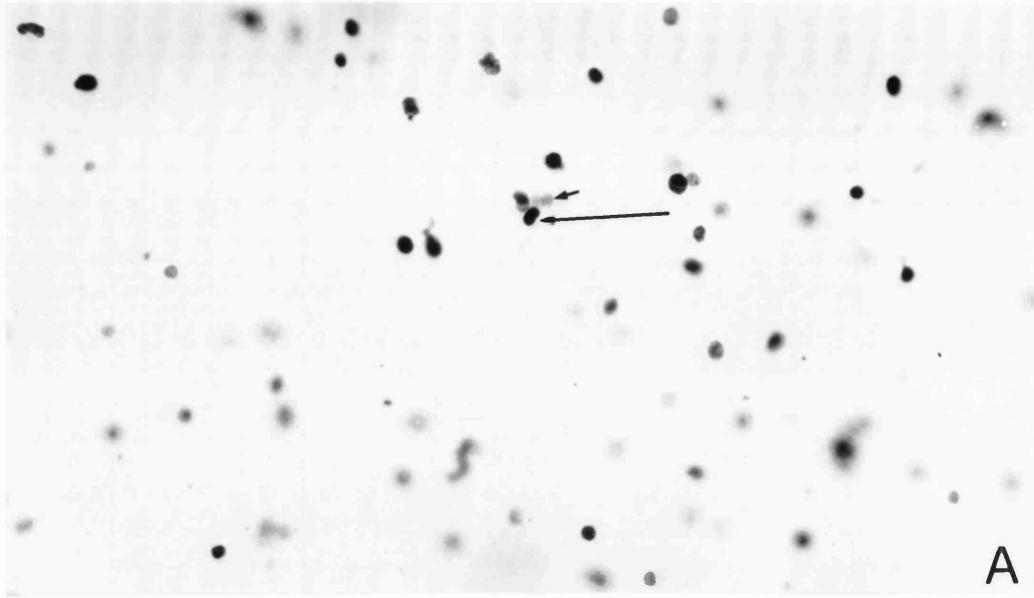


Figure 18

Proportions of the different cell types in acutely dissociated cells from rat cortices in 3 prenatal ages and at birth. No clear cut examples of neurons stained for Glu were evident at these ages.

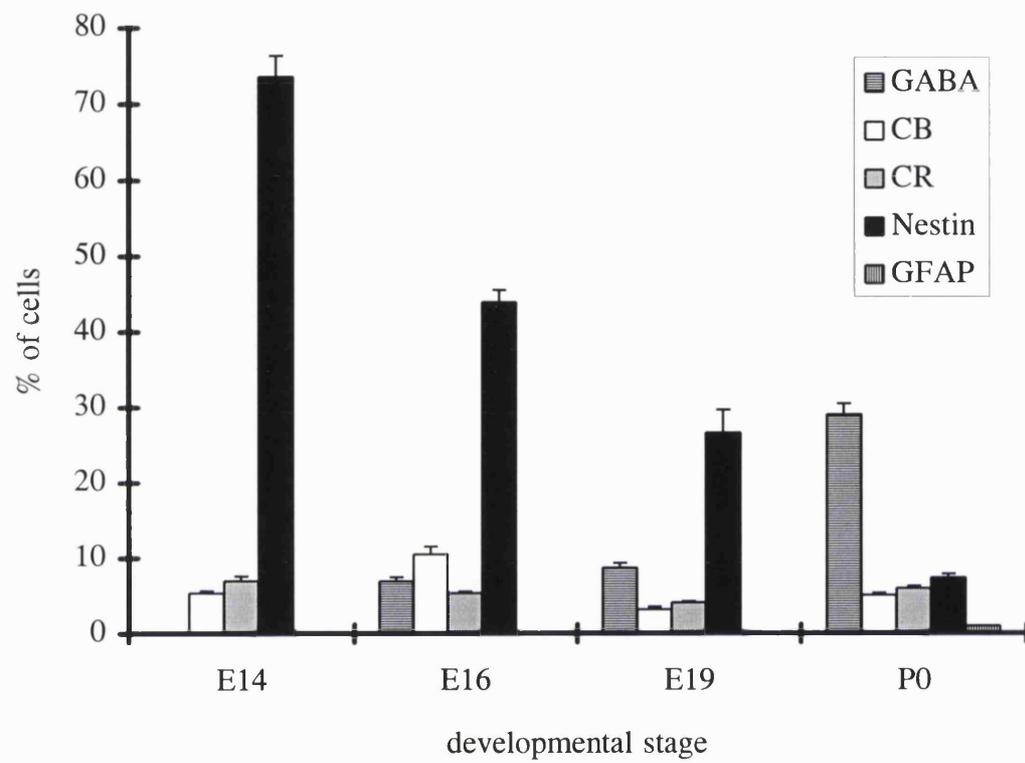


Figure 19

A. Proportions of cell types in E14 cultures kept under control conditions or exposed to 5-HT. The presence of the monoamine did not change any of the proportions in these cultures.

B. Proportions of cell types in E16 cultures kept under control conditions or in the presence of 5-HT, DA, NA or ACh. Serotonin significantly increased the proportion of Glu-containing cells, whereas the other neurotransmitters had no detectable effect.

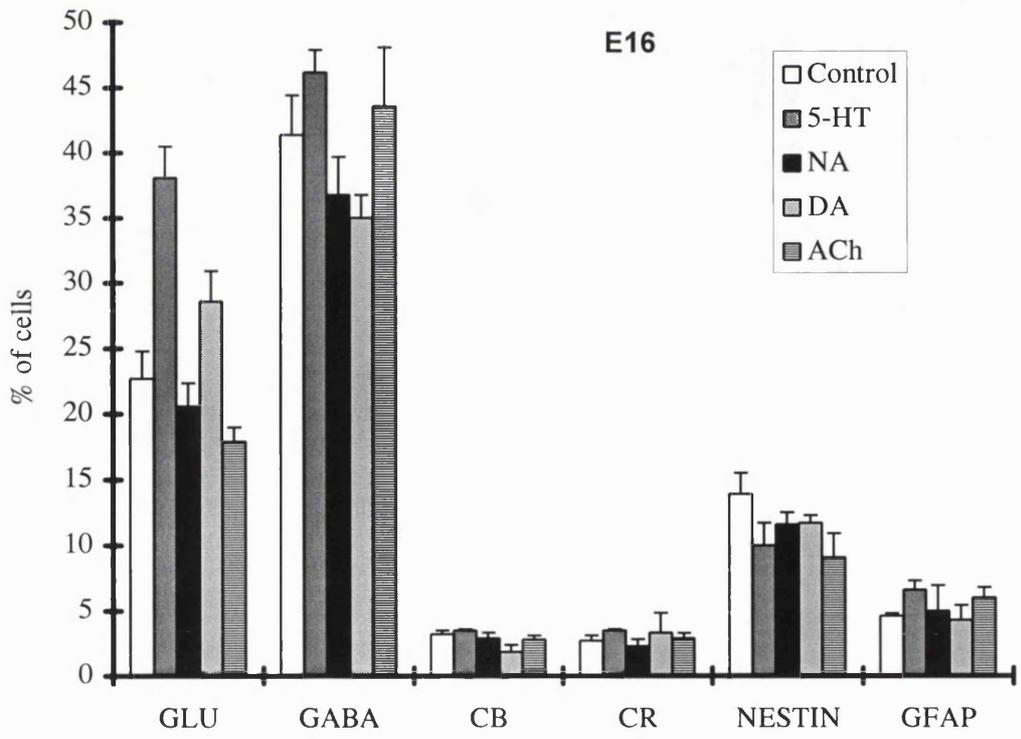
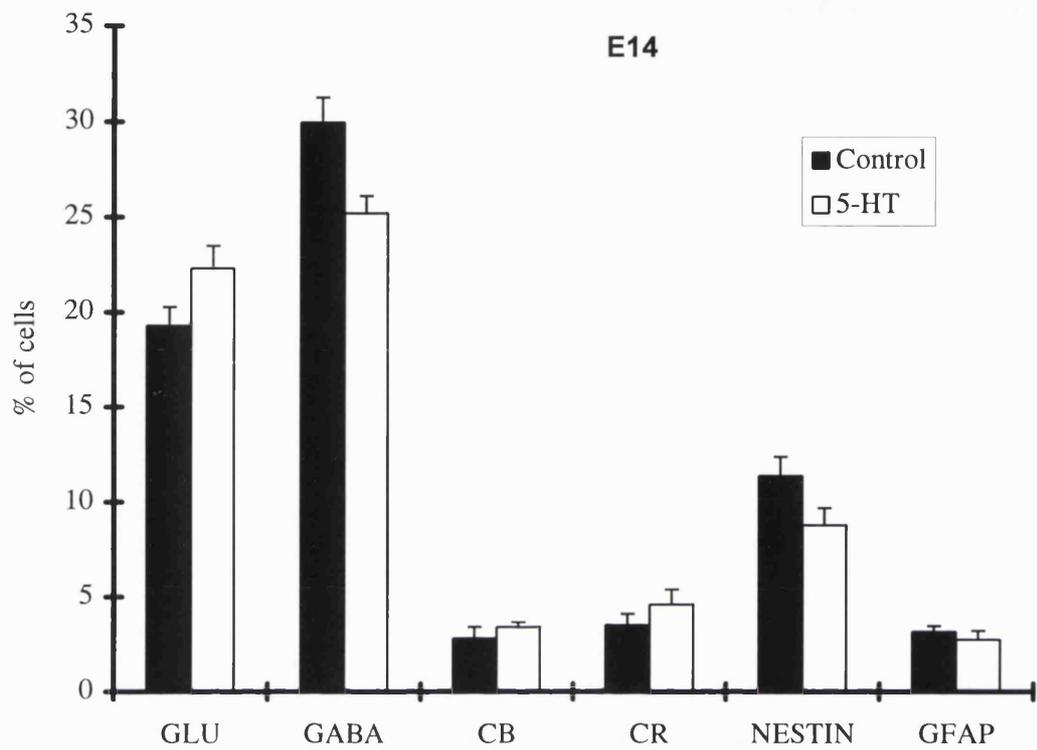


Figure 20

A. Proportions of cell types in E19 cultures kept under control conditions or in the presence of 5-HT. Proportions of various cell types were significantly changed as compared to E16 culture, but the presence of 5-HT had no detectable effect.

B. Proportions of cell types in P0 cultures kept under control conditions. The proportion of Glu-containing cells was somewhat higher than in E19 cultures, but generally no significant differences were found..

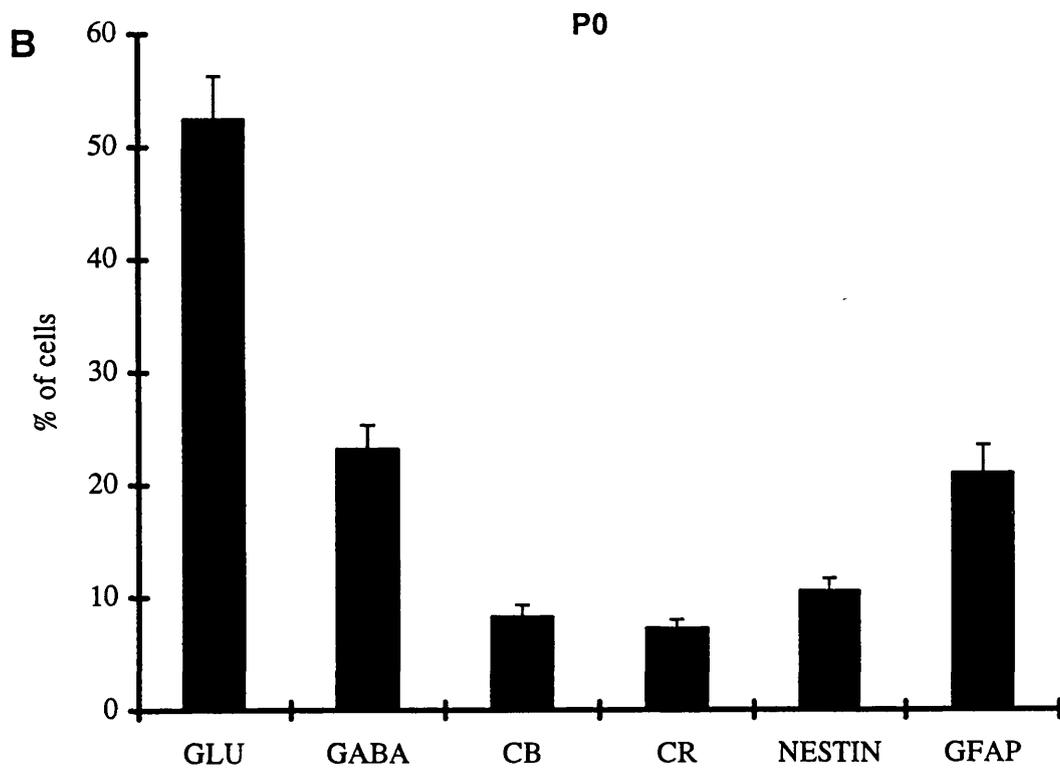
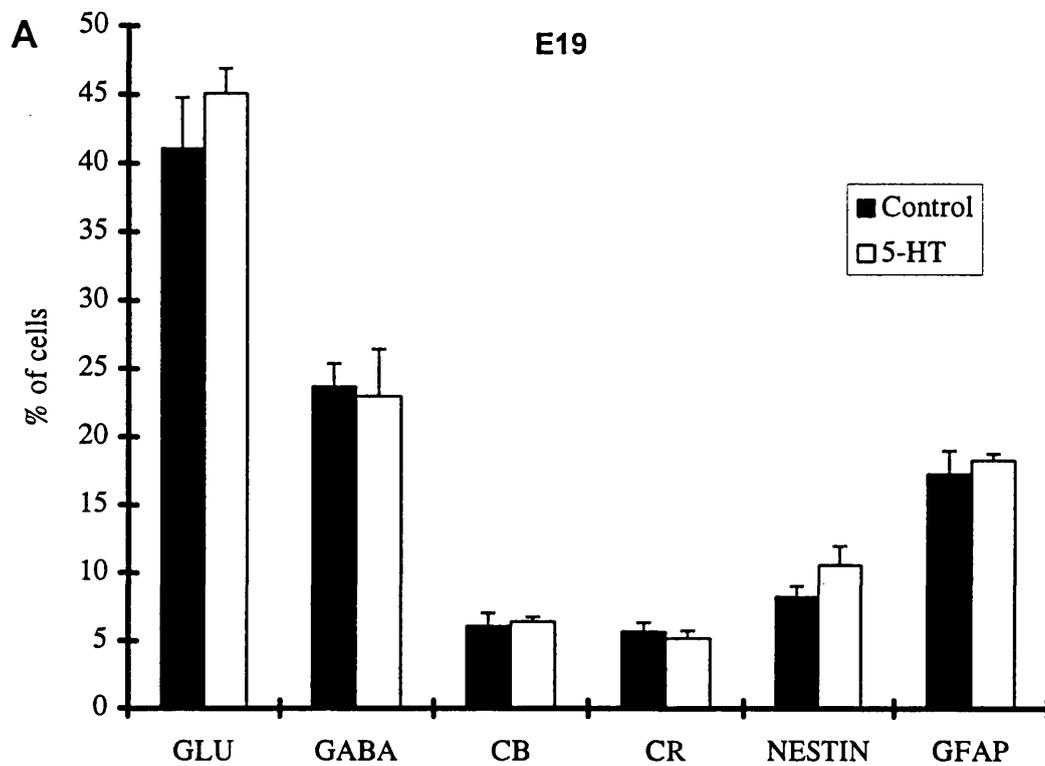


Figure 21

The proportions of Glu- and GABA-containing cells in control cultures prepared at different developmental stages, illustrating the opposing trends in the two cell populations. Culturing E16 slices for 10 instead of 7 DIV did not bring the proportion of Glu-containing neurons in these slices to the same level as in E19/7DIV cultures.

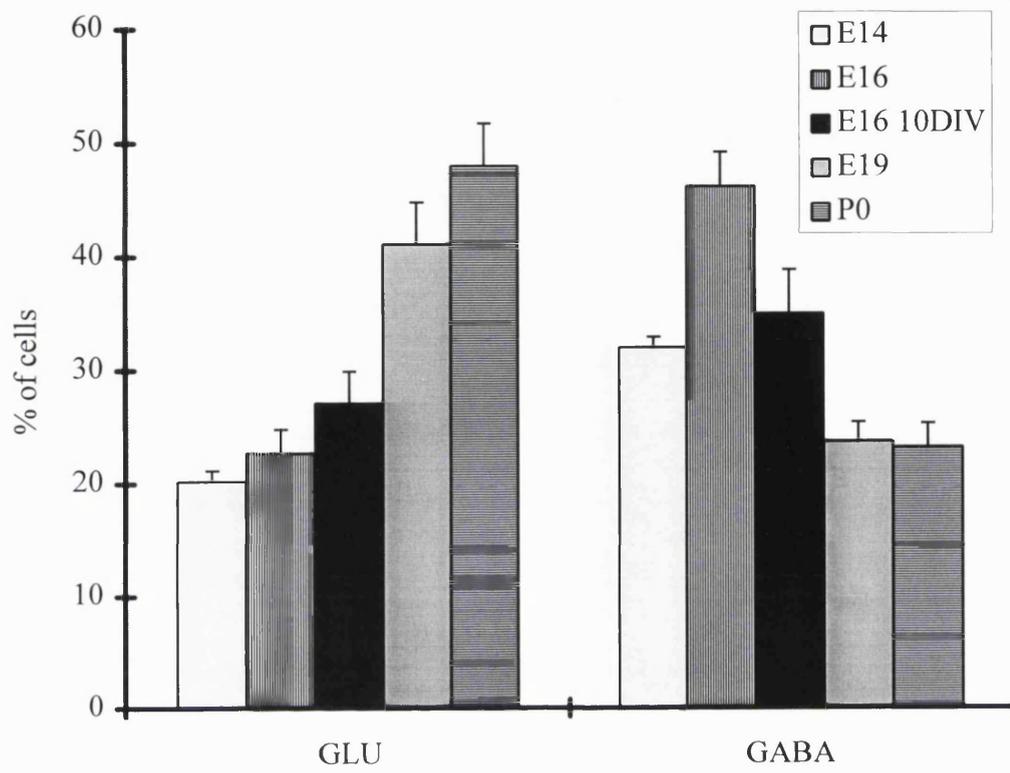


Figure 22

Exposing E16 cultures to 5-HT for the first 4 DIV was sufficient to significantly increase the proportion of Glu neurons. However, exposure to 5-HT during the last 3 DIV had no effect on the Glu cell population.

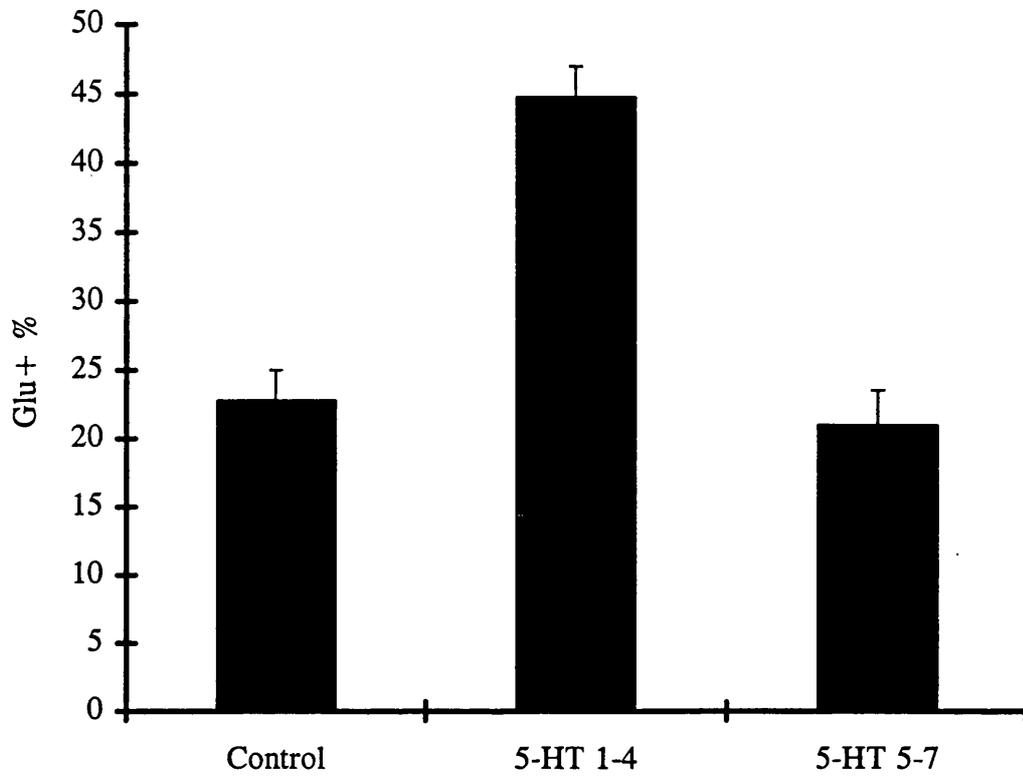


Figure 23

Dose-response curve illustrating the relation between 5-HT concentration in the culture and the proportion of Glu-containing cells. A significant increase in the glutamatergic cell population was first observed with concentrations around 80 μM while maximal effect was detected with doses 120–200 μM .

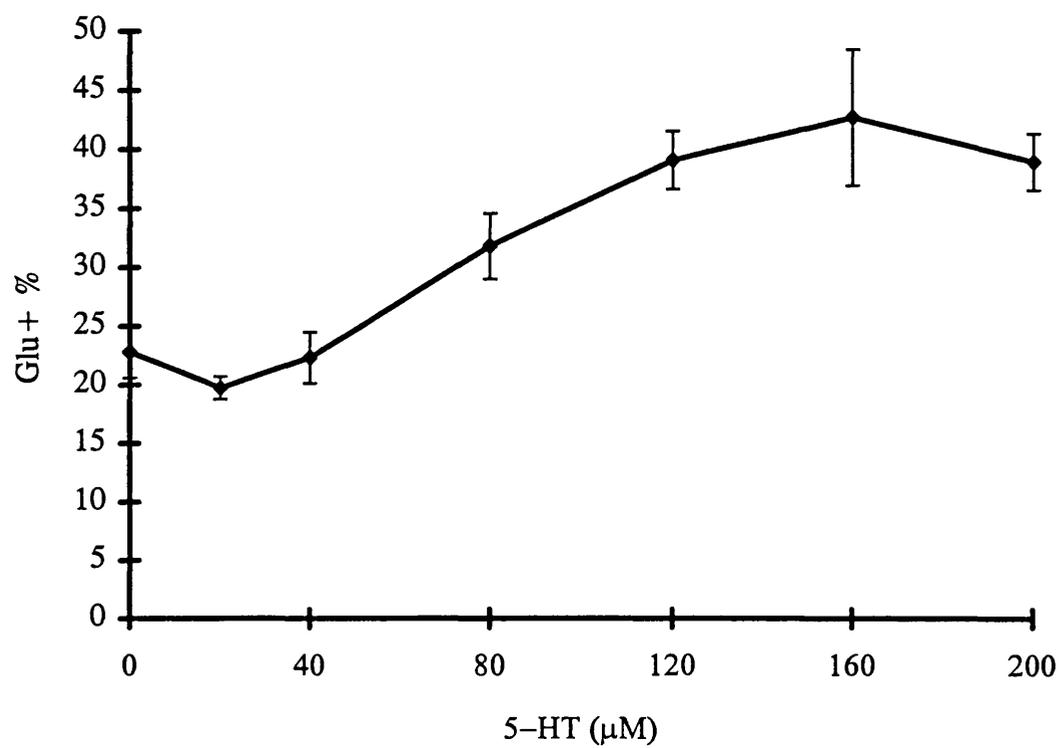


Figure 24 A. Cryostat section (10 μm) through a BrdU-exposed slice showing cells labelled for BrdU. Arrow points to one of the labelled cells. Scale bar = 30 μm .

B. BrdU incorporation expressed as a proportion of BrdU-immunoreactive cells.

Serotonin had no effect on BrdU incorporation.

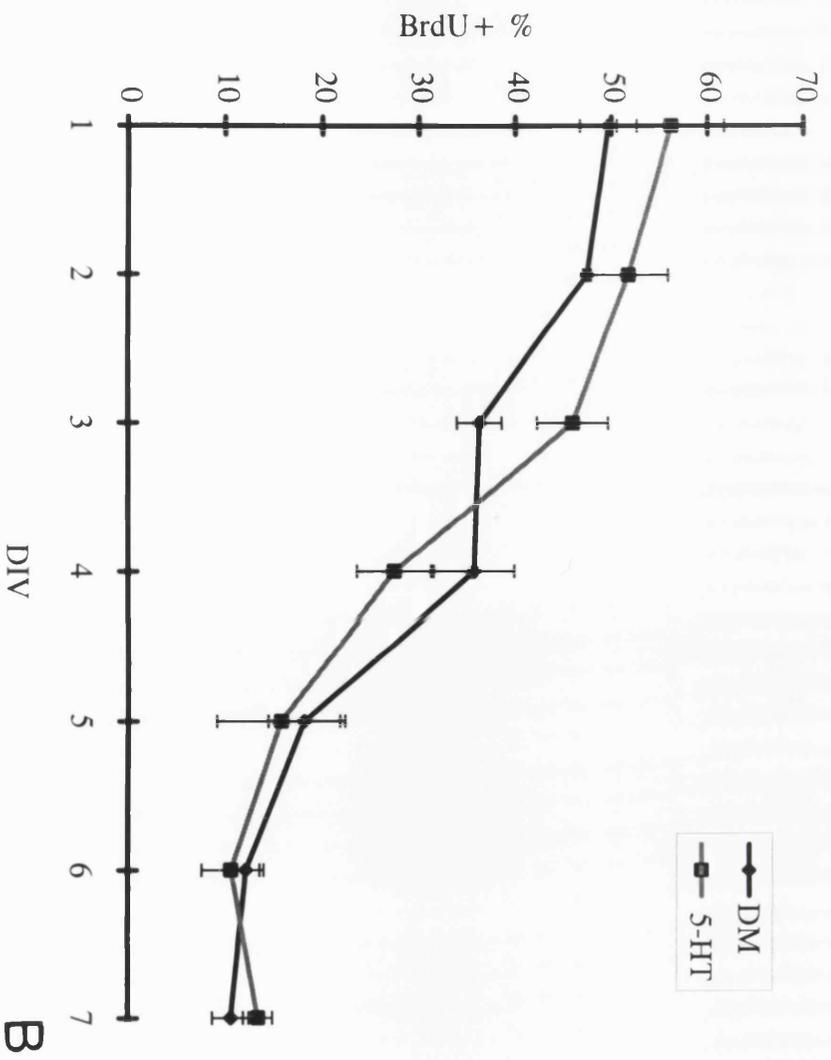
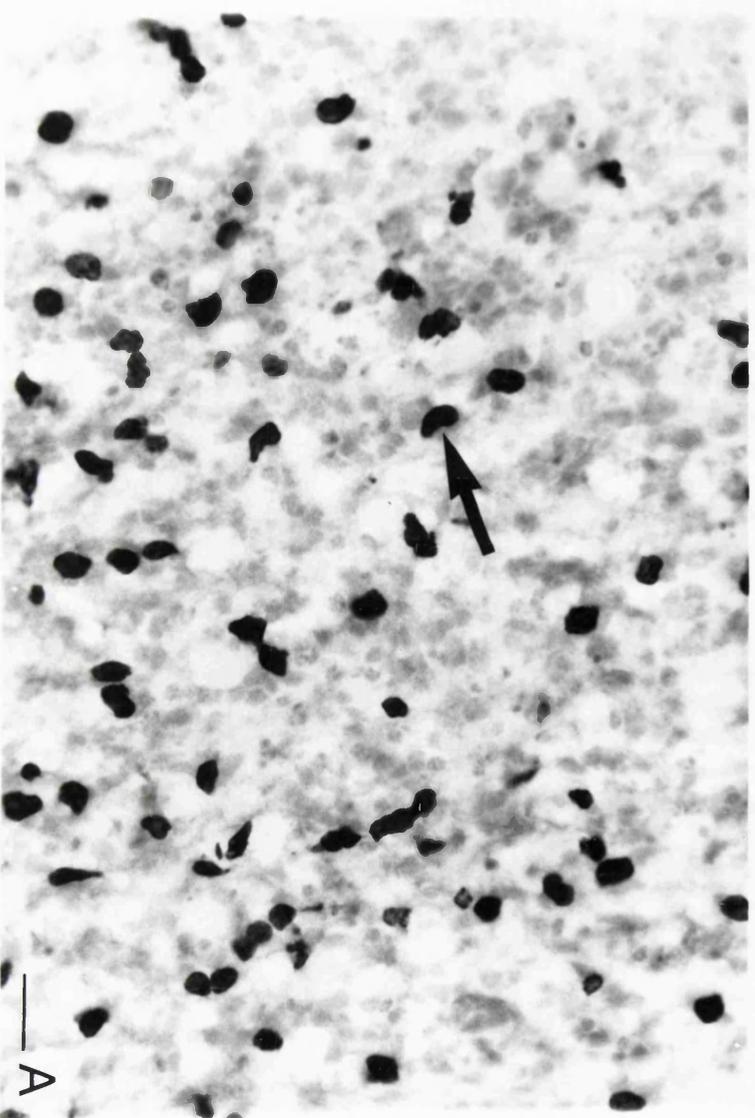


Figure 25

A. Serotonin-immunoreactive neurons in the raphe slice of a raphe-cortex co-culture.

B. Serotonin-immunoreactive processes growing into the co-cultured cortex. The arrow indicates a growth cone with extended filopodia.

Scale bar = 20 μm .

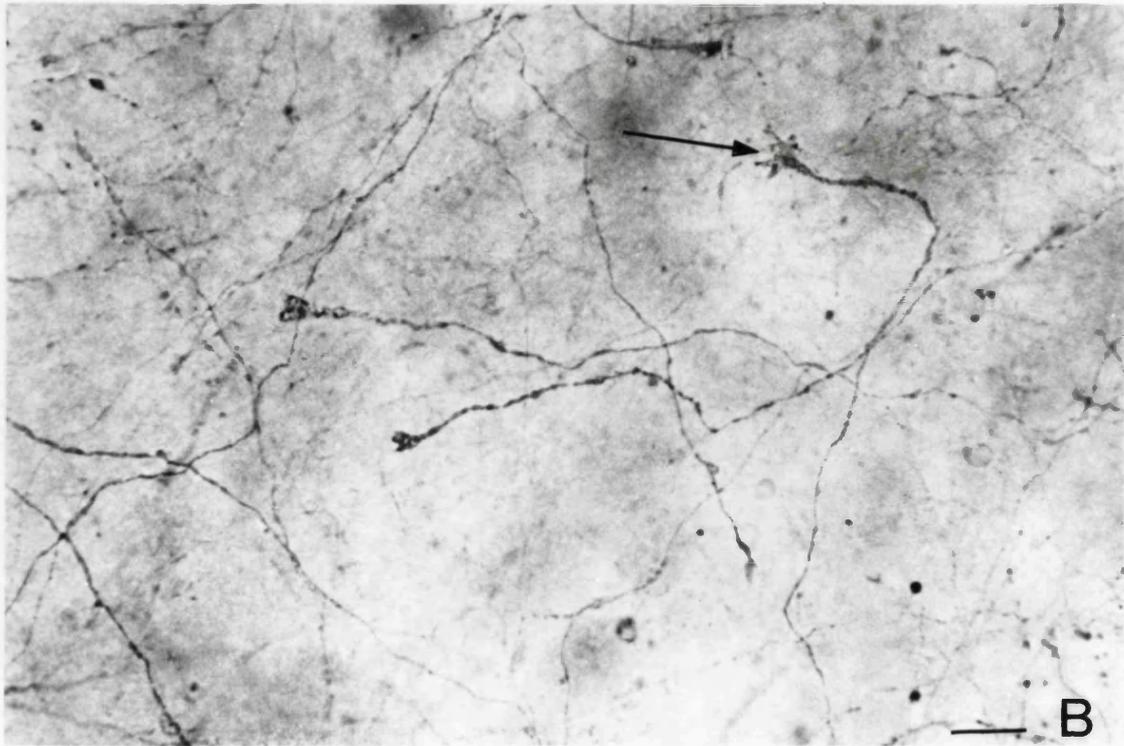
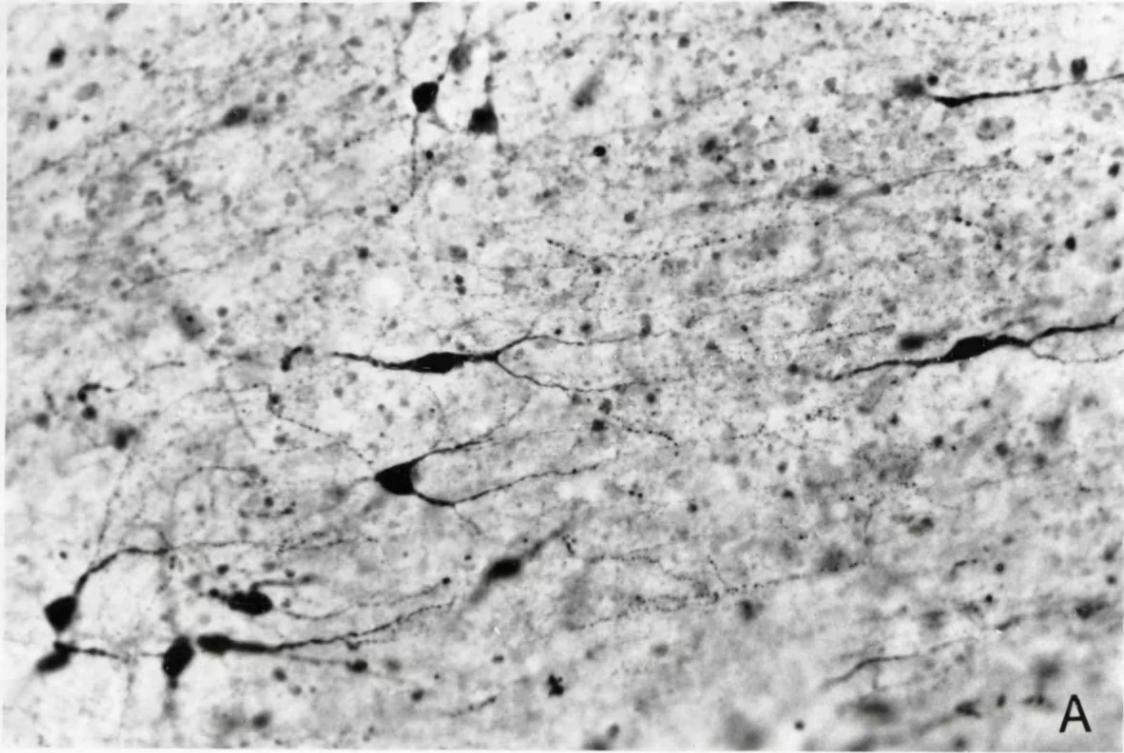


Figure 26

Camera lucida drawing of 5-HT immunoreactive processes through the thickness of the cortical slice of a cortex-raphé co-culture. Pia is at the top.

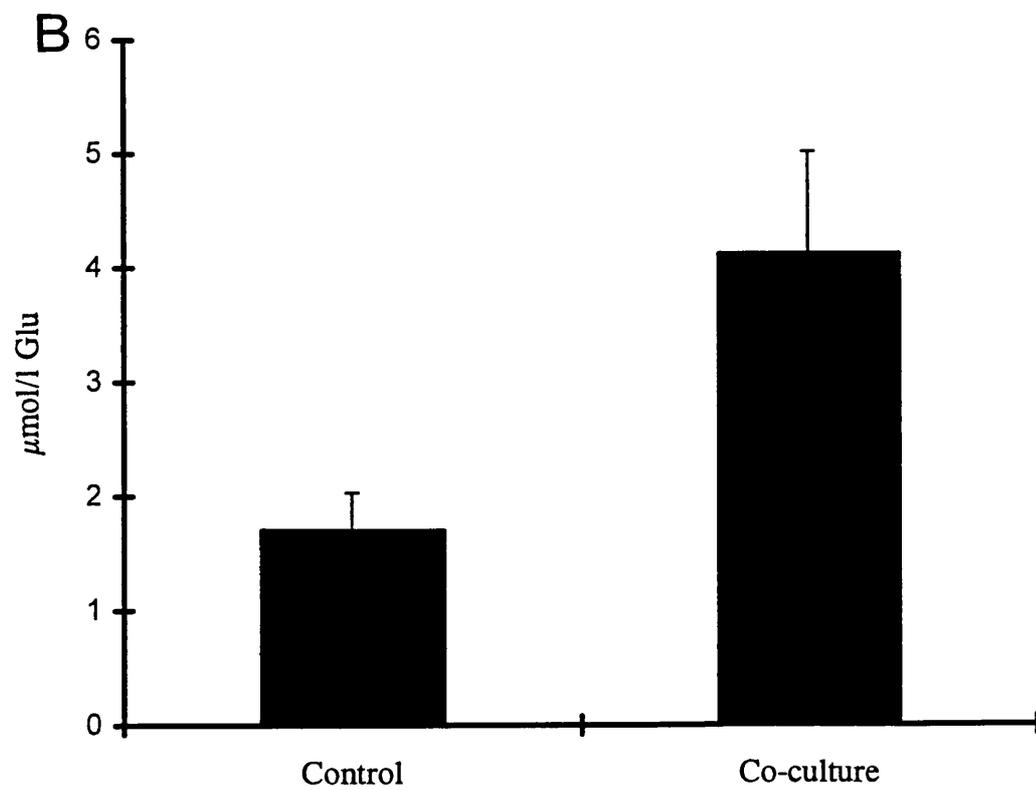
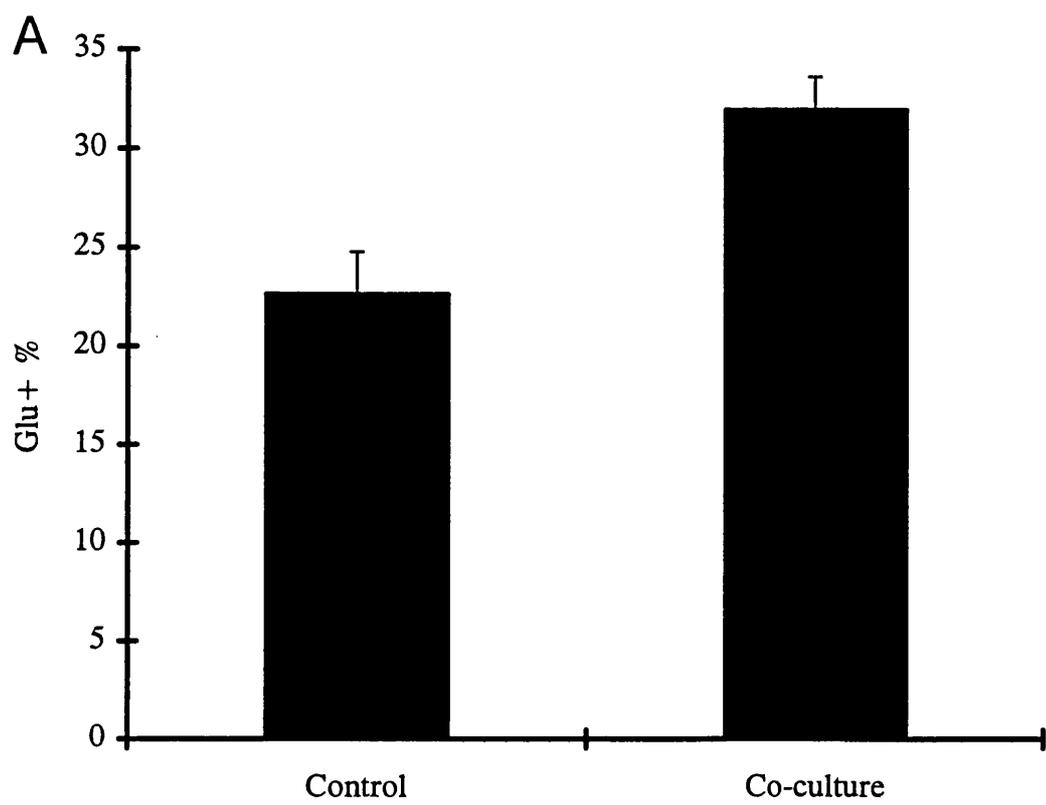
Scale bar = 100 μm .



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Figure 27

Co-culturing raphe slices with the cortex resulted in a significant increase in both the proportion of Glu-containing neurons (A) and in the Glu content of the cultures as assessed with HPLC (B).



4. DISCUSSION

The monoaminergic pathways are among the earliest axonal systems to invade the developing cerebral cortex (Lidov and Molliver, 1982a; Wallace and Lauder, 1983; Kalsbeck et al., 1988; Levitt and Moore, 1979; Verney et al., 1984). Due to their early arrival and widespread distribution throughout the cortex, these axonal systems are in a position to regulate ongoing developmental processes, i.e., cell generation, migration and differentiation. Studies have so far focused on these systems' effects on synapse formation, plasticity and the morphological maturation of cortical cells and their connections (e.g. Kasamatsu and Pettigrew, 1976; Chubakov et al., 1986; Lauder, 1990; Bennet-Clarke et al., 1994). The purpose of this study was to address the possibility that the monoaminergic innervation might participate in the processes of neurochemical differentiation of the cells of the developing cerebral cortex. Although ACh appears in the cortex at a much later stage than the monoamines (Dori and Parnavelas, 1989), it was also included in this study, as the possibility of it acting as a developmental signal has been raised in recent years (Bear and Singer, 1986; Höhmann et al., 1988, 1991; Gu and Singer, 1993; Liu et al., 1994) and as the transient presence of cholinergic structures in the embryonic cortex (Dori and Parnavelas, 1989) could potentially serve such a function.

The first part of this study constitutes a description of the neurochemical content and the morphology of the cellular populations present in slice cultures prepared at various stages of the development of the rat neocortex and kept for 7 days *in vitro*. Changes in the neurochemical composition and morphology were

examined over a period between E14-P0. The second part of the study is an attempt to identify possible alterations in the established parameters brought about by exposing the cultured slices to a number of neurotransmitters (5-HT, DA, NA, ACh) that are present in the cortical afferent systems. The sixteenth day of gestation was chosen as a starting point for the assessment of possible effects of these transmitters, as this stage corresponds to the period when neurogenesis is at its peak. At this stage, 5-HT demonstrated an effect on the neurochemical composition of the slices by significantly increasing the proportion of Glu-containing cells. No effect was observed with the other transmitters examined. After the effect of 5-HT was established, the responsiveness of embryonic cortical neurons to 5-HT was examined in a co-culture system comprising cortical slices and slices containing the raphe nuclei. The proportion of cortical Glu-containing neurons also increased in this system. This effect was additionally assessed and confirmed by measuring the Glu content of the cortical slices with HPLC. BrdU incorporation was shown not to differ between control cultures and slices exposed to 5-HT, precluding an effect of the monoamine on the proliferation of Glu-containing cell precursors. Finally, 5-HT was tested in cultures prepared from E14 and E19, but no effect was observed. As both E14 and E19 cultures were found to be unresponsive, the effect of 5-HT was presumed to be restricted to a specific time period and no 5-HT was added to cultures prepared at P0.

4.1. Methodological considerations

4.1.1. Selection of culture system

Relative to the *in vivo* condition, *in vitro* systems offer a comparative advantage in two major points: accessibility and reduction of variables. The latter can be a drawback as well as an advantage: it allows for an easier interpretation of the effects of varying conditions, but it also means that the system is significantly different to the *in vivo* situation and that any extrapolations should be made taking this difference into account. Organotypic cultures have been a useful tool in studying brain structures under controlled conditions since their first experimental application in the first decade of this century (Harrison, 1907). These preparations maintain the local microenvironment but are small enough to survive without capillary circulation, by simple diffusion of nutrients and catabolites between the preparation and the bathing medium and allow for easy accessibility and control of conditions. Organotypic slice cultures of the cerebral cortex have been shown to maintain histological organization and exhibit cell maturation (Caeser et al., 1989), migration (Roberts et al., 1992), preservation of barrelfield structures (Behen et al., 1991) and synaptic connectivity not dissimilar to that found in the intact cortex (Wolburg and Bolz, 1991). Appropriate connectivity has also been shown to be established between organotypic slices of the cortex and other brain structures (Bolz et al., 1990; Novak and Bolz, 1992; Yamamoto et al., 1992; Distler and Robertson, 1993) and layers already formed at the time of explantation persist *in vitro* (Annis et al., 1993). However, in prenatal cortical preparations cell migration takes place to a smaller extent than *in vivo* (Gillier and Price, 1993), and migrating

cells fail to form defined layers as they do in postnatal cultures (Götz and Bolz, 1992). Taken together, these studies demonstrate the adequacy of the local microenvironment for preservation of the cortical structure, but at the same time stress the importance of additional parameters in development that are missing from the microenvironment of the slices.

The slice culture method of Stoppini et al. (1991) was chosen for its simplicity and because it does not require the use of an adhesive medium such as a plasma clot, as is the case in the roller drum technique (Gähwiler, 1981). Gradual diffusion of the clot would mean that the medium could not be considered as defined, while dissociation of the slice after the end of the culture period would also be rendered more difficult by the presence of the clot. The overgrowth of the glial population observed with the roller drum technique (Del Rio et al., 1991) was also not desirable.

4.1.2. Concentration of neurotransmitters

Neurotransmitters were applied to E16 cultures at various concentrations before the working concentrations were finalized. The concentrations initially applied were 20 μM (5-HT) and 10 μM (DA, NA and ACh), at a range close to the physiological or minimally active concentrations (Chubakov et al., 1986; Cooper et al., 1986) and preliminary cell counts were performed. These revealed no significant effects, and subsequently concentrations 10 times higher were tested, in the range previously shown to cause toxicity in dissociated cell cultures (Graham et al., 1978; Rosenberg, 1988). In this case, signs of toxicity were observed in cultures exposed

to DA (and, to a lesser extent, NA), namely high proportions of dead cells (as observed under phase contrast after dissociation), fragility of the slices, and a brown discoloration of the slices and the medium. The autooxidation of DA and NA results in the production of toxic quinones and the stoichiometric generation of H_2O_2 and the free radicals $O_2^{\cdot -}$ and OH^{\cdot} , both of which processes contribute to the cytotoxicity in varying degrees (Graham et al., 1978). The brown discoloration observed after a few days of exposure to 100-150 μM DA is in keeping with the presence of a considerable amount of neuromelanin, produced by polymerization of the autooxidation end product indole quinone (Graham, 1978). Noradrenaline was less toxic than DA, in agreement with previous *in vitro* studies (Graham et al., 1978; Rosenberg, 1988), and 5-HT (a substance more stable than the catecholamines) was even less toxic, as previously demonstrated *in vitro* in dissociated cultures (Rosenberg, 1988), having deleterious effects only in concentrations higher than 250 μM . No ACh toxicity was observed in the 100-150 μM range. The toxic effects of the monoamines were manifest in concentrations somewhat higher than what has been reported by Rosenberg (1988) in dissociated cortical neurons. This is consistent with the finding, in the same study, that the toxic action (up to a certain neurotransmitter concentration) was dependent on cell plating density, an effect interpreted as a result of the actions of mechanisms for clearing and sequestering the monoamines as well as for inactivating the end products of their degradation. It is to be expected, then, that the toxicity threshold would be higher in slices, where cell density is higher than in dissociated cell cultures. Taking these factors into consideration, the concentrations used were 50 μM for DA, 100 μM for NA and ACh, and 200 μM for 5-HT. After an effect of

5-HT was established at 200 μ M, 40, 80, 120 and 160 μ M were also applied, so as to produce a dose-response curve. Application of 40 μ M 5-HT produced no changes in the proportion of the different cell types, while 120 mM and 160 μ M were as effective as 200 μ M. The presence of 80 mM of 5-HT in the medium resulted in a proportion of Glu-containing cells (31.8%) that was significantly higher than the controls ($p=0.05$), and at the same time lower than what was obtained with 200 μ M 5-HT, the difference being just below the limit of statistical significance ($p=0.06$) (fig. 23).

Another issue related to the concentration was the stability of 5-HT in the culture medium. High pressure liquid chromatography showed that the concentration of 5-HT decreased from 200 μ M to about 155 μ M in the 18 hours between the start of the culture and the first sampling, but did not change significantly over the following 24 hours, indicating that the neurotransmitter was fairly stable. The apparent paradox of the medium containing a somewhat higher (but not significantly so) concentration after 36 hours than after 18 hours, is explained by the fact that a different set of 3 cultures was sampled at each time point, so as to avoid causing stress to the cultures by removing considerable quantities of medium.

4.1.3. Immunohistochemistry and quantitation

Although cultures become considerably thinner during the 7 day culture period, they remain a few layers thick (also see Stoppini et al., 1991). As such, they can be stained for qualitative examination of cell morphology, but they are still too thick to be used for quantitation, as cells deeper in the slice cannot be stained and the

of the slices seems like the obvious choice, but the staining obtained with the anti-Glu antibody on cryosectioned cultures was of poor quality and was considered to be an inappropriate basis for reliable quantitation. The method used by Vaccarino et al. (1995) was chosen, as it was found to give a strong contrast between stained and unstained cells and facilitated rapid counting, with all cells being reduced to either stained or unstained spheres against a light background (figs. 16,17). For the counts to be consistent, quantitation for all cell-specific antigens was performed using the same system. Cryostat sections were used in the case of monoclonal antibodies against BrdU and TUJ1, as it was found that these would not stain agarose-embedded cells. Temperatures in the range 40⁰-50⁰ C were used in the preparation of the agarose slices. Such temperatures, though not excessive, could induce minor conformational changes to which antigenic reactivity is very sensitive (Ghélias and Yon, 1982). As monoclonal antibody reagents consist of immunoglobulins binding only to a specific epitope (Köhler and Milstein, 1975), such a change could be enough to abolish staining altogether.

4.2. Interpretation of the results

4.2.1. Glutamate-containing cells

The first appearance and subsequent development of the two neuronal cell types, characterized by the presence of either Glu or GABA, have been studied in the developing cortex of the rat *in vivo* (Van Eden et al., 1989; Parnavelas, 1992; Dori and Parnavelas, 1996) and in slice cultures (Götz and Bolz, 1994). In the present study, Glu-containing neurons could not be identified in acutely dissociated (not

cultured) cells from E14, E16 and E19 cortices, while cells from neonatal animals exhibited some degree of differential staining, though not to an extent that would allow for a reliable quantitation. This is in keeping with the previous finding that an increased background starts appearing around E20-P0, while clear-cut Glu-immunostaining is not present before P3 (Dori and Parnavelas, 1996). However, Glu production was initiated *in vitro* and Glu-containing neurons were present in slices prepared from all four developmental stages (E14, E16, E19, P0) and cultured for 7 days. Their proportion increased from 20% of all cortical cells in the early cultures to about 50% in slices taken from P0 rats. This is consistent with the relatively late appearance of Glu in cortical pyramidal neurons (Götz and Bolz, 1994; Dori and Parnavelas, 1996). This increase was more pronounced between the E16 and E19 stages.

The main finding to emerge from the present study is that exposure of E16 cortical slices to the indoleamine 5-HT increased the proportion of Glu-containing neurons (and the total Glu content), without affecting the proportions of other neuronal or glial cell types. Neither the catecholamines, NA and DA, nor ACh had any effect on the proportions of the different cortical cell populations.

The 5-HT innervation of the cortex originates in the mesencephalic dorsal and median raphe nuclei (Moore et al., 1978). Immunohistochemical studies of the development of the serotonergic system in the rat brain have shown that 5-HT containing neurons can first be detected in the raphe nuclei at E12. The axons of these neurons begin to elongate shortly thereafter and ascend through the medial forebrain bundle to enter the rostral telencephalon around E16-E17. (Lidov and Molliver, 1982a, b; Aitken and Törk 1988; Wallace and Lauder, 1983). Upon

arriving in the cortical anlage, the 5-HT axons enter as two tangential sheets, one above and one below the cortical plate. They then gradually arborize sending branches into all cortical layers. Specific 5-HT receptors have also been localized in the developing cortex of the rat (Whitaker-Azmitia et al., 1987; Leslie et al., 1992; Hellendal et al., 1993; Morilak and Ciaranello, 1993), with some subtypes (e.g. 5-HT₃; Johnson and Heinemann, 1995) expressed in neuroepithelial cells in the proliferative zones. Serotonergic receptors have been shown to be “functional” in embryonic life (Whitaker-Azmitia et al., 1987), supporting further a role for the serotonergic system in the early stages of cortical development.

Lineage studies have shown that the expression of the neurotransmitters Glu and GABA in cortical neurons is specified early in corticogenesis (Mione et al., 1994). This determination is likely to depend on the interaction between the cells' inherent properties and the local environmental signals to which these cells are exposed. The finding that the proportion of Glu-containing neurons did not change in E16 slices cultured for 10 instead of 7 days and the fact that this proportion was considerably lower than in slices of comparable “age” but prepared from older embryos (E19/7DIV) suggests that it is the stage of explantation rather than the age of the cells that determines the size of the Glu neuronal population. It also suggests that part of the cell population that would differentiate into glutamatergic neurons *in vivo* does not do so in this *in vitro* situation implying the importance of some extrinsic factor(s) that the cells are deprived of following explantation. The finding that cortical neurons explanted at E19 or P0 are capable of expressing the appropriate neurotransmitters *in vitro* is in agreement with data from dissociated cell cultures (Götz and Bolz, 1994), according to which dissociated cultures of

neurons prepared at this stage are capable of expressing Glu and GABA in the same proportions as *in vivo*, when they are left in culture for a more prolonged period of time.

A similar albeit somewhat smaller increase in the Glu-containing neuronal population was also observed in cortices cultured together with slices containing the raphe nuclei. Why is the effect in the co-culture experiment not as pronounced as that following addition of 5-HT into the culture medium? This may simply be due to differences in the concentration of 5-HT in the two experimental paradigms, with cells in the raphe nuclei requiring a certain time after placement into culture to re-establish a normal level of metabolic activity. Replenishment of the medium presumably also results in depleting the local 5-HT concentration built up at the points of release within the innervated cortical slice. Irrespective of the concentration of 5-HT available to cells in the slice, innervation of the cortex by 5-HT-containing axons resulted in more than doubling in the amount of Glu in the cortex as measured by HPLC. Serotonergic axons arising in the raphe invaded the cortex as thick and varicose fibers occasionally giving rise to short branches. Electron microscopical examination revealed that these axons did not engage in synaptic contacts with neuronal elements in the cortex, consistent with results of earlier studies of synaptogenesis (Wolff, 1978). This would suggest that 5-HT does not exert its effect through synapses in agreement with the notion of non-synaptic release of transmitters acting as regulatory factors early in development. The presence of synaptic connectivity between serotonergic afferents and cortical cells is well established in the adult (Papadopoulos et al., 1987 a, b), but the situation is different during the period of early corticogenesis. Non-synaptic release of 5-HT

from developing neurons has already been documented *in vitro*: In acute preparations, serotonergic elements innervating the rat hypothalamus exhibit uptake and spontaneous release of [³H]5-HT at E16, while K⁺-stimulated, Ca²⁺-dependent release first appears at E17, suggesting that at least part of the released 5-HT is stored in vesicles (Ugrumov et al., 1989). Serotonergic neurons from E14 rat embryos cultured for 3DIV have also been shown to release 5-HT in a Ca²⁺-dependent manner, while additional evidence for the intravesicular storage of 5-HT at these early stages is given by the demonstrated inhibition of [³H]5-HT uptake by nigericin (which affects the gradient across vesicle membranes) and reserpine (which competes for the amine transporter) (Reisert et al., 1989). This release can take place through varicosities (as proposed by Reisert et al., 1989) and/or growth cones, a phenomenon observed with a number of transmitters (Hume et al., 1983; Young and Poo, 1983; Lockerbie et al., 1985; Taylor and Gordon-Weeks, 1988). Isolated growth cone preparations from the developing rat brain have been shown to contain the synaptic vesicle protein synaptophysin as well as 5-HT-binding protein, specifically found in 5-HT-containing vesicles, from as early as E15 (Ivgy-May et al., 1994). In the same study, the ratio of 5-HT to 5-hydroxyindoleacetic acid in the isolated growth cone preparations suggested that 5-HT is protected from MAO, further supporting vesicular storage, while reserpine was shown to deplete 5-HT from the growth cones at E20 (but not E15). Taken together, these studies suggest that serotonergic neurons are capable of non-synaptic release of 5-HT from as early as E15-E16. The timing of the emergence of this early non-synaptic release is compatible with the notion of 5-HT participating in the

maturation of newly generated cortical neurons as suggested by the findings of this study.

Is the increased proportion of Glu neurons due to increased proliferation in the cortex after exposure to 5-HT? The selective mitogenic action of 5-HT on progenitors of glutamatergic cells is not supported by the experiments of BrdU incorporation, as levels of incorporation did not differ between the control and 5-HT treated groups. It is known that glial cells can accumulate Glu (Henn et al., 1974; Drejer et al., 1982) and the presence of more Glu-containing cells could reflect an increase in the glial cell population. However, this scenario can be easily ruled out, as: a) there is no increase in the GFAP-reactive cell population in the slices exposed to 5-HT, and b) the morphology of Glu-containing cells is that of immature neurons (fig. 7A) and clearly contrasted to the glial morphology as demonstrated by GFAP staining (fig.9B). Furthermore, it has been shown that cortical astrocytes are first labelled for glutamine synthase (the enzyme that converts Glu to glutamine) around the time of birth (Norenberg, 1983), making it unlikely that glial cells participate in Glu metabolism before that time. 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 cell population? Having established the effect as specifically neuronal, there are two possibilities that may account for this increase: a) Selective survival of glutamatergic neurons; and b) differentiation of newly generated neurons. The first possibility can be ruled out because cell counts did not reveal differences in the proportions of all other cell types between the control and 5-HT treated group. A selective survival effect should result in a lower proportion of all other cell types.

Instead, the only cells that showed a diminished proportion in the 5-HT treated cultures are the unstained ones (non- Glu, GABA, CB, CR, nestin, GFAP; 19B). This would then argue in favour of differentiation of immature neurons along the Glu pathway.

The hypothesis that 5-HT influences the onset of differentiation of prospective target neurons during embryogenesis was first put forth by Lauder and Krebs (1978) in a study in the superior colliculus and hippocampus of rats that had received the 5-HT depleting drug p-chlorophenylalanine (pCPA). They demonstrated that pCPA retarded neuronal differentiation (as assessed by a delay in the time of cessation of cell division) and suggested that increased differentiation could occur as a direct interaction between 5-HT axons and proliferating progenitor cells or due to circulating monoamines influencing dividing cells. However, as no effect was found in the rate of cell proliferation (fig. 24B), the results of this study suggest that 5-HT does not act on dividing cells, but rather on newly generated postmitotic neurons. The addition of 5-HT in the culture medium seemed to compensate for the isolation of cortical cells from the required signal(s), in that it increased the proportion of Glu-containing neurons to a level equal to that observed near the end of the period of neurogenesis.

Under control conditions, the proportions of Glu-containing cells obtained with E14 and E16 cultures are similar (figs. 20A,B). This is not surprising, given the fact that the neurochemical expression of the glutamatergic phenotype occurs relatively late (Dori and Parnavelas, 1996). If the serotonergic innervation participates in the maturation of the glutamatergic phenotype, as the results of this study suggest, then it is to be expected that no such influence will have been

exerted *in vivo* between the ages of E14 and E16, as the serotonergic innervation is absent before E16 (Lidov and Molliver, 1982a; Wallace and Lauder, 1983; Aitken and Törk, 1988). Why are these E14 cultures not affected by the presence of 5-HT in the medium? In situ hybridization detection of m-RNA transcripts for 5-HT_{1C}, 5-HT₂ (Hellendall et al., 1993) and 5-HT₃ receptors (Johnson et al., 1995) has demonstrated that the expression of these receptors is developmentally regulated. 5-HT_{1C} receptor transcripts are first detected in the telencephalon at E16 and 5-HT₂ transcripts at E14, while 5-HT₂ immunoreactivity is first detected in the cortex much later, at P0 (Morilak and Ciaranello, 1993); whether this delay reflects a time lag between the appearance of mRNA transcripts and the receptor protein or a difference in the sensitivity of the two methods, or both, is a matter of debate. 5-HT₃ transcripts are first present in small quantities in the cortical neuroepithelium at E14 (Johnson et al., 1995) and are found in progressively increasing quantities by E18, by which time they are present throughout the cortical anlage, mostly concentrated in the marginal and subventricular zones. It seems, then, that the presence of 5-HT receptors is, at the most, in its very first stages at E14. If the presence of neurotransmitter receptors in embryonic tissues serves an ontogenetic function, it is to be expected that 5-HT receptors would not be developed in E14 cells, as the serotonergic innervation of the telencephalon first appears 2-3 days later (Lidov and Molliver, 1982a; Wallace and Lauder, 1983; Aitken and Törk, 1988). In this context, it is interesting to note that, in the brain stem, 5-HT_{1A} receptor transcripts were first detected by PCR at E12, increasing to a maximum level at E14-E15 and then progressively diminishing until they become undetectable at E20 (Hillion et al., 1994). As the function of 5-HT receptors in the

embryonic brain stem is likely to be related to the (auto)regulation of serotonergic cells development, the early appearance and decline of the transcript level is in keeping with the first appearance of serotonergic cell somata around E12-E13 (Lidov and Molliver, 1982b; Wallace and Lauder, 1983; Aitken and Törk, 1988).

The findings of this study then suggest that 5-HT, acting through newly available receptors, may be one of the signals that immature cells in the cortex require to follow the correct differentiation pathway, without implying that this neurotransmitter is involved in the choice of the pathway.

4.2.2. GABA-containing cells

The early presence of GABA and GABA-containing neurons in the cortex has been demonstrated in a number of neurochemical (Coyle and Enna, 1976; Coyle, 1982) and immunocytochemical (Wolff et al., 1984; Van Eden et al., 1989; Cobas et al., 1991) studies. These immunocytochemical studies first detected labelled cells in the primordial plexiform layer at E14. At E15, the number of GABAergic cells is greatly increased appearing in the plexiform layer and the intermediate zone. Beginning at E16 and throughout the remaining period of gestation, GABA-containing neurons appear throughout all layers of the developing cortex including the ventricular and subventricular zones. After E19, while the number of GABAergic neurons in the cortical plate increases, those in the marginal zone and in the layers below the cortical plate diminish in number significantly.

In acutely dissociated (non-cultured) cortical preparations, some GABA immunoreactivity was apparent from E14, but quantitation was not possible, as in the case of P0 Glu staining. Easily discernible GABA-immunoreactivity appeared

from E16 onwards, in increasing proportions, reaching around 30% at the time of birth.

GABA-containing neurons in the slice preparations exhibited a pattern of development similar to that found *in vivo*. GABAergic neurons outnumber Glu-containing neurons, comprising about one third of all cells in the cultures prepared from E14 animals. Their proportion increased at E16, but declined to about 20% of the total cell population at E19 and P0. These figures are in agreement with previous findings in dissociated cell cultures, where GABA-containing cells have been found to make up between 64% (Deloulme et al., 1991) and 20% (Götz and Bolz, 1994) of the total cell population, depending on the age the cultures were prepared. GABA as well as Glu have been shown to influence several aspects of cortical neuronal development *in vivo* and *in vitro* (Lipton and Kater, 1989; Komuro and Rakic, 1993), and recent studies (LoTurco et al., 1995; Antonopoulos et al., 1997) have provided evidence that both amino acids regulate cell proliferation in the ventricular zone. However, the local environmental factors that may be involved in the regulation of GABA expression itself in the cortex are not known. The present study has demonstrated that the differentiation of the GABAergic phenotype is not influenced by the monoaminergic or cholinergic afferent systems. These results also preclude the possibility that the increased number of Glu-containing neurons following 5-HT application is a consequence of a choice neurons have between expressing Glu or GABA, as the increase of Glu-containing neurons following 5-HT application is not accompanied by a decrease in GABA-containing neurons.

4.2.3. Calcium-binding protein - containing cells

The calcium binding proteins, implicated in functions related to intracellular calcium buffering (Blaustein, 1988; Baimbridge et al., 1992) are thought to define subpopulations of cortical nonpyramidal neurons (Demeulemeester et al., 1989; Celio, 1990). Cells containing the calcium-binding proteins CB and CR were present in the acutely dissociated cells of embryonic cortices (fig. 18). The proportion of *CB*-positive cells increases from E14 to E16, presumably as a result of the addition of immunoreactive cells in the cortical plate to the first immunoreactive cells of the PPL. Both the proportion and the intensity of the stained cells diminishes at E19, as the number of positive cells in layer I and the subplate declines, their immunoreactivity is reduced, and the ratio of the *CB*-positive cells to the total cortical cell population is decreased as the cortical plate increases in size (Sánchez et al., 1992). The proportion of *CR*-containing cells was higher in E14 compared to the other two embryonic stages studied; the *CR*-positive cells found at E14 belong to the PPL, while the number of *CR*-immunoreactive cells subsequently appearing in the cortical plate has been found to be very small during all embryonic stages (Fonseca et al., 1995), so that the increase in the absolute size of the cortical plate results in the observed decrease in the proportion of *CR*-containing cells. Calretinin-positive cells in the cortical plate begin increasing in numbers on P0 (Fonseca et al., 1995), which is reflected in the somewhat increased proportion found at P0 in this study. The proportions of cells containing the calcium-binding proteins in the cortical slices were not affected by the presence of the neurotransmitters in the culture medium and exhibited a trend towards higher

values in cultures prepared at more advanced stages (figs. 19A,B; 20A,B); this increase presumably corresponds to the final wave of CaBP expression observed postnatally *in vivo* both in the case of CB (Sánchez et al., 1992) and CR (Fonseca et al., 1995), and demonstrates that these CaBPs are capable of expression without the need for external signals. However, lineage studies in the cerebral cortex (Mione et al., 1994) have shown that the expression of these proteins is not lineage dependent and would, therefore, be likely to be determined by environmental factors. Immunocytochemical studies in the cortex have demonstrated a close relationship between 5-HT fibers and nonpyramidal neurons containing CaBPs (Hornung and Celio, 1992). This prompted the suggestion that the expression of these proteins is influenced by the serotonergic afferents. Afferent connectivity might be important in establishing and stabilizing CaBP phenotypes after the waves of expression observed in early life (Sánchez et al., 1992; Fonseca et al., 1995). However, it is important to stress that an association does not necessarily imply a causal relation of this nature, as it could be that the presence of a particular CaBP induces the specific innervation rather than the converse. In any case, the findings of this study do not support the possibility of an inductive role of the afferent systems during corticogenesis, but suggest that such an influence, if it exists, is likely to be exerted at more advanced stages, when the neurons have reached their final positions and established their afferent contacts in the cortex. It could then have a role in the maturation of other aspects of the cells phenotype; the maturation of morphological parameters of neonatal CR-containing neurons *in vitro* has been reported to be promoted by the presence of 5-HT in the medium (Köstner and Hornung, 1995). The observed close association between 5-HT axons and

nonpyramidal neurons (Hornung and Celio, 1992) is unlikely to occur at the time of neurogenesis in the ventricular zone, and is most likely established later in development. A presumed inductive action of the afferent fibers could only be achieved between specific neurons and specific afferents and, consequently, only in the already organized cortex. In earlier stages, it is difficult to envisage how the presence of a certain signal substance in the extracellular environment could trigger the differentiation of a specific subgroup of cells towards a certain phenotype and not affect their neighbours, unless these cells have been rendered selectively responsive to the signal at an earlier stage, in which case the real differentiating event would be the appearance of this responsiveness. Such seems to be the case with Glu expression, as 5-HT is not likely to determine the Glu phenotype but rather to participate in its maturation as discussed earlier.

4.2.4. Nestin-containing cells

Nestin is an intermediate filament protein that is present in progenitor cells in the CNS (Lendahl et al., 1990), and the transition from a proliferating to a postmitotic state being accompanied by a rapid decrease in the levels of nestin m-RNA (Dahlstrand et al., 1995). Radial glial cells are also part of the nestin-positive population (Yachnis et al., 1993; Hurley and Streit, 1995), and nestin, being a cytoskeletal protein, has been shown to play an important role in their organization and morphological maintenance (Matsuda et al., 1996). In acutely dissociated (non-cultured) cortices, nestin-positive cells constituted the majority of the cellular population at E14, appearing in decreasing proportions in E16, E19 and P0 (fig.

18). In slice cultures, nestin immunoreactivity remained more or less constant at all stages, constituting on average about 10% of the total cell population (figs. 19A,B; 20A,B). This figure is presumably reflecting different cell populations at different times, including radial glial cells (in diminishing numbers at the more advanced stages) as well as glial progenitors, as glial cells continue to be produced up to 2 weeks postnatally (Ichikawa and Hirata, 1982). The appearance of radial glial fibers in the cultures was similar to that *in situ* in corresponding stages as visualized by the monoclonal antibodies RC1 and RC2 (Misson et al., 1988; Edwards et al., 1986, 1990; Misson et al., 1991). In E14 (fig. 6A) and E16 cultures (fig. 9A), radial glial fibers and cells mostly of monopolar morphology, were seen traversing the entire thickness of the cortical slice. In E19 slices, for the first time, multipolar morphological configurations appeared, suggestive of early astrocytes (fig. 12A). In cultures prepared at P0 nestin-positive filaments were still present, but they were not as dense, and their earlier distinctive linear arrangement was more or less distorted, while multipolar forms were once again evident (fig. 15A). It has been demonstrated in the mouse neocortex *in vivo*, that RC2 immunoreactivity is significantly reduced in the second half of the first postnatal week, while the remaining immunoreactive cells demonstrate a multipolar morphology (Misson et al., 1991); these forms persist into adulthood as cortical astrocytes. The changes in the morphology and density of radial glial elements in the slices seem, then, to correspond well with the time course of the phenomena associated with the gradual decline of the radial glial population *in vivo* and its transformation into adult type multipolar astroglial forms (astrocytes). Soluble factors from the embryonic forebrain have been previously demonstrated to be a

necessary prerequisite for the development of the radial glial phenotype in dissociated cell cultures (Hunter and Hatten, 1995), and the results of the present study suggest that the local cortical microenvironment in the slice culture is sufficient for the normal development of the radial glial cell population. Although the presence of nestin-positive cells after a week *in vitro* is in agreement with what has been previously reported in slices by Götz and Bolz (1992), the reported failure of these cells to further differentiate in that study is in contrast with the present findings, presumably as a result of the different culture system used by these authors (protocol according to Gähwiler, 1981, using 25% horse serum).

Nestin-positive cells did not differ significantly in proportion between the control slices and the slices cultured in the presence of added neurotransmitters. The finding that the proportion of these cells is not affected by 5-HT argues against a selective amplification of the Glu- cell progenitor population by the monoamine and is compatible with the results of the BrdU incorporation experiments. This finding also suggests the absence of any involvement of the neurotransmitters tested in the processes of the radial glial phenotype regression during the first postnatal week.

4.2.5. GFAP-containing cells

The astrocytic type of glial cells is characterized by the presence of GFAP (Bignami et al., 1972) which is, like nestin, an intermediate filament protein. Intermediate filaments, together with microfilaments and microtubules, constitute the cytoskeleton, a network involved in the regulation of cell shape, contractility,

locomotion and the compartmentalization of the cell interior (Fulton, 1984). GFAP is developmentally regulated, increasing sharply during the first two postnatal weeks (Pixley and De Vellis, 1984; Weir et al., 1984). As it was expected, cells immunoreactive for GFAP were not found in acutely dissociated (not cultured) cells from E14, E16 or E19, while only a very small proportion of P0 cells (< 1%) were GFAP-immunoreactive (fig. 18). However, GFAP expression was initiated *in vitro*, and immunoreactive cells were present in cultures prepared at all four developmental stages. Only a small number of these cells was detected in E14 or E16 cultures, whereas E19 and P0 slices exhibited a much higher proportion (figs. 20A,B; 21A,B). This numerical increase was accompanied by the presence of a more mature and elaborate morphology (figs 12B, 15B). This is in keeping with the postnatal maturation of astrocytes (Misson et al., 1991) and appearance of GFAP *in vivo* (Pixley and De Vellis, 1984; Weir et al., 1984), and demonstrates that the development of this cell type *in vitro* closely resembles its development in the corresponding *in vivo* stages. The substantial growth of the GFAP- containing population in later embryonic cultures is not in agreement with a previous study (Götz and Bolz, 1992) that had reported that only postnatal cultures demonstrate successful differentiation of the GFAP phenotype. As it has been already discussed, such discrepancies may be due to the different culture conditions used .

In this study, no effect of exposure to the different neurotransmitters was detected in the proportion of the GFAP-positive cells in the total cell population. Previous studies have suggested that 5-HT might be involved in the morphological maturation of astrocytes (Chubakov et al., 1986) as well as the modulation of GFAP expression (Prince et al., 1990). Given the method of quantitation used in

the present study, and the fact that the reported alterations were mainly concerning the distribution of gliofilaments in the processes (Prince et al., 1990), these sets of data can not be compared. If any correlation can be made, that could only be to suggest that any changes in the GFAP expression are not of a magnitude that would alter the proportion of cells detectable with immunohistochemistry.

4.2.6. Dopamine, Noradrenaline and Acetylcholine

The presence of DA, NA or ACh in the medium of the cultures did not have any effect in the makeup of the cell population of the slices, as assessed by immunostaining for cell-specific markers (fig. 19B). The presence of DA receptors in the cortex of the embryonic rat (Reinoso, 1996) and macaque monkey (Lidow, 1991), as well as the early innervation of the cortex by DA-containing fibers (Kalsbeek et al., 1988) suggest a possible role of this monoamine in prenatal cortical development. A regulatory role of DA in the processes of morphological maturation of cortical neurons has been suggested by the finding that exposure of rat fetal cortical neurons to DA or DA agonists *in vitro* affects morphological parameters of the cultured neurons in diverse ways, depending on the concentration of the catecholamine and the receptor specificity of the agonist used (Todd, 1992; Reinoso et al., 1996). Exposure of rabbits to cocaine *in utero* has been shown to lead to morphological alterations, mainly involving the dendrites of neurons in the anterior cingulate cortex of the animal in adulthood (Levitt et al., 1997). Cocaine binds to presynaptic transporters of the monoamines and blocks reuptake, thus increasing the synaptic concentration of DA, as well as of NA

and 5-HT. However, the effects of cocaine have been mainly attributed to changes in the dopaminergic system, as the limbic cortex, which was particularly affected by cocaine administration, is a target of dense dopaminergic innervation (Levitt et al., 1984, 1997). A role of DA in postnatal development has also been proposed (Kalsbeek et al., 1987, 1989). The results of the present study, in conjunction with earlier data, seem to indicate that, although DA has a role in the prenatal development of the cortex, that role is more likely to be related to the morphological rather than the neurochemical maturation of neurons.

Noradrenaline also first appears in the cortex during prenatal corticogenesis (E17) (Levitt and Moore, 1979; Verney et al., 1984), while adrenergic receptors are found even earlier, at E14 (Goffinet, 1986). This monoamine has been proposed to play a role in synaptogenesis (Blue and Parnavelas, 1982; Parnavelas and Blue, 1982) and cellular maturation (Morris and Slotkin, 1985), and to have a neurotrophic role (Felten et al., 1982). A role of NA in the processes of cortical plasticity has been suggested by a number of studies (Pettigrew and Kasamatsu, 1978; Kasamatsu, 1979; Kasamatsu and Pettigrew, 1979a, b, 1981; Daw et al., 1983; Kolb et al., 1992), but contested by other investigators (Brenner et al., 1983, 1985). The finding that a structurally normal cortex develops even after destruction of the noradrenergic system at E17 (Lidov and Molliver, 1982b) suggests that, irrespective of its involvement in plastic responses, NA per se may not be essential for the normal development of the cortex. As the results of the present study do not support any role of NA in the neurochemical maturation of cortical cells, it may be that the main developmental function of NA in the cortex is that of a mediator of

plasticity in response to external stimuli.

The late innervation of the cortex by ACh (Dori and Parnavelas, 1989) argues against it having a developmental role in embryonic life. There is a transient population of ACh- containing cells in the cortex around E17, while muscarinic and nicotinic receptors have been observed from E18 (Höhman et al., 1985) and E20 (Ostermann et al., 1995) respectively. However, even these early events occur slightly later than the corresponding phenomena in the case of the monoamines (Levitt and Moore, 1979; Lidov and Molliver, 1982a; Verney et al., 1983; Wallace and Lauder, 1983; Kalsbeek et al., 1988). What do the results of this study tell us about the possible role of ACh in corticogenesis? As no effect was observed in the proportions of any cell population, as identified with immunohistochemistry for cell-specific markers, these results suggest that ACh does not participate in the neurochemical maturation of cortical cells. However, by virtue of the timing of its emergence in the cortex, this neurotransmitter is in a much better position to regulate events related to cytoarchitecture and the maturation of cellular morphology postnatally, and morphological studies have shown that deprivation of the cholinergic input to the cortex can affect these parameters (Höhmann et al., 1988, 1991). The onset of the (second phase of the) cholinergic innervation of the cortex in the second postnatal week (Dori and Parnavelas, 1989) coincides with a period of marked maturation of cortical cells (Parnavelas and Lieberman, 1979), and may thus participate in the process of this maturation.

In conclusion, this study has provided evidence for a role of 5-HT in the neurochemical maturation of a major class of cortical cells, the glutamatergic

neurons. This effect is dose-dependent, selective for the specific cell population, and is also restricted to a specific time window. Other cortical afferent neurotransmitters tested do not share this effect of 5-HT on the developing cortical neurons. It is to be expected that different transmitters might have different roles in development, and there is a wealth of information available on the roles of these transmitters in various other aspects of cortical development. A single neurotransmitter can bind to a number of different receptors, and these receptors are each linked to one or more different second messenger systems that can mediate a variety of responses such as modulation of cell proliferation, gene expression, process outgrowth, secretion of growth factors from glia and cell viability (Lauder, 1993). The complementary and sometimes opposing effects of the stimulation of different receptors seem to explain how a small number of neurotransmitters can be used as signals for numerous, complex and interwoven developmental events. Deciphering these developmental events will bring us closer to a more comprehensive understanding of the developing cortex (and indeed the whole of the developing organism) as a multivariate system, where the effect of a change in one of the parameters is the induction of more changes in others; it is the integrated effect of these changes, not the isolated actions of single effectors, that shift the system to its next developmental stage.

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