A SEARCH FOR FACTORS CONTROLLING THE FORMATION AND MAINTENANCE OF CONNECTIONS BETWEEN THE THALAMUS AND CORTEX IN VITRO.

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ABSTRACT

Abstract of thesis submitted for the degree of Doctor of Philosophy entitled:

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Although the development of the nervous system is continuous, the neurones that constitute the nervous system proceed through specific developmental stages. Such stages included: birth, differentiation, migration, the formation of efferent and afferent connections, and the organisation of these connections based on exogenous and endogenous cues. Alternatively, a neuron may undergo "natural occurring" cell death at various points during development.

When considering the complexity of the nervous system, it is not surprising that our knowledge, as to the factors that control these development stages, is limited. One significant obstacle for experimentally addressing these questions, for practical and/or ethical reasons, has been the animal itself. Thus, the advent of tissue culture techniques has greatly facilitated research in this area.

In addition to <u>in vivo</u> DiI tract-tracing experiments, I have used three different <u>in vitro</u> techniques: (i) co-cultures of organotypic explants on a two-dimensional collagen substrate, (ii) organotypic explants co-cultured within a three-dimensional collagen gel, and (iii) co-cultures of dissociated cells. Because serum is known to contain many trophic factors, only serum-free medium was used for all of these experiments. Using the above <u>in vitro</u> techniques, I investigated questions concerning the development of connections between the thalamus and cortex.

These experiments yielded the following results. (i) When thalamocortical and corticothalamic efferent fibres began to grow <u>in vivo</u> at embryonic day 15 (E15), conserved diffusible target-derived factors enhanced their neurite elongation <u>in vitro</u>. (ii) The survival of E15 thalamic and cortical neurones was independent from target-derived

trophic support, since both the thalamus and cortex promoted their own survival in vitro, possibly through the production of endogenous trophic factors. However (iii), between E17 and postnatal day 2, a time coincident with the arrival of their fibres to their target, the survival of thalamocortical and corticothalamic neurones was dependent on factors released from their target. (iv) The production of growth promoting factors within the cortex increased with postnatal age and was at least partially regulated by afferent activity. And finally (v), it seems that the recognition/stop-signalling molecules expressed within these targets are also highly conserved since they were recognised in inter-species co-culture experiments. Thus, in general, the development of both thalamocortical and corticothalamic efferents are highly influenced by factors produced by their target cells in vitro. In vivo, these factors may play various roles during the different developmental stages from promoting neurite outgrowth and cell survival, to regulating the reorganisation of connections.

DISCLAIMER

Aside from the experiments generating the data illustrated in Fig. 6 Chapter 2 which were performed in collaboration with Suzanne Rennie, and the culture experiments concerning cell migration and subplate cell death presented in Chapter 5 done in collaboration with Katy Gillies, all the experiments presented in this thesis were performed by me (R. Beau Lotto) alone under the direct supervision of Dr. David Price and secondary supervision of Mayank Dutia.

R. Beau Lotto

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CONTENTS

GENERAL INTRODUCTION

<u>An inspiration: "Use It or Lose It"</u>

When I was at Berkeley, one of my professors (Dr. Strohman) used to tell me that we each have an incredible physical and mental potential. The only way to realise this potential, he used to say, was by placing oneself within a stringent, i.e. challenging, environment which the body will then adapt to. If, on the other hand, one remains within a less challenging environment, either mentally and/or physically, the body will also adapt to this, i.e. deteriorate. So, basically, his point was: "use it or lose it".

Memory and its loss are obvious manifestations of this concept, and both demonstrate that the brain is able to adapt to changes in its internal and/or external environment. The brain is, therefore, plastic (i.e. changable). Plasticity can be manifested in several different ways: (i) neurones can either survive or die, (ii) support cells, like glia, can proliferate (as in Einstein's brain: Diamond et al., 1985), or degenerate, (iii) neuronal morphologies can increase or decrease in complexity, (iv) and the connections between neurones can increase or decrease in strength (such as long-term potentiation, or long-term depression).

One of the first discoveries of plasticity was made at the neuromuscular junction. Tello (1917) and Boeke (1932) first made the observation that a major difference exists in the innervation pattern of neonatal and adult muscle fibres; while neonatal fibres are polyneuronally innervated, adult fibres are innervated by only one axon. Thus, it could be suggested from these studies that, during the course of development, many connections are lost and/or rearranged in the PNS.

Plasticity is also known to occur in the CNS, the most dramatic example of which was discovered by Hubel and Wiesel in the early 1960s. Hubel and Wiesel observed that in the new-born kitten and in some primates cells within layer four are driven by both the right eye and left eye (Wiesel and Hubel, 1963). However, after several months raised in normal conditions, these cells become responsive to either the right or the left eye exclusively. These cells are clustered in groups, and are termed ocular dominance

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columns. Under normal conditions these columns are equally spaced, i.e. they share the same amount of territory. However, if one eye is covered (monocular deprivation) during the critical period early in development, reducing the level of afferent activity, the columns corresponding to the active eye expand in size at the expense of the deprived eye (Hubel and Wiesel (1970). After the critical period, much of this plasticity is lost. Thus, these pioneering experiments demonstrate that the very young CNS is plastic.

Work from Marion Diamond's laboratory, who was my advisor when I was an undergraduate at Berkeley, demonstrated that plasticity within the CNS can occur in adult animals. In her experiments (Connor and Diamond, 1982; Connor et al., 1982), she housed adult rats in three different environments: (i) a deprived environment -- no "toys", (ii) an enriched environment -- with "toys" that were changed every few days, and (iii) an over-enriched environment -- with many "toys" that were changed every day. She then, anatomically, assessed the size of the dendritic trees of the cells within the cortex. She found that the dendritic trees of neurones from rats raised in an "enriched" environment grew in size, whereas those from the first group, the "deprived mice", actually shrunk. Interestingly, the dendritic morphology of cells from the "over-stimulated" group resembled those from the deprived group.

It is currently believed that the process of organisation results from a competitive process in which activity is integrally involved. The most prominent theory concerning synaptic competition (of which plasticity is a manifestation) was devised by D. O. Hebb in 1949 before the above observations were made. Basically, Hebb suggests that those synapses whose activity is correlated will be reinforced, while those which are not will be weakened (Hebb, 1949). But the question remains what these fibres are competing for. One possibility is that these fibres compete for limiting amounts of factor, called chemotrophic factors, produced by their target cells, and theory described by D. Purves and J. Lichtman in 1984.

Chemotrophism

For the purpose of my thesis I am defining trophic factors as molecules that promote neurite growth and/or neuronal survival (unless otherwise specified). The first

trophic factor, called Nerve Growth Factor (NGF), was discovered by Rita Levi-Montalcini during the 1950s. However, the discovery of NGF was, in actuality, a long process extending over many years, and resulted from the work of many prominent researchers.

The story begins with E. Bueker in the late 1940s. Bueker was interested in the effects of target tissue on sensory neuronal survival, research which was in its infancy at the time. Bueker surmised that tumour tissue, which can be grown in large quantities, may, when transplanted into a host animal near the spinal cord, act as a sensory target, possibly promoting the survival of the neurones that innervated it. This is exactly what happened; Bueker observed an increase in the number of innervating sensory fibres at the level of the transplant, and an increase in the size of the contralateral dorsal root ganglia.

Levi-Montalcini and Hamburger pursued Bueker's findings, confirming that the sarcoma was in fact releasing a diffusible factor that promoted the growth of sensory peripheral neurones (Levi-Montalcini and Hamburger, 1951, 1953). They called this factor nerve growth factor. Over the course of several years, Levi-Montalcini and Cohen set about characterising nerve growth factor. Using a tissue culture assay for NGF, they serendipitously discovered in the late 1950s that snake venom contained a high concentration of NGF. This finding was integral in isolating NGF since it gave researchers a rich source of the, as yet unknown, factor. It was another ten years before the NGF molecule was isolated and characterised. Using a preparation from salivary glands (Bocchini and Angeletti, 1969), Angeletti and Bradshaw (1971) reported the amino-acid sequence of NGF.

Since the discovery of NGF, several other related trophic factors have also been revealed: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and 4/5 (NT-4/5) (see review by Barde, 1989; Korshing, 1993). Collectively, they are referred to as the neurotrophins. Other families of trophic factors also exist such at the heparin binding factors of which fibroblast growth factor (FGF: both acidic and basic forms) is a member (see review by Walicke, 1989). In addition to these more prototypical agents,

other molecules, such as neurotransmitters, have also been shown to have trophic affects (Lipton and Kater, 1989).

Although, for the purpose of my thesis, I defined trophic factors above as purely growth and/or survival promoters, their functions extend far beyond these two basic actions. For instance, trophic factors regulate mitosis, migration, neuronal sprouting, maturation, immediate early gene transcription, glial proliferation, axonal guidance and more (see reviews by Thoenen et al., 1987; Davies, 1988; Barde et al., 1989). The response of a cell to a particular factor will depend on the developmental state of the cell in question. For instance, at one stage, a trophic factor may promote mitosis, whereas later in development, the same factor may promote neuronal survival. A case in point is that of the trigeminal ganglionic cells. In this system, the target cells of the trigeminal axons release chemotropic factors that guide the ganglionic neurites towards their target. However, guidance can only be demonstrated with neurones that have yet to reach their target in vivo. After innervation, this chemotropic response is lost (see review by Davies, 1987). In addition to altering their response to one factor, neurones may also respond to different trophic factors at different times during development (see review by Davies, 1994). Which factor a neurone responds to depends on its constituent of receptors. For example, each member of the neurotrophin family binds to specific tyrosine kinase receptors (trk): NGF binds to trk-a, whereas BDNF binds to trk-b, and NT-3, 4/5 bind to trk-c and possibly a and b as well (see review by Schlessinger and Ullrich, 1992).

The production of trophic factors in the PNS and CNS is dynamic. In the PNS, the production increases postnatally (see review by Barde, 1989). This increase does not seem regulated by afferent activity. The production of many trophic factors in the CNS also increase postnatally, while some decrease (Large et al., 1986; Whittemore et al., 1986; Maisonpierre et al., 1990; Castren et al., 1992; Matsuyama et al., 1992). However, unlike the PNS where activity and growth factor production are negatively correlated, neuronal activity in the CNS appears to increase trophic factor production (Zafra et al., 1990; Isackson et al., 1991; Lu et al., 1991; Castren et al., 1992; Gall, 1992; Patterson et al., 1992; Riva et al., 1992).

To date, most of the data concerning chemotrophism have derived predominantly from research on the peripheral nervous system. The role trophic factors play in the development of the central nervous system (CNS) is poorly understood. Furthermore, the limited research on the CNS has been focused primarily on the hippocampus and basal ganglia (since it is these two areas that are affected during degenerative disorders such as Alzheimer's disease). I, on the other hand, have focused my research on another critical area of the brain, namely the reciprocal connections between the thalamus and cortex. These connections are primarily involved in transmitting sensory information, such as visual evoked responses, arising from peripheral sensory organs.

Is there any reason to believe that trophic factors are involved in regulating the development and/or plasticity of thalamocortical and corticothalamic connections? Before addressing this question, I will give a general overview to this system, focusing my discussion on the development of the visual system.

Development of the thalamocortical connections

Aside from olfaction, the thalamus receives input from all the senses and relays this information to different areas of the cortex. The neurones that constitute the murine thalamus are generated between embryonic day 13 (E13) and E14 (Rennie, 1993). Between E14 and E15, these neurones send axons to the internal capsule. How they navigate this pathway is not known. In the internal capsule, thalamocortical axons interdigitate with the descending corticothalamic fibres (De Carlos and O'Leary, 1992) and then continue through the presumptive white matter of the telencephalic hemisphere to their correct cortical area. How thalamocortical fibres navigate through the white matter, and what gives rise to the specificity of connections between different nuclei of the thalamus and different cortical areas is not know; however, several theories have been proposed (see McConnell et al., 1989; Molnar and Blakemore, 1991; O'Leary et al., 1994). In rodents, thalamic fibres reach the subplate (a transient layer below the developing cortex: see below for a discussion of this layer) between E16 and E17. In the kitten, ferret, and primate, thalamocortical fibres then "wait" within this layer before invading the overlying cortical plate (see review by Allendoerfer and Shatz, 1994). In

rodents however, the existence of a waiting period is debated (see Catalano et al., 1991). Nonetheless, after E17, thalamocortical fibres begin their radial innervation into the cortex. Shortly after birth, most of the thalamic fibres have terminated within layer 4 (Lund and Mustari, 1977). At this time, a stage of natural occurring cell death ensues within the thalamus (see review by Finlay and Pallas, 1989).

After thalamocortical axons have reached their target, they undergo a stage of refinement (although this is debated in rodents). In the case of the visual system this results in the segregation of inputs from the two eyes within layer 4 into ocular dominance columns. During a "critical period" early in development the distribution of ocular dominance columns (or, for example, the barrel fields in the somatosensory system) is changeable. In other words, during this period, these connections are able to adapt to changes in both the level and pattern of activity arising from the eyes. For instance, if activity from one eye is eliminated (or significantly reduced or even altered) it is reflected in a rearrangement in the patterning of ocular dominance columns.

Development of the cortex and corticothalamic connections

One of the largest sources of input to the thalamus arises from the cortex. Before the cortex is formed, however, the wall of the telencephalic vesicle is composed of the pre-plate and ventricular zone (site of mitosis). Postmitotic cortical cells from the ventricular zone migrate into the pre-plate and form a cell dense layer called the cortical plate that divides the preplate into both the marginal zone above (future layer 1) and the subplate layer below. As mitosis and migration continue, the cortical plate thickens. The cells that give rise to layer 6 are generated first followed by those constituting layer 5 etc. Thus, the layers of the cortex are formed from inside to outside.

Each layer of the cortex sends and receives a unique set of connections. Layers 2, 3 and 5 send and receive connections to and from other cortical regions. Layer 4, as stated above, is the primary recipient layer for thalamic afferent input. Layer 5 and 6 project subcortically (layer 5 to the superior colliculus, pons, and spinal cord; layer 6 to the thalamus). However, the first subcortical projections arise from subplate neurones.

The subplate is a transient structure that disappears within the first few postnatal weeks in mammals including mice (Wood et al., 1992; Gillies and Price, 1993). It has been demonstrated in kittens that the lateral subplate axons pioneer the pathway between the cortex and, at least, the internal capsule creating a scaffold for axons from both medial subplate and layers 6 and 5 to follow (McConnell et al., 1989). This suggestion has been supported by lesion experiments to the subplate which (i) prevented the thalamocortical axons from entering the cortex (reviewed by Allendoefer and Shatz, 1994) and disrupted the targeting of 50% of the corticothalamic axons (McConnell et al., 1994). How the pioneering subplate neurones find the internal capsule in not known. Like the thalamus, shortly after birth cells within the cortex (and particularly within the subplate) undergo a stage of naturally occurring cell death (Ferrer et al., 1989). What causes their death, and/or prevents the death of the cortical cells is unknown.

Aims of My Research

I posed above the question of whether there are reasons to believe that diffusible target-derived trophic factors are involved in the development of the reciprocal connections between the thalamus and cortex. To date there is little direct evidence for this. However experiments have demonstrated that many of the identified trophic factors have been isolated in both the thalamus and cortex. Of these factors, FGF, NGF, and BDNF have been shown to promote the survival of either cortex or thalamus (Ghosh et al, 1994; Walicke, 1989). In addition, infusion of high levels of NGF into the lateral vesicle prevents the deleterious effects resulting from monocular deprivation (Domenici et al., 1991; Domenici et al., 1992; Maffei et al., 1992). Furthermore, in vitro and in vivo experiments suggest that cortical neurones release diffusible proteins that promote the growth of E15 thalamic neurones (Lotto and Price, 1994; Rennie et al., 1994) and the survival of postnatal ones (Cunningham et al., 1987; Hisanaga and Sharp, 1990). On the other hand, recent experiments by Bolz et al. (1992) are at odds with this. Instead, these authors have suggested that the thalamus releases tropic (i.e. guidance) factors that guide the axons of cortical neurones towards its source. However, this interpretation is arguable since the appropriate chemotropic experiments were not performed (see Tuttle

and O'Leary, 1993). In my experiments, I sought the existence of cortical- and thalamically-derived trophic factors and to elucidate their roles in the development of the reciprocal connections between these two structures.

I have chosen to use tissue culture, not only because organotypic and dissociated culture techniques have been well established for this system, but because one of the greatest obstacles confronting developmental neuroscientists is, ironically, the animal itself, as the vast complexity of the animal is the greatest barrier to research. Thus, one needs a way of reducing the complexity of an already overwhelming system to address questions about the system itself. One obvious approach is to remove the complication of the animal, and place the tissue of interest (the developing nervous system in this case), in a controlled, potentially isolated environment.

Tissue culture was created by Ross G. Harrison (Harrison, 1907, 1910). It was born out of an attempt to answer one of the greatest questions of his and our time, namely, how axons are established. The debate centred around two schools of thought: those who believed that axons grew by linking individual segments together -- a process they believed was dependent on Schwann cells -- and those who believed that axons were formed by growing from their parent cell body. In addressing this question, results obtained from in vivo experiments were limiting. Thus, it was necessary to observe axons during the process of formation itself. To this aim, Harrison placed pieces of neural crest cells from frog embryos, which lack Schwann cells, into drops of lymph, and was able to observe axonal extension under the microscope. Harrison's observations settled this issue (obviously in favour of the latter group), and at the same time confirmed Cajal's belief that growth cones were the site of axonal extension.

Since the pioneering work of Harrison, tissue culture has become an integral part of developmental neuroscience. However, it was not until Maximov extended Harrison's work of organotypic cultures of the CNS tissue which Levi-Montelcini used for the experiments described above that tissue culture became fully appreciated (in fact Maximov coined the termed "organotypic"; see review by Gahwiler, 1988). Organotypic

cultures have the advantage of maintaining in vivo connectivity in culture. Yamamoto et al. (1989) was the first to perform co-culture experiments of thalamus and cortex.

Chapter outlines

The following is a brief explanation of each of the chapters in my thesis:

CHAPTER 1:

There were two points to these experiments: (i) to determine the location of the posterior thalamus and cortex, the two tissues that I have used for my culture experiments, and (ii) to determine the timing of thalamic and cortical afferent formation in vivo.

CHAPTER 2:

The purpose of these experiments was to determine whether the cortex releases diffusible trophic factors that promote the growth of thalamic neurones in culture and whether the production of such factors changes with age and/or with afferent activity.

CHAPTER 3:

In the previous chapter, the existence of diffusible cortically-derived trophic factors was confirmed. I then sought to determine whether these factors, as well as those responsible for target recognition, are conserved between divergent mammalian species.

CHAPTER 4:

The previous chapters on growth promotion response of E15 thalamic neurones in the presence of cortically-derived trophic factors, and whether the production of these factors changes with age of the cortex. In the experiments described in this chapter, I have looked to see whether the response of thalamic neurones to cortical factors changes with thalamic age.

CHAPTER 5:

To this point, my experiments have concerned the effects of the cortex on the development of the thalamus. In this chapter I have asked the question of whether the opposite is true, i.e. whether thalamic factors influence the development of the cortex. I have used the same two criteria as in previous chapters for these experiments, namely growth and survival promotion.

CHAPTER 1:
DiI TRACT-TRACING LOCATING THE POSTERIOR THALAMUS AND POSTERIOR CORTEX.

ABSTRACT

1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was placed in the eyes of embryonic day 14 (E14) and E16 mice to determine the timing of thalamic and cortical innervation, as well as the exact location of the posterior thalamus and cortex. The posterior thalamus receives its first afferent input from retinal ganglionic cells at E14. and from the posterior cortex at E16. The posterior cortex receives its first afferent input from the posterior thalamus at E16.

INTRODUCTION

Most of the experiments presented in this thesis involved the dissection of the posterior thalamus and posterior cortex. However, dissecting the correct area from the fetal mice brain can be difficult since it lacks many of the characteristic landmarks present in adulthood. Thus, it was imperative to locate the thalamus and cortex within the embryonic brain. Furthermore, in subsequent experiments concerning growth promotion and neuronal survival, it was important to know the exact timing of thalamocortical and corticothalamic development. Therefore, I conducted anatomical tract-tracing experiments of retinogeniculate, geniculocortical and corticogeniculate pathways at E14 and E16.

METHODS AND MATERIALS

Labelling of fixed embryos was done to define the region from which thalamic and cortical explants were taken. Time-mated BALB/c mice were deeply anaesthetised with urethane (0.3ml of a 25% solution in normal saline, i.p.). E14 and E16 foetuses were removed by caesarean section, and decapitated. The heads were placed in 4% paraformaldahyde for 24 hours, and crystals of DiI were then pushed into both right and left eyes, or only into the right eye, filling the entire optic cup. Fetal heads were stored in fixative in a cool dark environment for 9 months. After this time, the brains were carefully dissected from the skulls and the hemispheres removed. Using an inverted fluorescene microscopy (with a rhodamine filter), observations were made on wholemounts and hand-sectioned slices of the hemispheres and thalamus.

RESULTS

Figs. 1.1a-c show the developmental state of retinogeniculate axons at E14. As seen in these photomicrographs, ganglionic axons have grown beyond the optical chiasm.
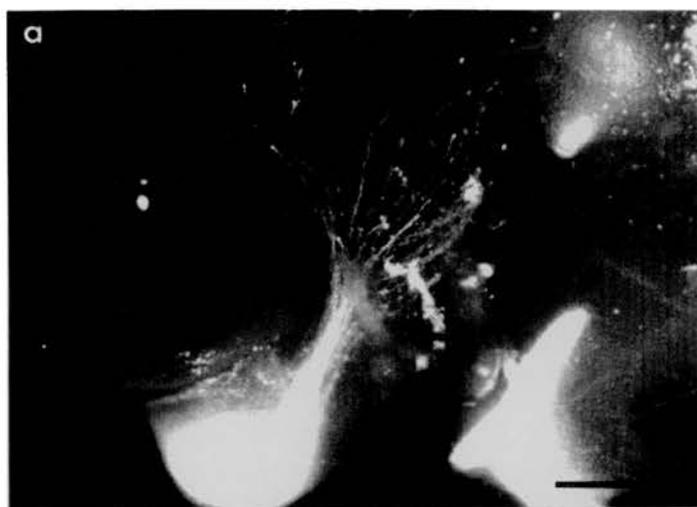
Fig. 1.1 (a) An overview of retinogeniculate axons coursing beyond the optic chiasm towards the posterior thalamus (whole mounts of E14 murine brain with cerebral hemispheres removed). Rostral is to the left and caudal to the right of the photograph. (b & c) Retinogeniculate axons shown at a higher magnification. These axons turn towards the posterior thalamus once they reach the anterior ventral thalamus. In b and c, rostral is to the right and caudal to the left, while dorsal is up and ventral, towards the optic chiasm, is down. Scale bar: a, 500μm; b & c, 50μm.
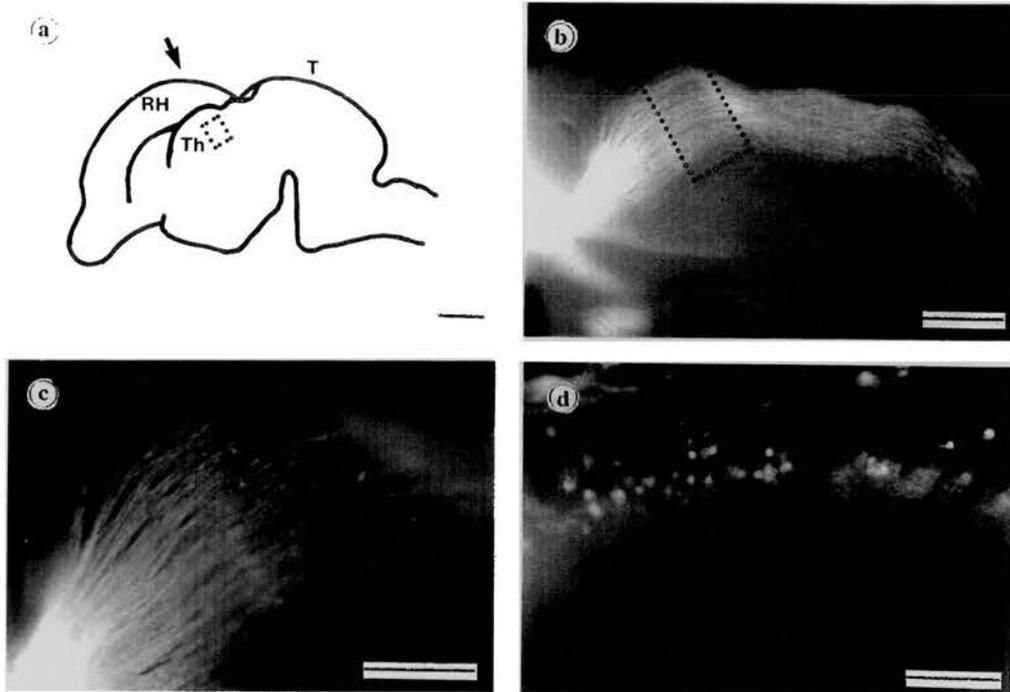
Fig. 1.2 (a) Diagram of an E16 mouse brain viewed from the left; the left cerebral hemisphere has been removed to reveal the thalamus (Th; RH, right hemisphere). The dotted box is centred on the LGN and outlines the region of the posterior thalamus explanted for culture; it corresponds to the area outlined in (b). The arrow indicates the location of the cortical section shown in (d). (b-d) Photomicrographs of fibres and cells labelled with DiI placed in both right and left eyes. Axons entering the dorsal diencephalon fan out in the region of the LGN (outlined in b and shown at higher magnification in c); many continue to the tectum (T). (d) DiI was transported from retinogeniculate to corticothalamic axons, resulting in the retrograde labelling of cortical cells in the deep layers of the developing visual cortex (where this transport occured is not known). This is the region of the hemisphere taken for culture. Scale bars: a, 1mm; b, 500μm; c, 250μm; d, 250μm.

These axons are tightly fasiculated until they reach the base of the thalamus. There they spread out from one another. Shortly after fanning out, they turn towards the posterior thalamus. At this age, few fibres have reached the lateral geniculate nucleus (LGN).

As shown in Fig. 1.2b,c, crystals of DiI placed in the eyes confirmed the location of the LGN in fixed E16 mice. The explanted region of the thalamus is outlined in Fig. 1.2a and corresponds approximately to that shown in Fig. 1.2c. This region was centred on the LGN, but because it included neighbouring nuclei, I refer to the explants as "posterior thalamus" throughout this thesis.

Interestingly, in these experiments the DiI must have been transneuronally transported, either at the thalamus or outside the thalamus when corticothalamic and retinothalamic axons converge, since labelled fibres, which would not normally be labelled, were labelled in the internal capsule extending caudally towards the occipital pole. It was clear that, at this embryonic age, relatively few geniculocortical fibres had reached the visual cortex, in agreement with the findings of others in the mouse (Ferrer et al., 1992). At least some corticogeniculate axons had reached their target since I observed retrogradely labelled cells in the lower layers of the developing visual cortex itself (presumably layer 6 and/or the subplate). The transneuronal transport of DiI from the retinogeniculate to the corticogeniculate pathway confirmed the location of the visual cortex in the occipital pole (no labelled cells were found in other cortical areas such as the somatosensory cortex). Thus, I am confident that the cortical explants used in this study included visual cortex, but I refer to them as being from the posterior cortex because I cannot rule out the inclusion of non-visual cortex.

DISCUSSION

In these experiments, I determined not only the location of the posterior thalamus and posterior cortex, but I also confirmed that the LGN receives its first retinogeniculate afferent input around E14. In agreement with others (Ferrer et al., 1992), the first corticogeniculate afferent input occurred around E16. Also around E16, the posterior

cortex receives the first of its afferent input from the posterior thalamus. The relevance of these observations will be discussed in subsequent chapters.

CHAPTER 2:

THE STIMULATION OF THALAMIC NEURITE OUTGROWTH BY CORTICALLY-DERIVED GROWTH FACTORS IN VITRO; THE INFLUENCE OF CORTICAL AGE AND ACTIVITY.

ABSTRACT

Recent in vitro experiments have provided useful insights into the development of connections between the thalamus and the cortex. While most of these previous studies focused on neurite guidance and target recognition, my experiments used a serum-free culture system to examine the possible roles of unidentified diffusible cortically-derived growth factors. I demonstrated that occipital cortical explants release diffusible growth factors that enhance neurite outgrowth from explants of the posterior thalamus (the region around the developing lateral geniculate nucleus). The amount of thalamic outgrowth was dependent on the age of the co-cultured cortical slices. My results suggest that there is an overall increase in the release of cortically-derived growth factors during the first three postnatal weeks in mice; this parallels known postnatal increases in the production of several identified growth factors. I found evidence for two peaks in the release of cortically-derived growth factors during the general upward trend, the first at around postnatal day 6 (shortly after thalamocortical innervation of layer 4) and a second between postnatal days 14 and 18 (just after eye-opening). The increased release of cortically-derived growth factors was not found when cortical slices were from mice that had been dark-reared from birth, suggesting that neural activity may be important for enhancing release. Other regions of the central nervous system, including the cerebellum and medulla, were also capable of stimulating some thalamic outgrowth; neither additional explants of the thalamus nor hepatic explants enhanced outgrowth. Fibroblast growth factor is one substance that is distributed preferentially among those tissues that were stimulatory in my experiments. Its level of transcription is known to increase in the brain during the first three postnatal weeks and to be influenced by neural activity. At low

doses, fibroblast growth factor greatly increased outgrowth from isolated posterior thalamic explants. Nerve growth factor, another candidate molecule, was less effective. Overall, my results complement the in vivo observations of others on the synthesis of identified growth factors in the cortex and the factors that influence their production. They suggest that growth factors may influence thalamic neurones, and indicate that fibroblast growth factor, and possibly nerve growth factor, are two candidates for molecules mediating the in vitro effects.

INTRODUCTION

In recent years, interactions between developing cortex and thalamus have been studied in organotypic and dissociated co-cultures (Yamamoto et al., 1989; Bolz et al., 1990; Hisanaga and Sharp, 1990; Molnar and Blakemore, 1991; Yamamoto et al., 1992; Bolz et al., 1992; Novak and Bolz, 1993; reviewed by Tuttle and O'Leary, 1993; Bolz et al., 1993). Many of these studies concerned the processes by which growing cortical or thalamic axons recognise their correct targets once they have invaded co-cultured tissue. The possibility of trophic and growth-promoting interactions between these structures in vitro has received less attention. In fact, some previous experiments failed to demonstrate such interactions, perhaps because the explants were grown in the presence of large quantities of serum (Bolz et al., 1992). Serum is known to contain many growth factors that may mask growth-promoting or trophic interactions (Esber et al., 1973; Honn et al., 1975; Annis et al., 1990). Other studies, using serum-free medium, have indicated that cortical slices do release as yet unidentified diffusible factors that enhance neurite outgrowth from thalamic explants (Rennie et al., 1994; Lotto and Price, 1994). In line with these findings, Hisanaga and Sharp (1990) demonstrated that growth factors released from cortical cells have a trophic effect on dissociated thalamic cells; these workers reported that this interaction is not specific to cortex and thalamus, and that other neural structures including cerebellum also have a trophic effect on the thalamus. The evidence for a release of cortically-derived growth factors (CDGFs) in vitro is consistent with in vivo observations. First, experiments by Cunningham et al. (1987) demonstrated that some axotomized thalamocortical cells can be rescued from death by the addition of medium pre-conditioned by the cortex to the brain of lesioned animals.

The in vivo significance of these growth-promoting and trophic interactions is far from clear. For instance, in vivo cell death in the thalamus takes place before their axons seggregate into ocular dominance columns. Therefore, cell death probably does not play a role in the organisation thalamocortical organisation. However, infusion of high levels of nerve growth factor (NGF) into the visual cortex can prevent the deleterious effects of

Table 1 The numbers of cultures in each experimental paradigm.

FIRST EXPLANT	SECOND EXPLANT	n
Effect of age of occipital cortex		
E16 thalamus	none	18
E16 thalamus	P0 occipital cortex	8
E16 thalamus	P2 occipital cortex	8
E16 thalamus	P4 occipital cortex	11
E16 thalamus	P6 occipital cortex	11
E16 thalamus	P8 occipital cortex	8
E16 thalamus	P10 occipital cortex	12
E16 thalamus	P14 occipital cortex	8
E16 thalamus	P18 occipital cortex	8
E16 thalamus	P28 occipital cortex	8
E16 thalamus	P38 occipital cortex	7
Specificity of interactions		
E16 thalamus	none	30
E16 thalamus	P2 occipital cortex	28
E16 thalamus	P2 cerebellum	42
E16 thalamus	P2 medulla	12
E16 thalamus	P2 liver	8
E16 tectum	none	8
E16 tectum	P2 occipital cortex	8
Dark-rearing		
E16 thalamus	P22 occipital cortex (normal)	12
E16 thalamus	P22 occipital cortex (dark-reared)	12

visual deprivation on thalamocortical afferents (Domenici et al., 1991; Domenici et al., 1992; Maffei et al., 1992). Therefore, these factors may maintain both thalamic survival and be a source for competitive interaction that yields ocular dominace columns.

Other identified growth factors, such as fibroblast growth factor (FGF), NGF and brain derived neurotrophic factor (BDNF), are produced by cortical cells both pre- and postnatally (Large et al., 1986; Whittemore et al., 1986; Maisonpierre et al., 1990; Castren et al., 1992; Matsuyama et al., 1992). The production of several of these growth factors increases with age and may be modulated by neuronal activity (Zafra et al., 1990; Isackson et al., 1991; Lu et al., 1991; Castren et al., 1992; Gall, 1992; Patterson et al., 1992; Riva et al., 1992).

But despite all of this evidence, the links between the in vivo and in vitro findings are tenuous. For example, it is not known whether the growth factors that have been identified in the cortex in vivo are those that promote neurite growth in culture. Nor is it clear whether the production of CDGFs in vitro is influenced by the age and activity of the cortex, as in vivo. In these experiments, after confirming that diffusible factors released by cortical slices do enhance neurite outgrowth from co-cultured thalamic explants, I determined how the release of such factors changes with the postnatal age of the cortex. In addition, I assessed whether the level of neural activity in the cortex prior to culture influences its growth-promoting effect by taking tissue from mice that had been dark-reared from birth. Since these experiments provided clues to the identity of the diffusible factors involved in promoting thalamic growth in vitro, I assessed the growth-promoting actions of two factors (FGF and NGF) on thalamic explants.

MATERIALS AND METHODS

Organotypic cultures

Occipital cortical slices, and slices of cerebellum, medulla and liver, were taken from BALB/c mice between postnatal day 0 (P0, the day of birth) and P38 to determine the effects of co-culturing occipital slices of different ages with E16 thalamic explants,

and to assess the specificity of the effect of the cortex on the thalamus (see Table 1 for full details). Mice were killed with an overdose of sodium pentobarbitone (30mg i.p.), immediately decapitated and their brains (and in a few cases their livers) were quickly removed and placed in oxygenated Hank's balanced salts (Sigma) on ice. The occipital cortices, cerebellar hemispheres, medullae and livers were isolated and sliced coronally at 350 μm with a McIlwain tissue chopper. Slices were placed in pre-incubated (at 37°C) defined serum-free culture medium (Romijn et al., 1984). The medial telencephalic wall of the occipital lobe, containing mainly visual cortex (see Results), and the other tissues were all dissected to a similar size (2.5-3.5 mm^2). Four cortical, cerebellar, medullary or hepatic slices were plated on each of a series of collagen treated membranes (Costar, Transwell-COL) suspended in wells filled with defined serum-free medium (Romijn et al., 1984).

Thalamic explants, and in a few cases tectal explants (Table 1), were obtained at E16 from the foetuses of time-mated BALB/c mice anaesthetised with urethane (as above). E16 mice were removed by ⌐aesarean section and decapitated. The brains were removed, and either the posterior thalamus, containing the lateral geniculate nucleus (LGN), or the tectum was carefully dissected out in oxygenated Hank's balanced salts on ice. The exact region included in the thalamic explants is described in Results. Some culture wells were set up with either 2 or 4 posterior thalamic explants, or 4 tectal explants, but no other tissue (Table 1); the others each contained 4 posterior thalamic explants with 4 slices of cortex, cerebellum, medulla or liver (Table 1). In all cultures, the tissues were plated at a distance (1.5 mm); thus, fibres growing from the explants never touched, eliminating the possibility of enhancing growth by factors dependent on direct contact. Tissues were cultured at 37°C in 95% humidity for 3 days. At the end of the culture period, cultures were fixed in 4% paraformaldahyde in 0.01M phosphate buffer. In a few cases, small crystals of DiI (relative to the size fo the thalamic explant) were placed in the thalamic explants and left for at least 2 weeks to label the growing neurites.

Dark Rearing Experiments

Two litters of mice, both born on the same day, were used. One litter was exposed to a standard 12 hour light-dark cycle; the other was reared in a light-proof room from just before birth. On P22, occipital cortical slices from either the normal-reared or dark-reared mice were co-cultured with E16 posterior thalamic explants from normal mice (Table 1). After three days, explants were fixed.

Assessing the health of the cortical slices

Propidium iodide is a vital dye that preferentially enters damaged cells (Macklis and Madison, 1990; Adams et al., 1993). I used it as an easy and rapid method for screening cultured explants and ensuring that cell damage and death were not excessive. Propidium iodide was added to the cultures (final concentration, 10 μg ml^{-1}), 2h before the end of the 3 day culture period. Immediately the culture period was complete, the cortical explants were photographed under the fluorescence microscope and then fixed. A few cultures containing propidium iodide were photographed but then left on the bench at room temperature for 2h, and re-photographed before being fixed. In addition to the use of propidium iodide, I stained some of the cortical slices (washed in phosphate buffered saline and fixed for 10 minutes in 90% ethanol and 10% formalin rather than in 4% paraformaldehyde) with one or other of two fluorescent nuclear dyes, bisbenzimide (Hoechst 33342; final concentration, 5 μg ml^{-1}) or acridine orange (final concentration, 1μg ml^{-1}) (Latt and Stetten, 1976; Sumner, 1988). The cortical slices were stained overnight at room temperature, then washed with phosphate buffered saline, mounted on glass slides and analysed with both confocal and standard fluorescence microscopy. Hoechst was viewed through a UV filter and acridine orange through a fluorescene filter.

Posterior thalamic explants cultured in FGF and NGF

E16 posterior thalamic explants (n=28), prepared as described above, were cultured for 3 days in medium supplemented with 5 or 40 ng ml^{-1} FGF (a combination of acidic and basic forms; Sera-lab) or 10 or 40 ng ml^{-1} NGF (Sigma). The growth factors were dissolved in sterile serum-free culture medium before being added to the wells. After three days, the cultures were fixed.

Fig. 2.1 Photomicrographs of DiI labelled neurites emerging from posterior thalamic explants cultured for 6 days on collagen-coated membranes; the neurites end with broad growth cones. (a) Most of the neurites that emerge from the explants are guided by the closely spaced shallow grooves on the filters (orientated left to right in this Figure and visible in Figs. 3 and 5). They grow in a relatively straight and orderly fashion. (b) Neurites that emerge at an angle or perpendicular to the grooves may initially cross them, but many eventually reorientate and grow along the collagen grooves. Scale bar, 500µm
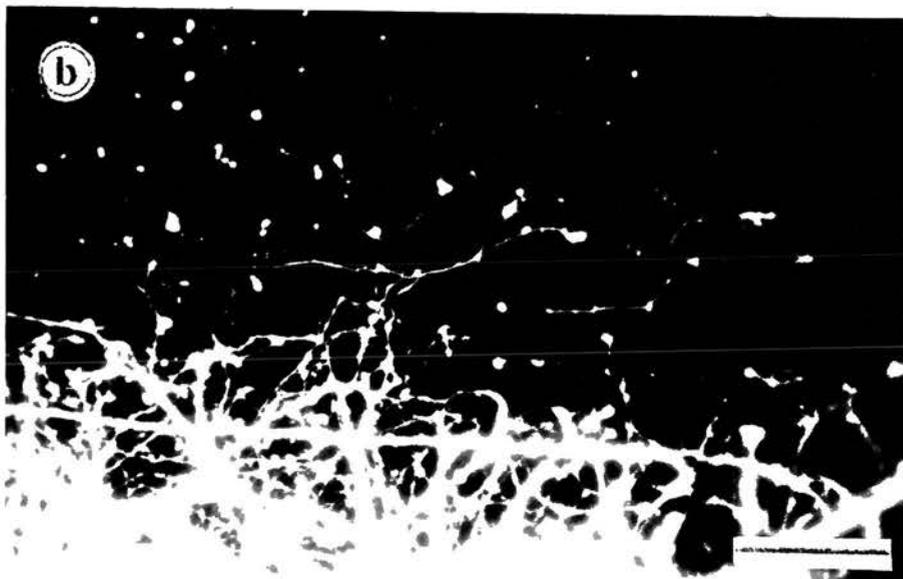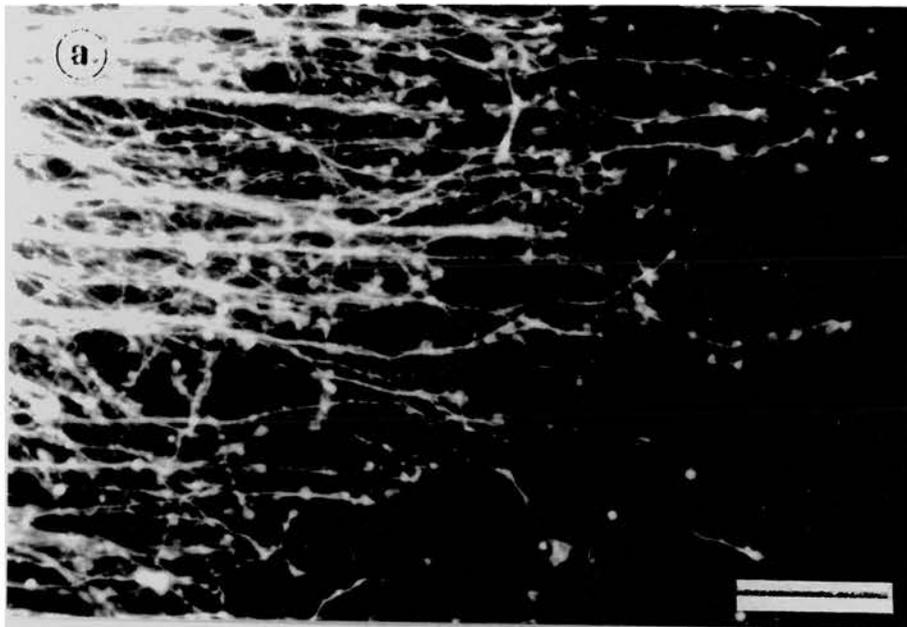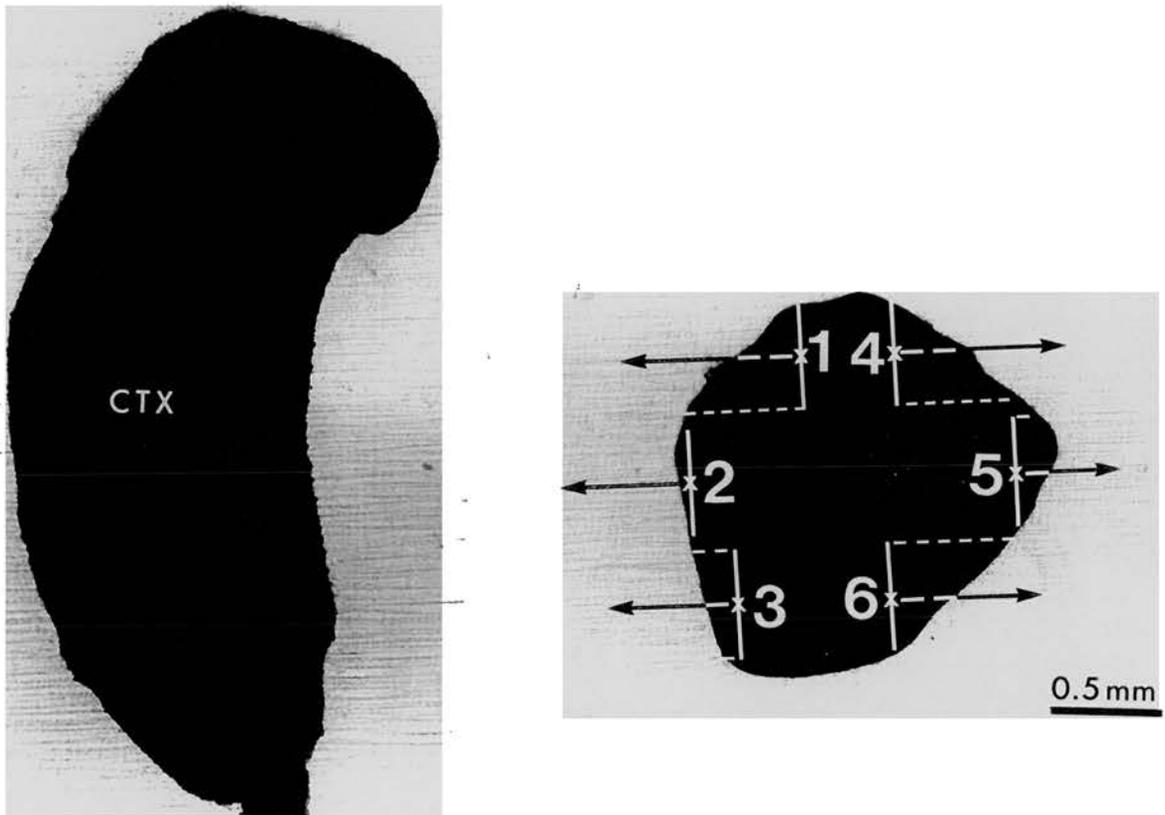
Fig. 2.2 Phase-contrast photomicrograph of a typical co-culture (CTX, cortex). The scheme used to quantify outgrowth is indicated on the thalamic explant: six 500 µm windows (three on each side of the explant), perpendicular to the grooves on the collagen-coated membranes, were used to assess density of outgrowth; the arrows indicate the positions at which neurite lengths were measured.
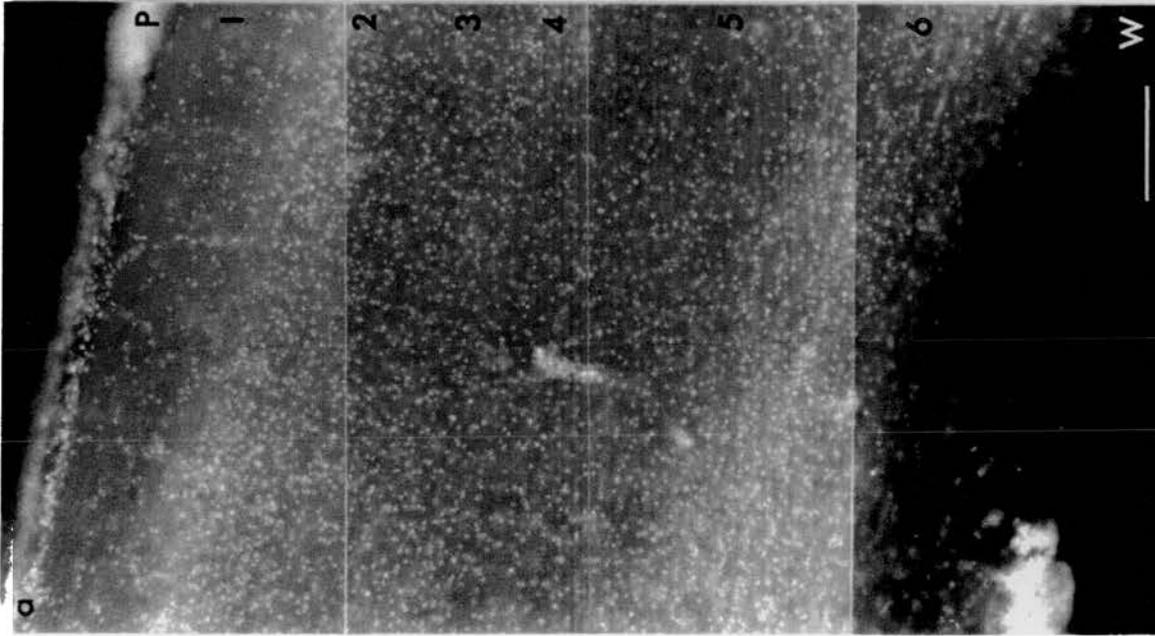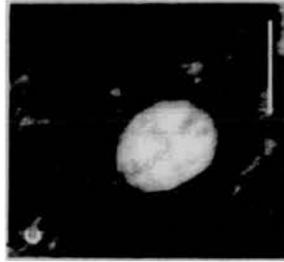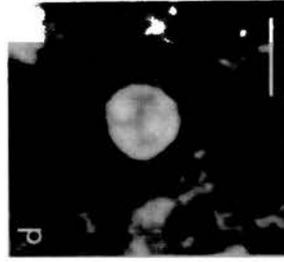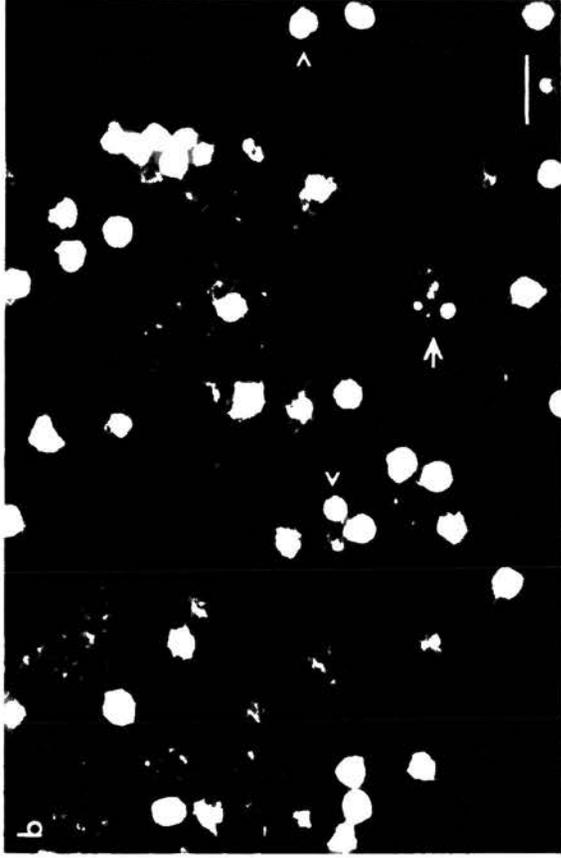
Fig. 2.3 Fluorescence photomicrographs of P22 cortical explants that had been cultured for 3 days and then fixed and stained with bisbenzimide. (a) Although the cortical slices spread slightly during culture, the integrity of the tissue has been well preserved and the six major layers of the cortex are clearly visible (P, pia; W, white matter). (b) A representative overview at an intermediate magnification, taken with a confocal microscope; the optical section is 1 μm thick, and is through the middle of the cortical slice. The vast majority of the nuclei appear as intact, uniformly bright disks (resembling those in acutely dissected cortical slices); a few show signs of early (arrowheads) and late (arrow) apoptosis (i.e. chromatin condensation and nuclear disintegration). (c,d,e) The types of cell observed in the cortical slices are exemplified in these high magnification confocal views: the vast majority had a healthy appearance (c), while others were in the early (d) or late (e) stages of apoptosis. Scale bars: a, 200 μm; b, 20μm; c, d & e, 10μm.

<u>Quantification</u>

During the manufacture of the Transwell-COL membranes, most of the collagen fibres orient themselves in one direction and, as a result, they generate parallel equally-spaced narrow grooves (35μm wide). This furrowed structure channels most of the neurites emerging from the explants so that they grow in a well-ordered parallel fashion (Fig. 2.1a); where neurites emerge at an angle to the grooves, they usually reorient to follow the grooves (Fig. 2.1b). I found this an invaluable feature of the membranes, allowing quantification. Density and length of neurite outgrowth were measured under phase-contrast (x20 objective); I used phase-contrast because DiI did not always label every neurite (although DiI labelled material gave better photographs). The quantifications were carried out through the microscope, following the scheme illustrated in Fig. 2.2. For each explant, (as described by Rennie et al., 1994) I counted the numbers of neurites crossing six 500μm windows, three equally spaced on each side of the explant, at its edge and oriented perpendicular to the grooves on the filters. When fascicles were encountered and could not be resolved into individual neurites, the number of neurites within each fascicle was estimated based on the width of the fascicle as compared to the width of individual neurites. In fact, tight fascicles that could not be resolved were rare; almost none were wider than 20μm. It is very likely that I underestimated the numbers of neurites in tight fascicles, since neurites may have been growing on top of each other, but since I detected no tendency for such fascicles to occur with greater frequency in any particular paradigm, the underestimates would apply equally in all cases. The length of the neurite emerging closest to the midpoint of each window was measured. For each experimental paradigm, data from all six windows for all explants were combined for statistical comparisons (Student's t-test).

RESULTS

<u>Viability of cultured cortical slices</u>
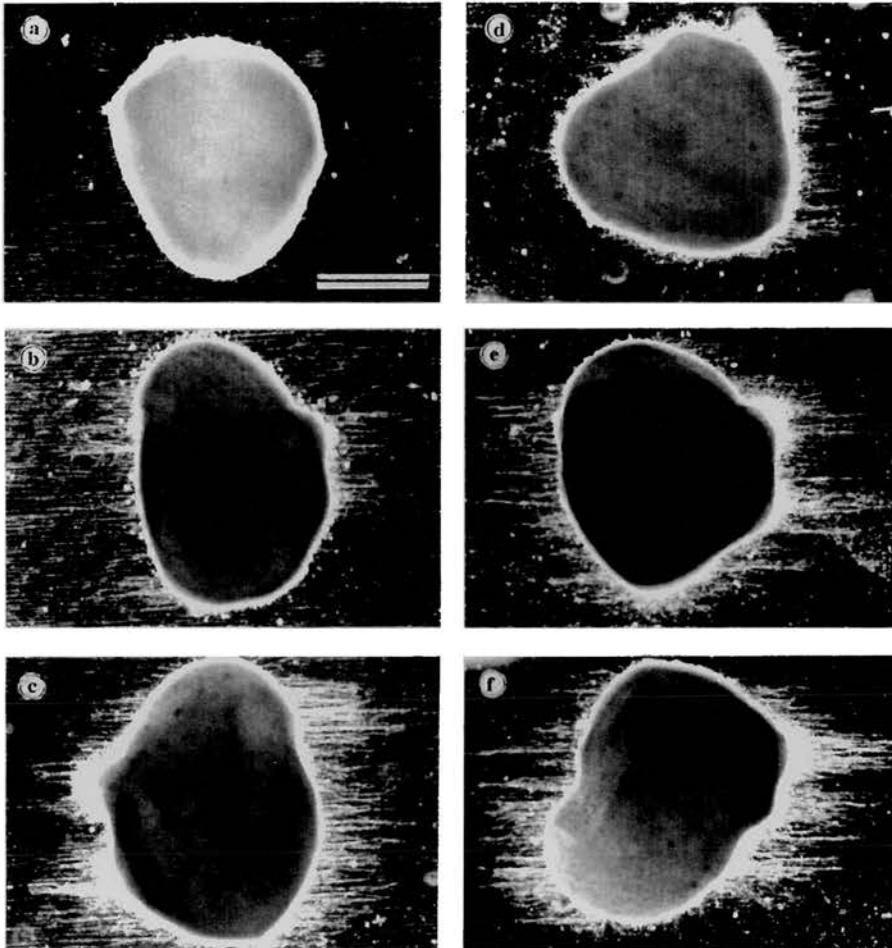
Fig. 2.4 Phase contrast photomicrographs of E16 thalamic explants cultured (a) alone, or with cortical slices from (b) P4, (c) P6, (d) P10, (e) P14, (f) P20 embryos. Neurites are phase bright. Scale bar, 500μm.
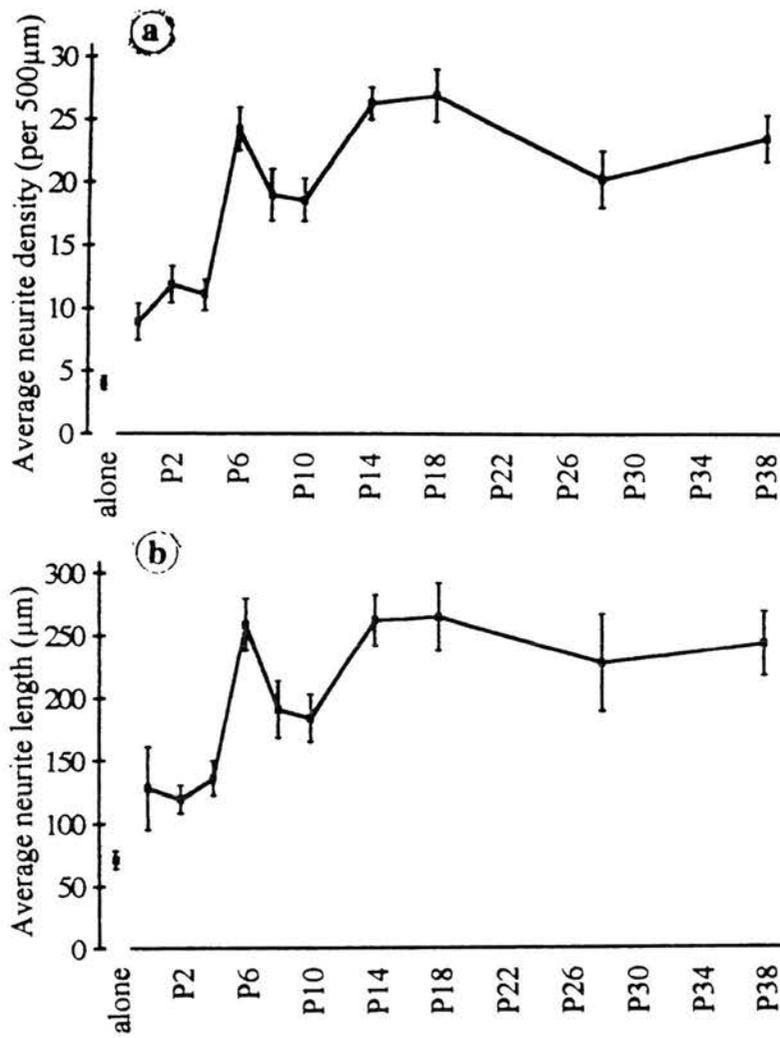
Fig. 2.5 Graphs plot (a) the average density and (b) the average length of neurites growing from E16 thalamic explants cultured alone or with cortical slices of different ages (all of equal volume; see material and methods). Values are ± standard error of the mean.
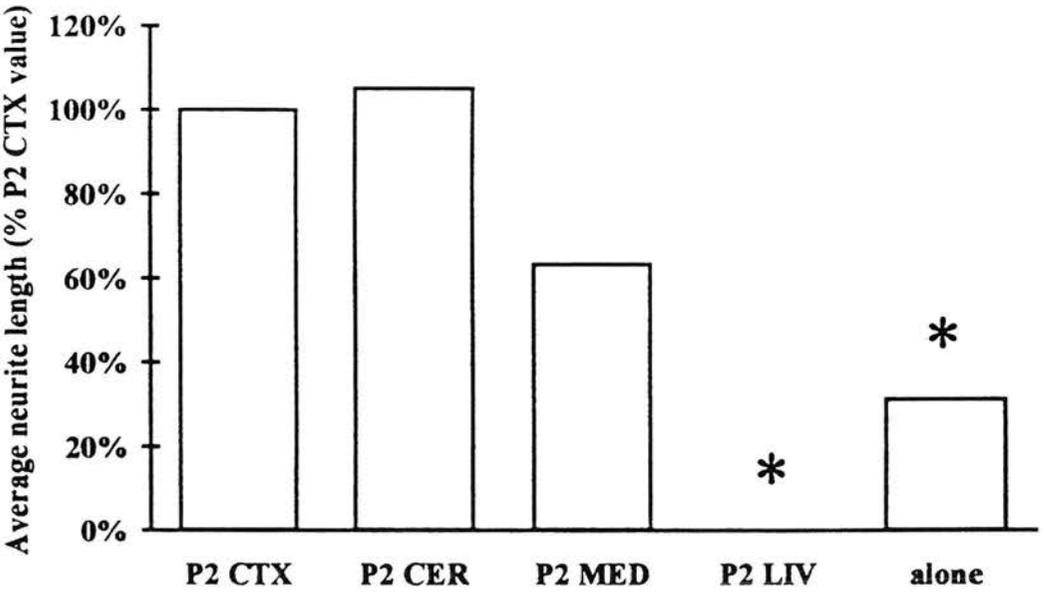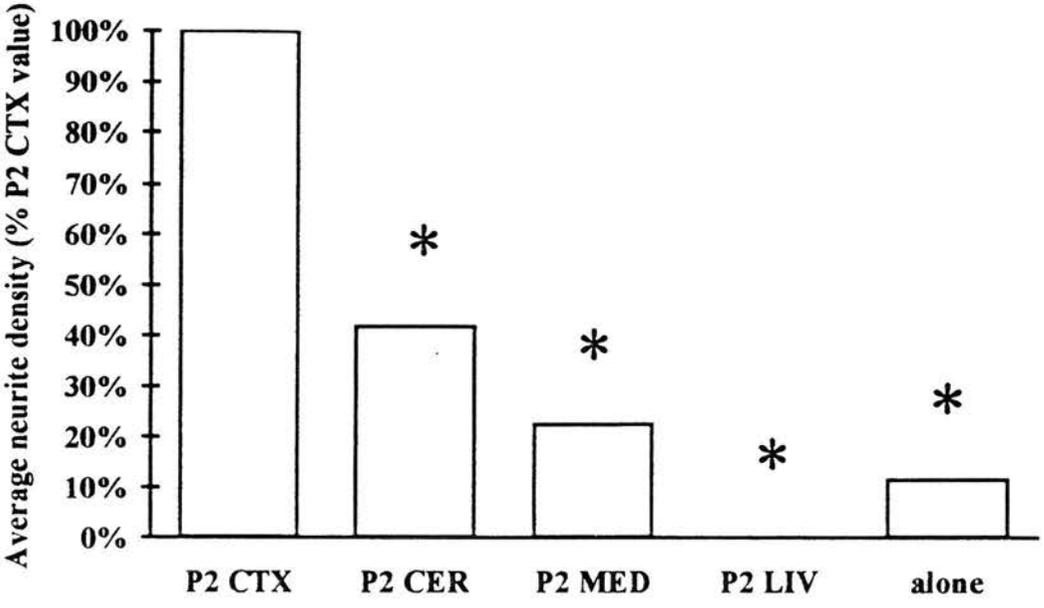
Fig. 2.6 Histograms of (a) the average density and (b) the average length of neurites from E16 thalamic explants cultured with P2 occipital cortex (P2 CTX), P2 cerebellum (P2 CER), P2 medulla (P2 MED), P2 liver (P2 LIV) or alone (n = 8 each). Asterisks indicate values that were significantly lower than those obtained with P2 occipital cortex (all p<0.01, Mann-Whitney test). Data taken from Rennie (1994).

Previous workers have reported the successful culturing of brain slices from rats aged up to 20 days postnatal (Gahwiler, 1981), although most studies have used material from younger animals (e.g. Bolz et al., 1992; Molnar and Blakemore, 1991; Yamamoto et al., 1989 and 1992). The work of Caesar et al. (1989), which indicated that cortical tissue from rats aged over 11 days postnatal could not be maintained with the roller-culture method, raised a concern about whether the older cortical slices used in my study would remain healthy after 3 days in vitro. Certainly, in none of the cultures reported here were there any of the overt signs of disintegration that appear rapidly in cortical explants that have died due to technical problems (e.g. accidental exhaustion of the gas supply to the incubator). Figure 2.3a shows a P22 cortical explant stained with bisbenzimide; the tissue remained intact and the laminae were well preserved. These cells were viewed at intermediate and high magnifications with the confocal microscope (Fig. 2.3b-e). Figure 2.3b shows a representative field of view; the vast majority of the nuclei were of normal shape and size and stained fairly homogeneously with bisbenzimide or acridine orange, as shown at high magnification in Fig. 2.3c. This appearance was similar to that of bisbenzimide or acridine orange stained cells in acutely dissected cortical tissue from animals of the same age, and similar to that described in previous studies of normal cells stained with these dyes (e.g. Gordon and Parker, 1981). However, some of the cells in these 3 day cultures showed the morphological appearances characteristic of the early or late stages of apoptosis (i.e. condensation of the chromatin into several discrete masses with, at later stages, the distortion and disintegration of the nucleus; Kerr et al., 1972). Examples are seen in Fig. 2.3b (arrows/arrowheads) and, at higher magnification, in Fig. 2.3d and e. Overall, it appeared that no more than about 20% appeared apoptotic (early or late stage).

The use of bisbenzimide and acridine orange allowed me to assess the appearances of the nuclei of the cells in my cortical slices. To assess the integrity of the cell membranes, I added the vital dye propidium iodide to living cultures before fixation. Propidium iodide preferentially enters cells with damaged membranes; the amount of dye that enters if proportional to the degree of damage (Macklis and Madison, 1990; Adams

et al., 1993). In cortical cultures of all ages, propidium iodide labelled cells were scattered throughout the cortical depth. Many of the unhealthy cells labelled by propidium iodide in these cortical explants were probably damaged during the surgical removal of the tissue, since it has been previously observed (while repeatedly viewing individual cultures grown with propidium iodide in the culture medium from the outset, using a low-light detector on a fluorescence microscope with an incubated stage; Rennie et al., 1994) that most propidium iodide labelled cells appear early in the culture period and that very few of them are cleared before 3 days. I estimated (i) the numbers of cells that would have been present in cortical explants of different ages when they were placed in culture (calculated from a knowledge of the density of cells in 40 μm frozen sections of the cortex and the volume of the cortical slices) and (ii) the numbers of propidium iodide labelled cells per slice after culture (counted through the full depth of the slices), I estimated that averages of 10-20% of the cells became unhealthy in the cortical explants, irrespective of their age. To check directly the accuracy of my conclusion that the majority of cells in cultures were viable, a few P22 cultures containing propidium iodide were photographed but then left on the bench at room temperature for 2h, and re-photographed before being fixed. In these cases, both the density of propidium iodide labelled cells and the intensity of label in individual cells increased dramatically as the cultures died (as described previously by Macklis and Madison, 1990). Measurements of the densities of propidium iodide labelled cells indicated a 4 fold increase in the numbers of propidium iodide labelled cells after 2h on the bench compared to the numbers in cultures fixed immediately after culture. This result is in good agreement with my previous conclusions, and suggests that most cells in the cultures had membranes that were sufficiently intact to exclude propidium iodide at the end of the culture period.

The use of propidium iodide provided an easy and rapid method for screening cultured explants and ensuring that cell damage and death were not excessive. In the course of these experiments I observed a set of six cultures (comprising thalamus with P22 cortex from normally reared animals; all six were carried out simultaneously) in which a very high proportion of cortical cells was stained with propidium iodide and the
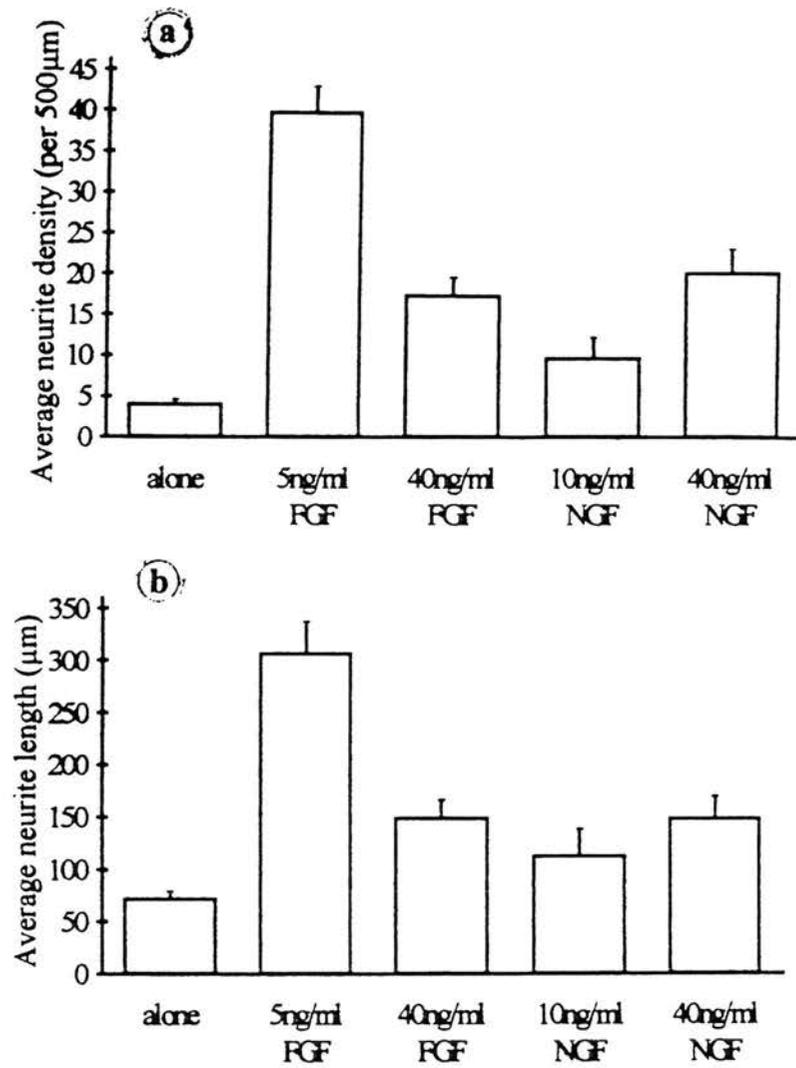
Fig. 2.7 Graphs plot (a) the average density and (b) the average length of neurites extending from thalamic explants cultured in serum-free medium alone or supplemented with FGF (5 or 40ng ml^{-1}) or NGF (10 or 40ng ml^{-1}). Values are + standard error of the mean.

reduction in average length (p<0.05). The average density of outgrowth in co-cultures with cortex from dark-reared animals was 14.5 neurites per 500μm ($\pm$ 1.6 s.e.m.); the average length was 99.1μm ($\pm$ 10.5 s.e.m.). These values are closest to those observed during the first few days after birth, prior to eye-opening (see Fig. 2.5).

<u>Addition of FGF and NGF to posterior thalamic explants</u>

Based on the results above, and for reasons given in Discussion, I assessed the effects of adding FGF and NGF to the culture medium on the outgrowth from posterior thalamic explants. The quantified results are shown in Fig. 2.7. Outgrowth was greatly increased over controls (almost 10-fold for density and about 4-fold for length) in the presence of 5ng ml^{-1} FGF (p<0.001 for density and length); there was an effect of 40ng ml^{-1} FGF, but it was less marked (p<0.001 for density and length). NGF had a stimulatory effect at 10ng ml^{-1} (p<0.002 for density and p<0.05 for length) and a slightly greater effect at 40ng ml^{-1} (p<0.001 for density and length).

DISCUSSION

My results suggest that the occipital cortex releases diffusible factors (referred to here as CDGFs) that enhance the growth of posterior thalamic axons. Further, they indicate that the level of CDGF release increases with the postnatal age of the occipital cortex, and that reduced neural activity in the occipital cortex blocks this increase.

The evidence that cultured cortical slices release diffusible factors that enhance the growth of neurites from thalamic explants is overwhelming. I believe that the key to demonstrating this interaction <u>in vitro</u> is to use a serum-free culture system, to avoid the possible masking effects of growth factors that are present in serum (Esber et al., 1973; Honn et al., 1975). In addition, to demonstrate that the interaction is mediated by diffusible molecules, it is essential to eliminate any contact between the co-cultured tissues; thus, although Molnar and Blakemore (1991) suggested a growth-promoting influence of the cortex on the thalamus in culture, they were unable to rule out growth-

promotive or growth-permissive effects mediated by direct contact. My previous work has shown that increased outgrowth from thalamic explants cultured alone can be stimulated by preconditioning the serum-free medium, but not by preconditioning the collagen substrate, with cortical slices (Lotto and Price 1994; Rennie, 1992; Rennie et al., 1994). Thus, I conclude that CDGFs have an active effect, stimulating thalamic cells rather than being permissive for outgrowth onto the collagen. I do not yet know the identity of the CDGFs released in my cultures, but they are unlikely to be simple, ubiquitous molecules that might come from any cultured tissue to enrich the medium, since neither neurite density nor length was significantly enhanced by increasing the number of thalamic explants cultured together in a well.

There is evidence from a number of other related studies that CDGFs influence thalamocortical development. Hisanaga and Sharp (1991) have demonstrated that CDGFs enhance the survival of postnatal thalamic cells in vitro. Cunningham et al. (1987) showed that, whereas LGN cells die in vivo when axotomized (Finlay and Pallas, 1989), the addition of medium preconditioned with cortical slices to the site of the lesion rescues many of them. Domenici et al. (1991 and 1992) and Maffei et al. (1992) demonstrated that infusions of high levels of NGF prevent the effects of visual deprivation on thalamocortical maturation during the critical period. Thus, CDGFs may be important in vivo for both the growth and survival of thalamocortical projections during development.

In my experiments, the amount of posterior thalamic neurite outgrowth increased significantly with the age of the mice from which occipital cortical slices were taken. It is most likely that the amount of thalamic outgrowth relates directly to the concentration of CDGF in the medium; in support of this, other experiments have shown that the growth-promoting effect of medium preconditioned with cortex is greater the longer the conditioning period (Lotto and Price, 1994; Rennie, 1992; Rennie et al., 1994), and increasing the numbers of cortical slices of any one age proportionately increases the effect on the thalamus (my unpublished observations). In my experiments, the volume of the cortical slices was kept constant at all ages. In vivo, cortical volume continues to increase and the overall density of cortical cells decreases postnatally. Thus, in my

cultures, cortical slices from older mice contain fewer cells than those from younger mice and my result cannot be an artefact caused simply by an increase in the number of cells in the cultured cortical slices. On the contrary, it is likely to be an underestimate of the extent of the change in CDGF release.

I suggest that the increased CDGF release with age results from a change in the properties of the cortical cells. Based on previous studies both in vivo and in vitro, it is probable that the CDGF-releasing cells in the cortex are neurones (Rennert and Heinrich, 1986; Cunningham et al., 1987; Ayer-LeLievre et al., 1988; Hofer et al., 1990; Hisanaga and Sharp, 1991). For instance, Hisanaga and Sharp (1991) have shown previously that factors released from purified cortical neuronal cultures rescues postnatal thalamic neurones.

During the general increase in the release of CDGF with age, two steep rises stand out, one between the ages of P4 and P6 and the other between P10 and P14. The first of these rises is likely to follow closely the termination of geniculocortical fibres in layer 4 (Lund and Mustari, 1977; Molnar and Blakemore, 1991). The increased release of CDGF may result from enhanced production stimulated by thalamocortical synaptogenesis with layer 4 cells, perhaps reflecting the arrival of spontaneous afferent activity driven from the retina (Galli and Maffei, 1988). Galli and Maffei (1988) have demonstrated the rat retina in vivo that spontaneous activity is present from at least E15/16. Consistent with this idea, increased neurotrophin release occurs coincident with afferent arrival at targets in the peripheral nervous system (Davies et al., 1987; Rohrer et al., 1988). The second sharp rise between P10 and P14 coincides with the opening of the eyes. This temporal relationship suggested to me that the rise may be caused by increased neural activity resulting from eye-opening, and indeed I did find that dark-rearing prior to culture prevented the enhanced effect of older cortical slices. I suggest that decreasing neural activity by dark-rearing blocks the normal increase in CDGF production, and that the normal age-related increase of CDGF release may be partially regulated by afferent activity. In support of this view, there is in vivo evidence that the levels of production of various identified growth factors in the brain are related to levels of afferent activity

(Zafra et al., 1990; Isackson et al., 1991; Castren et al., 1992; Gall, 1992; Patterson et al., 1992; Riva et al., 1992).

Levels of mRNA for several growth factors increase with the age of the postnatal brain, in a remarkably similar way to the age-related increase in CDGF release reported here. The production of FGF and NGF in the brain parallel my results particularly closely (compare my Fig. 2.3 with Fig. 1 of Large et al., 1986 and Thomas et al., 1991). There is previous evidence suggesting a role for these two molecules in thalamocortical development: the survival of E18 dissociated thalamic cells can be enhanced in the presence of FGF (Walicke, 1989; Hisanaga and Sharp, 1990) and, as mentioned earlier, infusions of high levels of NGF in vivo influence the plasticity of geniculocortical projections (Domenici et al., 1991 and 1992; Maffei et al., 1992). Furthermore, both molecules are synthesised in the cortex (Large et al., 1986; Thomas et al., 1991), are released from neurones (although the exact mechanism of release of acidic and basic FGF is unclear; Stock et al., 1992) and have their mRNA production influenced by neural activity (Zafra et al., 1990; Riva et al., 1992). In the present study, I found that both FGF (a mixture of acidic and basic forms) and NGF can enhance posterior thalamic growth in vitro. I found that 5ng ml^{-1} of FGF had a greater effect than 40ng ml^{-1}, in agreement with previous reports on the inhibitory effects of FGF at high concentrations in other systems (Morrison et al., 1986; Walicke et al., 1988). NGF had much less effect than FGF, and it promoted more growth at higher concentration. Thus, it remains possible that one or other of these molecules mediates the growth-promoting effects of the occipital cortex on the thalamus.

My finding that thalamic outgrowth is stimulated not only by cortical slices but also by other neural tissues is in agreement with Hisanaga and Sharp's (1990) report that both target and non-target tissue can have a trophic effect on thalamic neurones in culture. These observations are compatible with a possible role for molecules such as FGF and NGF, that are known to have widespread distributions in the developing nervous system. For example, in late embryonic rats, basic FGF is distributed throughout

the telencephalon (where it is in neurones), is located in fibres in the diencephalon (i.e. it is presumably transported through, rather than released by, the thalamus) and is found only at very low levels in the liver (Gonzalez et al., 1990). This distribution correlates well with my findings that thalamic explants are stimulated to grow by several brain tissues that are rich in neurones, but not by additional thalamic explants nor by liver. Similarly, NGF is found at high levels in the neocortex of the adult and developing rat, at intermediate levels in the medulla, diencephalon and cerebellum (note that although levels in the cerebellum are relatively low in the adult, they are higher in the neonate), and at very low levels in the liver (Large et al., 1986; Shelton and Reichardt, 1986). Recent work by Allendoerfer et al. (1994) has demonstrated that the receptors for neurotrophic factors (trkB, trkC and p75) are present in the developing central nervous system, including the LGN and the tectum. Thus, again my finding that the occipital cortex can stimulate the tectum as well as the thalamus is compatible with a role for known neurotrophic factors in the interaction described here.

My experiments with FGF and NGF must be interpreted with caution. Certainly, they strengthen the possibility of the involvement of FGF and NGF in thalamocortical axonal growth, but they allow me to reach no firmer conclusion on the nature of the CDGFs released in vitro. Further, NGF at high concentrations binds to BDNF receptors (trkB; Rodriguez-Tebar et al., 1990). I have not tested BDNF in my system (and I know of no other studies on whether thalamic cells are responsive to BDNF), but it may be at least as good a candidate as NGF. BDNF transcription in the cortex increases with postnatal age (Maisonpierre et al., 1990), and is affected by activity in thalamocortical fibres (Castren et al, 1992). In fact, Castren et al. (1992) have proposed that BDNF, rather than NGF, is involved in the segregation of geniculocortical afferents to form ocular dominance columns.

Two important questions remain. First, what are the roles of CDGFs in vivo, and what is the significance of the increase in their levels of release postnatally? In my culture system, the manifestation of the effects of CDGFs is a promotion of thalamic neurite growth. Similarly in vivo, the effects of CDGFs may be to enhance the growth of

thalamic axons, either as they grow towards the cortex and/or as they arborize within it. Furthermore, because the production of trophic factors in the cortex commences at E13 (Large et al., 1986; Thomas, et al., 1991) these factors may serve to initiate thalamic outgrowth. Given the fact that thalamic outgrowth is not stimulated exclusively by the cortex, but also by other nearby neural structures, it is rather unlikely that the diffusible factors identified in this work are involved in any simple way in guiding axons to specific targets. It seems more likely that they operate on thalamic axons that have already located their targets (through some other mechanism), and that they are important in later processes such as synaptic maintenance and the reorganisation of connections. A clue to the possible significance of the postnatal age-related increase in the levels of CDGF release is that it coincides closely with the timing and duration of the critical period (Rothblat et al., 1978; Rothblat and Schwartz, 1979; Stafford, 1984; Maffei et al., 1992). It has been suggested that aspects of cortical connectivity are generated as afferents compete for factors that are limited in supply (Purves, 1988; Maffei et al., 1992). Thus, I propose that the levels of growth ιactors in the cortex are crucial for determining the degree of neuronal plasticity. When growth factors are in limited supply (i.e. in the early postnatal period), fibres compete; when levels of growth factors are high (i.e. near the end of the critical period and continuing into adulthood), competition is diminished and plasticity is less evident. One implication of this hypothesis is that plasticity is a graded phenomenon, rather than all-or-nothing, and that the gradual transition from a state of high plasticity to one of low plasticity during normal development may not be irreversible. In support of this notion, there is clear evidence that significant plasticity can be manifest in the adult central nervous system (Kaas et al., 1990; Gilbert and Wiesel, 1992).

CHAPTER 3:

EVIDENCE THAT MOLECULES INFLUENCING AXONAL GROWTH AND TERMINATION IN THE DEVELOPING GENICULOCORTICAL PATHWAY ARE CONSERVED BETWEEN DIVERGENT MAMMALIAN SPECIES.

ABSTRACT

The general architecture of the visual system is similar for all species of mammal. To determine if the development of connections in the visual system might be under the influence of conserved molecules, I co-cultured explants of the murine lateral geniculate nucleus with slices from either murine or feline occipital cortex. Neurite outgrowth from embryonic murine geniculate explants was significantly enhanced by slices of new-born mouse occipital cortex or kitten visual cortex, or by medium previously conditioned by these slices. Slices of similar volume but from sites other than occipital cortex had less or no effect on the murine geniculate explants. Fibres from murine geniculate explants grew freely on cortical slices from the kitten. They terminated mainly in layer 4, and also in layer 6, in both murine and feline visual and frontal cortical slices, irrespective of whether they entered through the white matter or pial side. Only the deep layers of the kitten's cortex sent projections to co- cultured murine geniculate explants. I suggest that the diffusible factors released by the cortex that stimulate the growth of axons from the lateral geniculate nucleus, and the molecules that mark specific cortical laminae as targets for ingrowing afferents, are conserved in divergent species. I also found that murine geniculate axons grew freely on feline cerebellar slices. It is known from previous co-culture experiments that rodent geniculate axons are inhibited on rodent cerebellum, and I suggest that the inhibitory factors involved are not conserved.

INTRODUCTION

During the development of the mammalian visual system, the lateral geniculate nucleus (LGN) receives inputs from the retina and sends projections to the visual cortex, mainly to layer 4 but also to layer 6. In vitro experiments have indicated that the recognition of specific layers is mediated by membrane-bound molecular cues (Yamamoto et al., 1989; Yamamoto et al., 1992; Molnar and Blakemore, 1991; Bolz et al., 1992; Gotz et al., 1992). Diffusible molecules (most likely proteins) released by the visual cortex promote the growth of geniculocortical neurones and could provide a resource for which afferents from the right and left eye compete to form ocular dominance columns (Cunningham et al., 1987; Domenici et al., 1991; Hisanaga and Sharp, 1990; Lotto and Price, 1994; Rennie et al., 1994; Maffei et al., 1992) The molecules that control the development of the geniculocortical system are unknown.

In this study, I sought evidence for the interspecies conservation of these molecules for two main reasons. First, such information will later help me to identify the molecules involved, for example by narrowing the range of candidates. Second, given the broad range of organisation and complexity among cortices from different mammals, a useful approach to studying cortical development is to concentrate on features that are common to many species rather than specific to only one. Although there are archetypal cortical morphologies, there is little evidence on whether the developmental processes that generate them are controlled by conserved molecules. Based on results from other systems, any assumption that they are must be viewed with scepticism. For example, motor neuronal organisation and development are similar in the chick and the mouse, and in both species motor neurones are dependent on muscle-derived growth factors for their survival. Whereas evidence in the mouse suggests that the growth factors are neurotrophin-3 and/or brain derived neurotrophic factor, chick motor neurones are not responsive to these molecules (Arakawa et al., 1990; Henderson et al., 1993). In this study, I co-cultured organotypic LGN explants from the mouse either at a distance from or touching slices of murine or feline cortex in serum-free medium. The aim was to

assess whether neurones in the mouse LGN are able to use signals from the cat cortex. My experiments represent a simplified in vitro form of interspecies neural transplantation.

MATERIALS AND METHODS

I made organotypic cultures (n=180) comprising explants of embryonic murine LGN either alone, with slices of murine occipital cortex or liver, or with kitten visual cortex, frontal cortex, cerebellum or medulla in defined serum-free medium. LGN explants were taken on embryonic day 16 (E16), since this is the age when LGN outgrowth reaches the cortex in vivo (Catalano et al., 1991; De Carlos and O'Leary, 1992). I have found that older LGN explants do not survive in culture, perhaps due to extensive denervation. All tissues other than LGN were from either postnatal day 2-6 (P2-6) mice or P1 kittens; at these ages in both species, cortical layer 4 cells have completed their migration and afferent ingrowth has just begun (Lund and Mustari, 1977; Shatz and Luskin, 1986; Blakemore and Molnar, 1990; Catalano et al., 1991; De Carlos and O'Leary, 1992; Ghosh and Shatz, 1989).

Cultures to assess diffusible growth factors

Pregnant mice at E16 were anaesthetised (0.3 ml urethane, 25% in normal saline, i.p.); foetuses were removed and decapitated. Explants containing the LGN were dissected from the dorsolateral thalamus, as described before (Molnar and Blakemore, 1991; Yamamoto et al., 1992). Prior tract-tracing experiments with 1,1'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) placed in the eye of fixed foetuses confirmed the LGN's exact location in the mouse. 18 LGN explants were cultured alone. 12 were co-cultured with 350µm slices of either P2 mouse occipital cortex, 8 with liver (obtained immediately after decapitation) or one or three additional E16 murine LGN explants (n=8). 12 LGN explants were also co-cultured with P1 kitten visual cortex, 12 with frontal cortex, or 12 with medulla (from kittens anaesthetised with ketamine, 22mg kg^{-1} i.m. and Saffan, infused i.v. at 0.03ml min^{-1}); all slices were cut to a standard

Fig. 3.1. Fluorescence photomicrographs of DiI labelled geniculate neurites growing (a) through a feline cortical slice and (b) onto a collagen membrane. The photograph in a is of a confocal section and provides an example of a neurite count. In tranches through the cortex from pia to white matter, short lines of consistent length were drawn parallel to the pial surface (i.e. they were roughly at right angles to the trajectory of the geniculate fibres), at intervals of 50 μm between the pia and white matter. The numbers of labelled neurites crossing each line were counted. In this example, those crossing a line corresponding to the bottom of the photograph were counted; arrows indicate where neurites cut across the line, and the dots indicate the numbers counted (28 in this case). To continue the counting process, further lines would be drawn at 50 μm intervals above and parallel to the bottom of the picture. Scale bar: a, 50μm; b, 100μm.
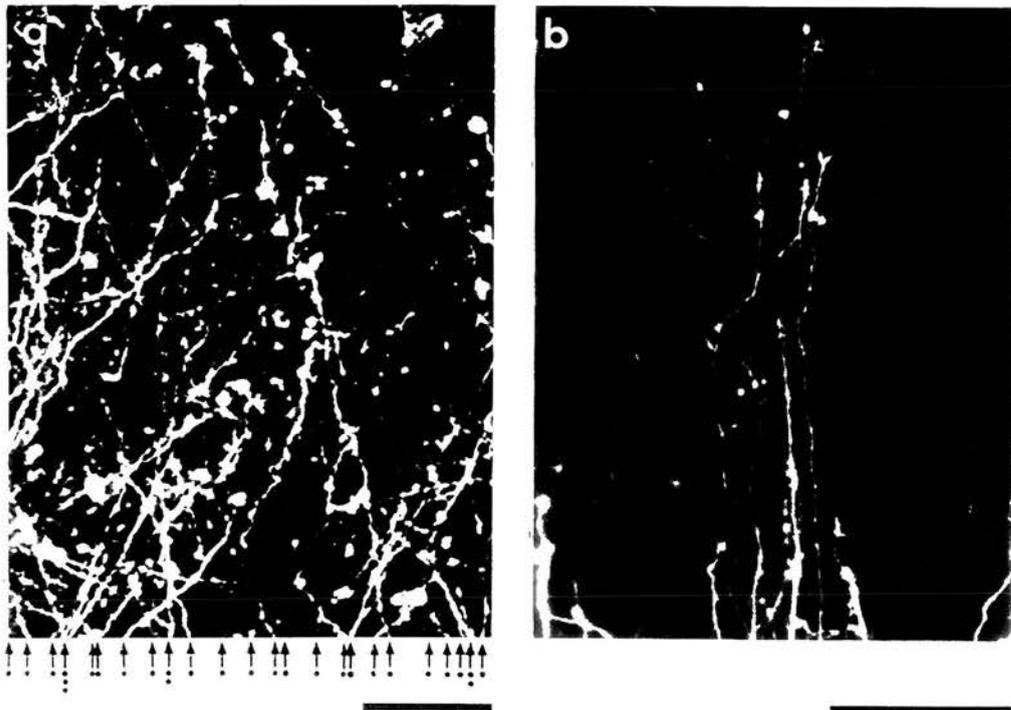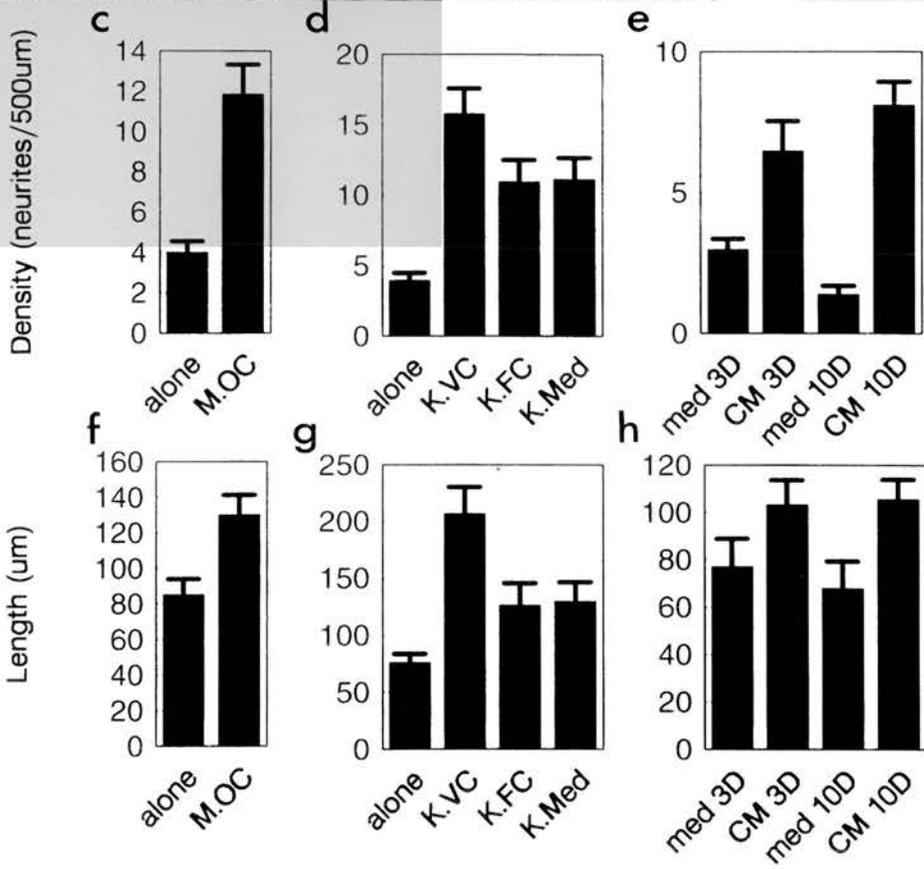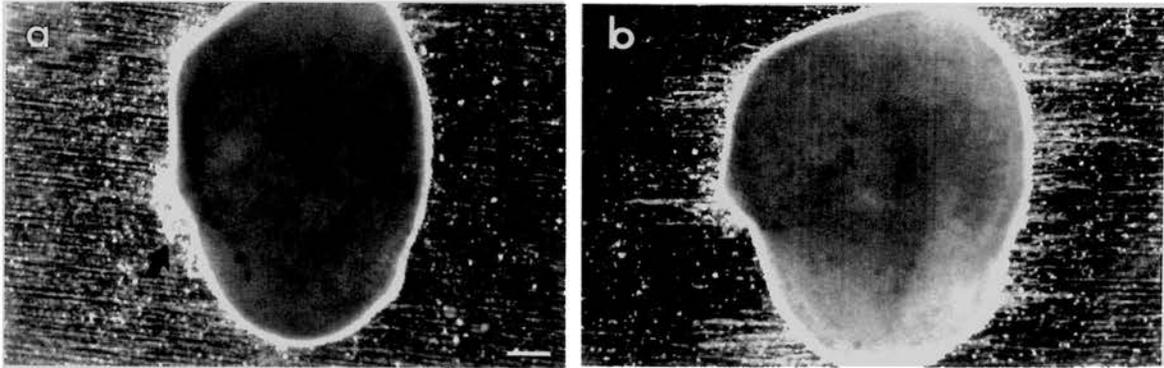
volume. Cultures were for 3 days in serum- free medium (Romijn et al., 1984) at $37^{\circ}C$ with 5% CO_2 on collagen filters (Costar, Transwel-Col). A 1-2mm gap was left between explants; neurites from the LGN explants were never allowed to reach the co-cultured tissues nor any outgrowth from them. This was essential to avoid direct influences of factors bound within the co-cultured explants and to allow quantification. Serum was not used in these experiments as it is known to contain many growth factors and substrate molecules in variable amounts (Esber et al., 1973; Honn et al., 1975).

To condition medium, $350\mu m$ slices of P1 kitten visual cortex were cultured alone for 3 or 10 days. The medium was then transferred to new culture wells, and E16 murine LGN explants were added and cultured for 3 days (n=24). As controls, LGN explants were cultured alone in medium that had been incubated for 3 or 10 days (n=24). To assess whether diffusible factors released by the cortex bind to the collagen substrate and make it more favourable for outgrowth, I cultured slices of murine occipital cortex alone for 3 days, then removed them, replaced the culture medium with fresh medium and cultured LGN explants on the conditioned substrates for 3 days (n=12).

All explants were fixed in 4% paraformaldahyde. Some were labelled with small crystals of DiI, placed in the LGN explants and left to label for 30 days. Most outgrowth followed the parallel grooves on the collagen filters (Fig. 3.1b and 3.2b), and this aided quantification by keeping neurites straight. The density and length of outgrowth were measured with phase-contrast (x20 objective): for each explant, I counted the numbers of neurites crossing six $500\mu m$ windows, three equally spaced on each side of the explant at its edge, oriented perpendicular to the grooves on the filters. The lengths of the neurites crossing closest to the midpoints of each window were measured. For each experimental paradigm, data from all six windows for all explants were combined for statistical comparisons (Student's t-test).

To ensure that outgrowth was axonal rather than glial, which can have an appearance similar to that of neurites (Torran-Allerand, 1990), I reacted explants with anti-neurofilament antibody (Affiniti Research Products NA1223 1:50 dilution). Binding

Density (neurites/500um)

c
14
12
10
8
6
4
2
0
alone   M.OC

d
20

15

10

5

0
alone K.VC K.FC K.Med

e
10

5

0
med 3D CM 3D med 10D CM 10D

Length (um)

f
160
140
120
100
80
60
40
20
0
alone   M.OC

g
250

200

150

100

50

0
alone K.VC K.FC K.Med

h
120

100

80

60

40

20

0
med 3D CM 3D med 10D CM 10D

Fig. 3.2 Phase-contrast photomicrographs of cultured embryonic murine LGN explants after 3 days (a) alone and (b) with P1 kitten visual cortex (a similar appearance was seen with P2 mouse occipital cortex); arrow in (a) indicates dissociated cells. These photomicrographs show low magnification (x4 objective) overviews; quantifications in c-h were carried out at higher magnification (x20; see Fig. 1b). Scale bar, 250µm. (c-h) The mean ($\pm$s.e.m.) neurite density and length of outgrowth from LGN explants were increased by co-culturing with P2 mouse occipital cortex (M.OC) (c, $p < 0.0001$; d, $p < 0.01$), P1 kitten visual cortex (K.VC) (e and f, $p < 0.001$), frontal cortex (K.FC) (e, $p < 0.001$; f, $p < 0.01$) or medulla (K.Med.) (e, $p < 0.001$; f, $p < 0.01$). The amounts of outgrowth with kitten visual cortex were greater than with kitten frontal cortex (e, $p < 0.05$; f, $p < 0.01$) or medulla (e, $p < 0.05$; f, $p < 0.01$). (g,h) The mean values for outgrowth in medium conditioned for 3 or 10 days with P1 kitten visual cortex (CM 3D or CM 10D) were greater than those in control medium (med 3D or med 10D) (g, $p < 0.001$ for both; h, $p < 0.01$ for CM 10D, n.s. for CM 3D).

of the primary antibody was detected with a fluorescein-conjugated goat anti-mouse secondary antibody (follow the precedure in Rennie et al., 1994).

Murine LGN explants cultured alone (n=4) or cultured with P1 kitten visual (n=4) cortex for 3 days were wax-embedded, sectioned at 10µm and Nissl-stained. I measured the densities of pyknotic cells (dead or dying cells, with small dense fragmented nuclei and little or no cytoplasm) and healthy cells in 12 sections from each explant.

Cultures to assess target recognition

E16 murine LGN explants were co-cultured for 7 days in serum- free medium touching either the pial or the white matter surface of 350µm slices of P2-6 mouse occipital cortex (n=12) or P1 kitten visual cortex (n=6), frontal cortex (n=6) or cerebellum (n=6) and then fixed in 4% paraformaldahyde. LGN explants were labelled with DiI crystals placed at a distance from the cortical or cerebellar border (to avoid direct contamination of the slices with diffused dye). I deliberately made large DiI deposits with the aim of labelling all of the ingrowth, and so obtaining complete and accurate assessments of the innervation patterns. Cortical slices were counterstained with m-phenylene diamine.

After DiI labelling, co-cultured explants were mounted on glass slides and viewed with a confocal microscope. For each co-culture, I sampled the densities of labelled neurites at a range of distances between the LGN explant and the pia or white matter (depending on the orientation of the culture) in two or three tranches equally spaced along the cortical slice. For each tranch, I made montages of photographs for three 1µm thick optical sections, one midway through the depth of the cortical explant, one 5µm above and one 5µm below this plane. Where the intensity of labelling was high (i.e. approaching the LGN explant), the sensitivity of the confocal microscope was reduced to obtain quantifiable images (note that this procedure did not invalidate the analysis since all data were expressed as relative values, see below). Lines of equal length were drawn across each montage parallel to cortical laminae (and perpendicular to geniculate fibres) at intervals of 50µm between the LGN explant and the pia or white matter. The total numbers of neurites crossing each line were counted, as illustrated in Fig. 3.1a; in none of
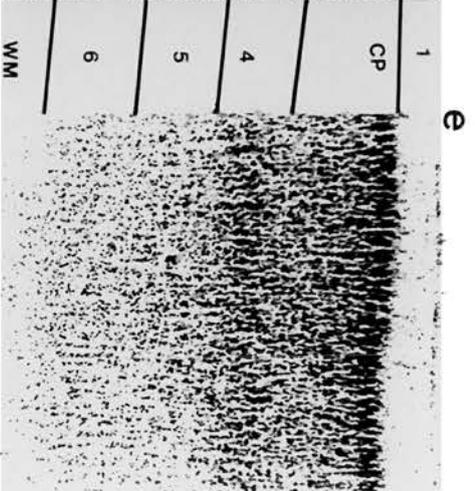
a

LGN

6|WM
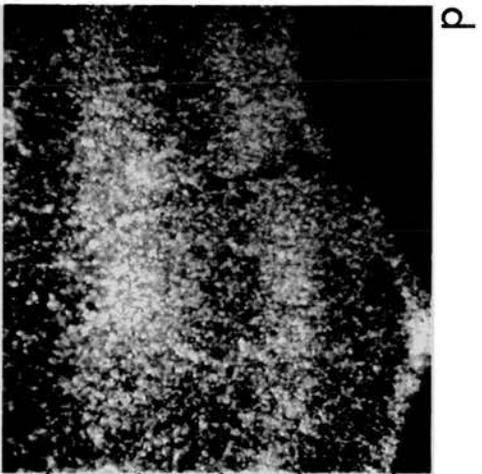4|5
CP

b

LGN

CP
4|5

c

LGN
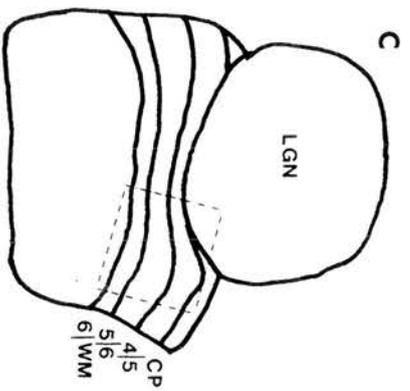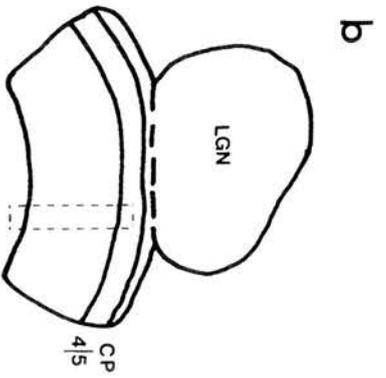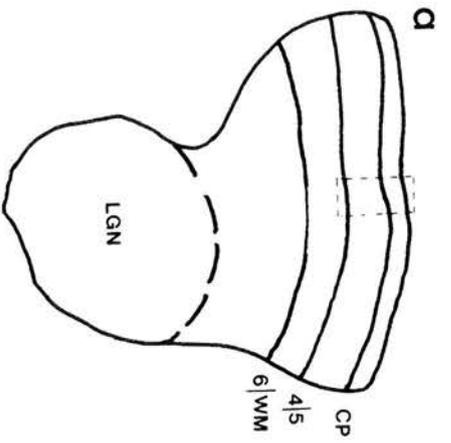
5|6
4|5
6|WM
CP

d

e

WM
6
5
4
CP
1

Fig. 3.3 (a,b,c) Camera lucida drawings illustrating the types of co-culture used to assess cortical innervation: murine LGN explants were cultured against (a) the white matter (WM) or (b,c) the pial surface of slices from visual (a,b) or frontal (c) cortex of P1 kittens. The most obvious laminar borders are marked (CP indicates the dense line of cells at the superficial aspect of the cortical plate, seen in d,e). Broken lines indicate the areas photographed in (d) and Fig. 5 (a-c). (d) Fluorescence photomicrograph of a counterstained explant from kitten frontal cortex; the murine LGN explant (which appears dark due to DiI label) has compressed the layers on the left (see c). (e) Counterstained section of the cortex of a P1 kitten: the most striking borders are between layers 4 and 5, between the superficial aspect of the cortical plate and layer 1 and between layer 6 and the white matter. These are seen clearly in vitro (d). Layers 5 and 6 can be distinguished with care both in vivo and in vitro; the superficial aspect of layer 4 is still forming at this age, as cells continue to migrate into the remnant of the cortical plate to form layers 2 and 3 (Shatz and Luskin, 1986). Scale bar, 250µm.

the experiments did I find large fascicles that could not be resolved into individual neurites. Measurements made by two observers agreed to within 5%. For each depth in each tranche, the highest number recorded (taking into account changes in the sensitivity of the confocal microscope due to changes in the intensity of DiI labelling) was given a value of 1.0 and other values were expressed as fractions of this. Relative values at each 50µm interval at all three depths were averaged for each tranch. To eliminate possible bias, all the quantifications were done before counterstaining to reveal cortical layers.

RESULTS

Growth promotion

Neurite outgrowth from LGN explants cultured alone was sparse (Fig. 3.2a). However, both the density and the length of outgrowth were significantly increased in cultures containing P2 mouse occipital cortex (Fig. 3.2c,d). LGN explants did not grow significantly more when they were co-cultured with mouse liver or with one or three additional mouse LGN explants than when they were cultured alone. Nor was there any increase in the amount of outgrowth from LGN explants grown alone on the collagen substrates that had been conditioned with murine occipital cortex.

The density and length of outgrowth from murine LGN explants were also both significantly increased in the presence of P1 kitten visual cortex (Fig. 3.2b,e,f). When cultured with slices of P1 kitten frontal cortex or medulla (all of equal volume to the slices of visual cortex), murine LGN explants also grew more than when cultured alone but less than when cultured with kitten visual cortex (Fig. 3.2e,f). Murine LGN explants cultured for 3 days in medium conditioned with P1 kitten visual cortex grew more than those in control medium (Fig. 3.2g,h).

Outgrowth was immunoreactive with anti-neurofilament antibody. For this reason, and because the use of DiI revealed that the growing processes ended with growth cones, I was confident that the outgrowth was indeed axonal. In none of my
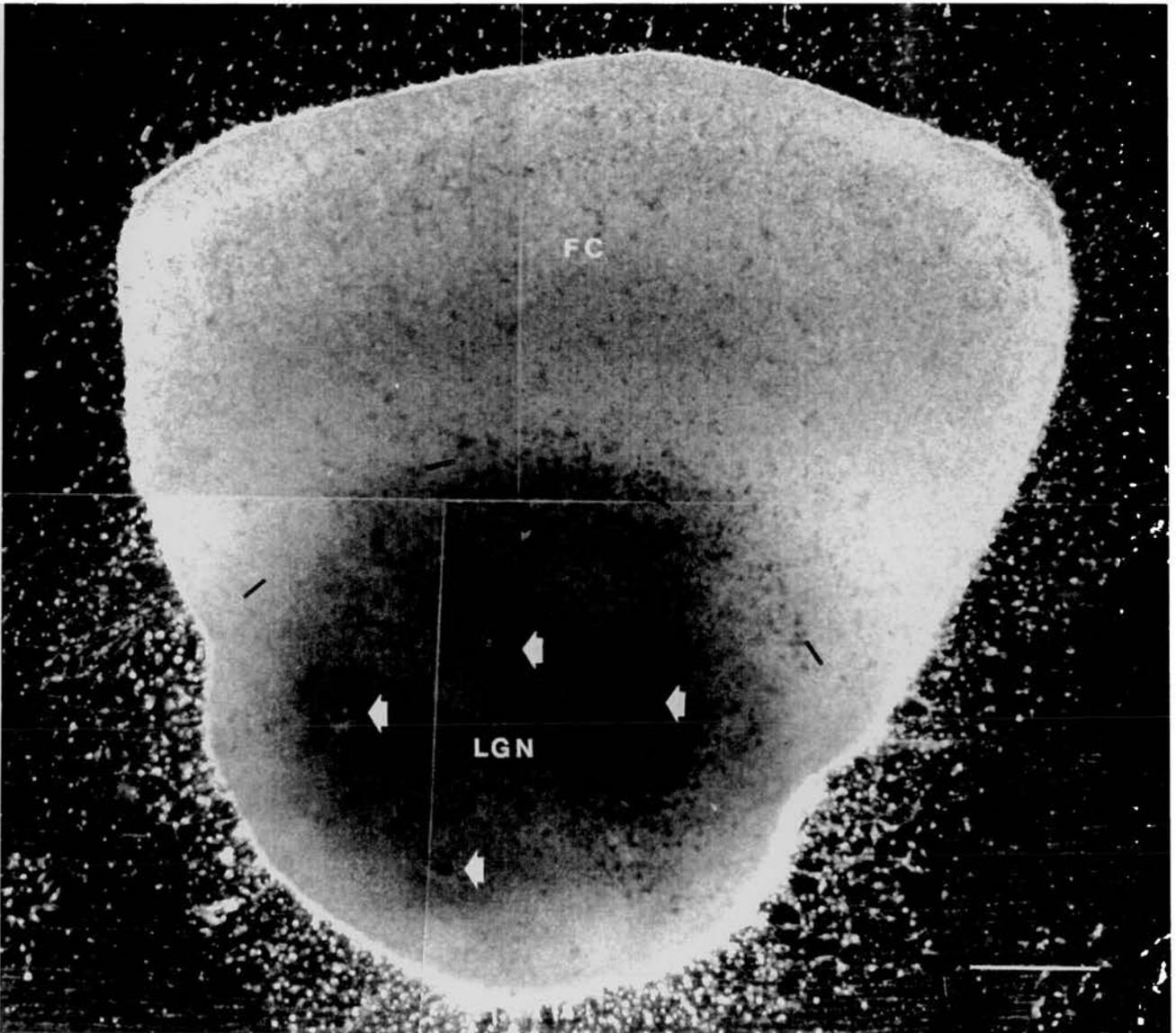
Fig. 3.4 A photomicrograph of an E16 murine LGN explant co-cultured touching a P1
kitten frontal cortical (FC) slice. Dashes indicate the border between the two tissues.
Arrows indicate DiI crystals deliberately placed at a distance from the cortical slice;
where the DiI has spread, it appears as dark smudges. Scale bar, 50μm.

a

—4/5
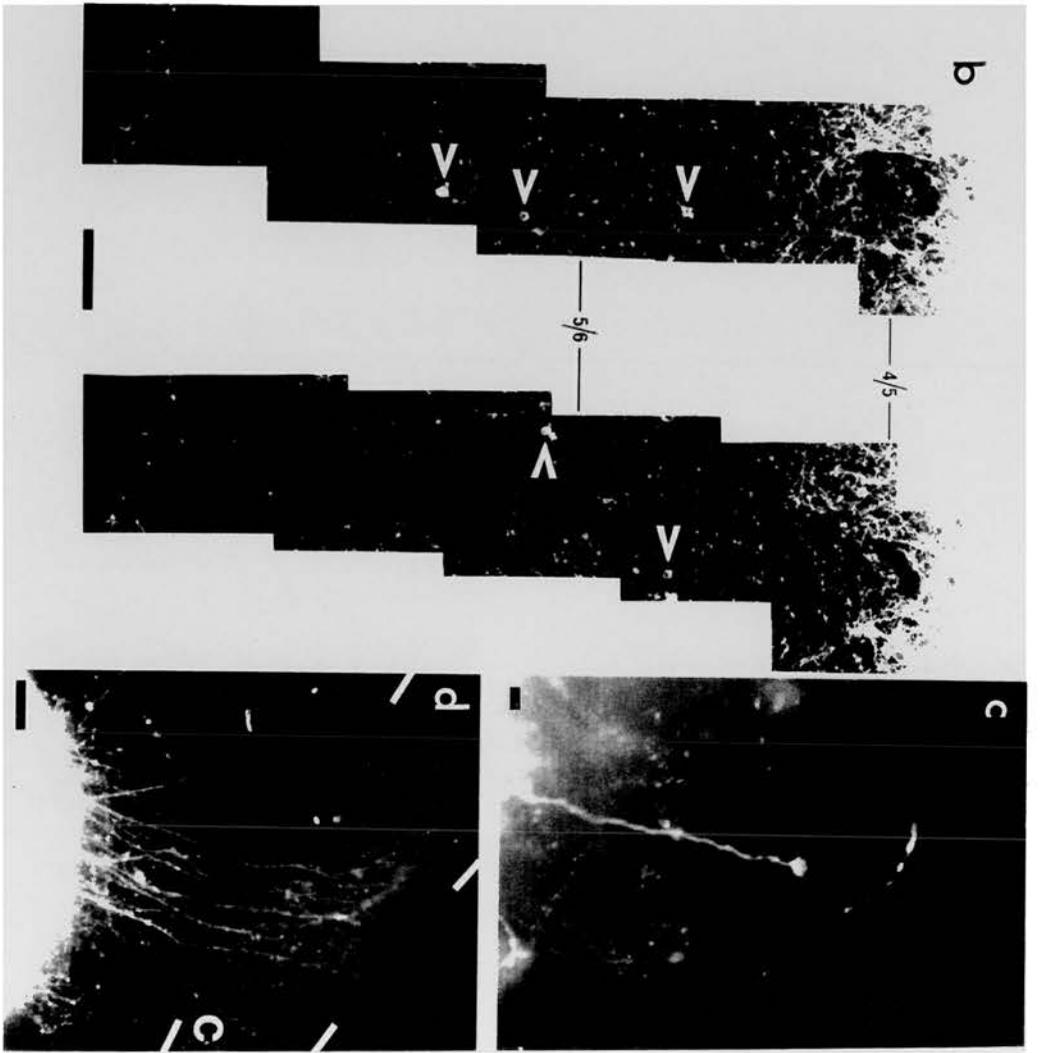
—CP/4

—PIA

b

—5/6—

—4/5—

c

d
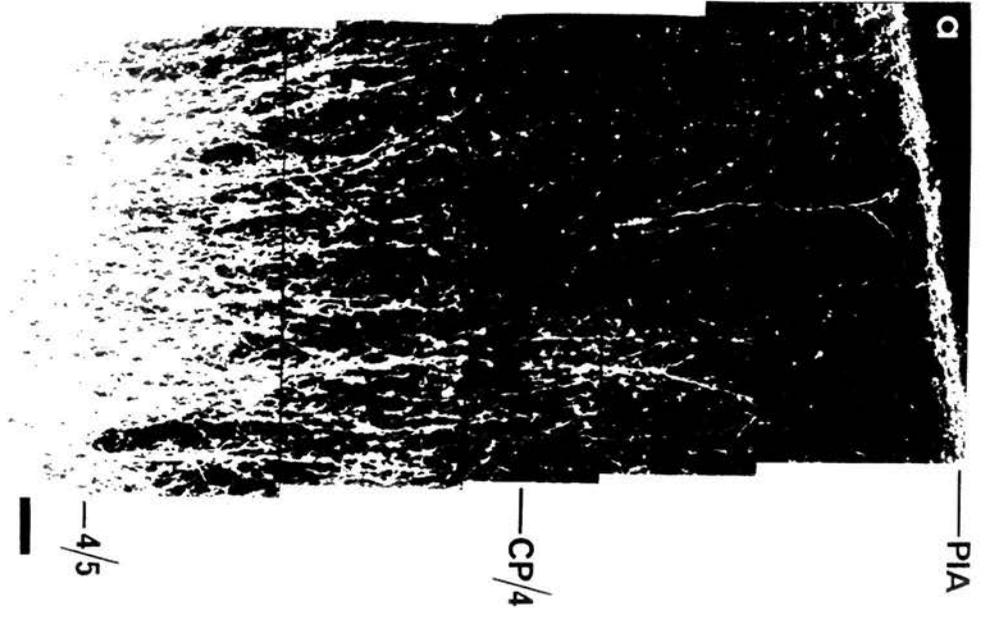
Fig. 3.5 (a) Montage of confocal photomicrographs of DiI labelled neurites from a murine LGN explant: the majority are terminating in layer 4 of a slice of P1 kitten visual cortex after entering through the white matter (this region is outlined in Fig. 3a). CP, cortical plate. (b,c) Two confocal sections from the region outlined in Fig. 3(b), separated in depth by 5µm: most LGN fibres are terminating in layer 4 of a slice of P1 kitten visual cortex after invading through the pia. There are many retrogradely labelled cells in the lower layers (arrowheads). (d) Photomicrograph of a DiI labelled growth cone in a cortical explant. (e) Fibres originating from a murine LGN explant (bright area) entering a slice of P1 kitten cerebellar cortex (marked c). Scale bars: a and d, 50µm; b and c, 100µm; e, 200µm.

experiments was there a significant difference between the amount of growth towards and that away from the co-cultured tissue.

Analysis of Nissl-stained sections revealed that murine LGN explants cultured either alone or with P1 kitten visual cortex were similarly healthy. Although it was clear that some cell death had occurred in both sets of explants (25-27% of cells were pyknotic), the vast majority of cells within these explants survived, suggesting that the cortex does not enhance neurite density indirectly by promoting cell survival, but rather by promoting neurite outgrowth.

Target Recognition

Previous experiments have demonstrated that outgrowth from explants of the embryonic rat LGN terminate selectively in layer 4 of slices taken not only from the visual but also from the frontal cortex of early postnatal rats (Yamamoto et al., 1989; Yamamoto et al., 1992; Molnar and Blakemore, 1991; Bolz et al., 1992; Gotz et al., 1992). By co- culturing murine LGN explants touching murine cortical slices, I confirmed this observation for the mouse. As illustrated in Fig. 3.3, I then co-cultured E16 murine LGN explants touching either the pial or the white matter surface of slices from P1 kitten visual or frontal cortex; as shown in Fig. 3.4, DiI crystals were placed in the LGN explants to label neurite growth (Fig. 3.5), which was then quantified (Fig. 3.6).

Neurites from the murine LGN explants grew freely and without extensive fasciculation through the white matter or pial surface of slices from P1 kitten visual or frontal cortex (Fig. 3.5a,b,c). They grew equally well on co-cultured cerebellar slices from the P1 kitten (Fig. 3.5d). In the cortical slices, the dense cortical plate presented no mechanical barrier to neurites entering through the pial surface. As they entered layer 4 of either visual or frontal cortex from either direction, their density fell precipitously and very few emerged from the other side (Figs. 3.5a,b,c and 3.6). Quantitative analysis suggested that ingrowth entering the visual and frontal cortex through the white matter terminated not only in layer 4 but also in layer 6 (Fig. 3.6a,c); most fibres entering through the pia terminated in or before layer 4, before they could reach layer 6 (Fig. 3.6b) I attributed the precipitous drops in neurite density in specific layers to termination, as

a

Relative neurite number

Visual cortical depth (mm)

PIA
CP
4
5
6
WM & LGN

b

Relative neurite number

Visual cortical depth (mm)

CP & L
4
5
6
WM

c

Relative neurite number

Frontal cortical depth (mm)

PIA
CP
4
5
6
WM & LGN
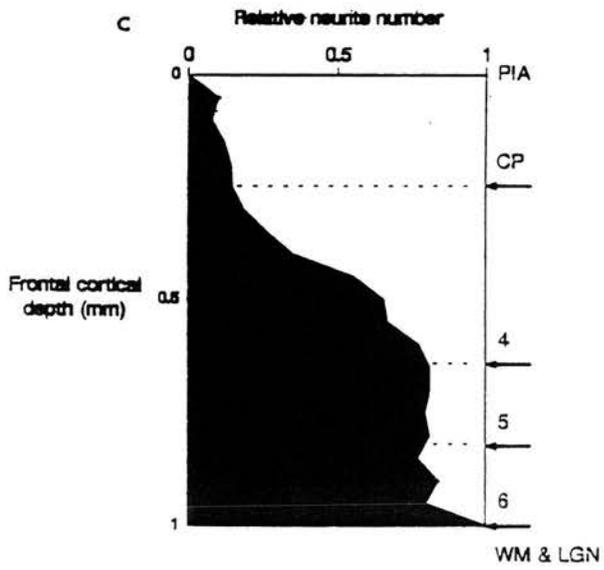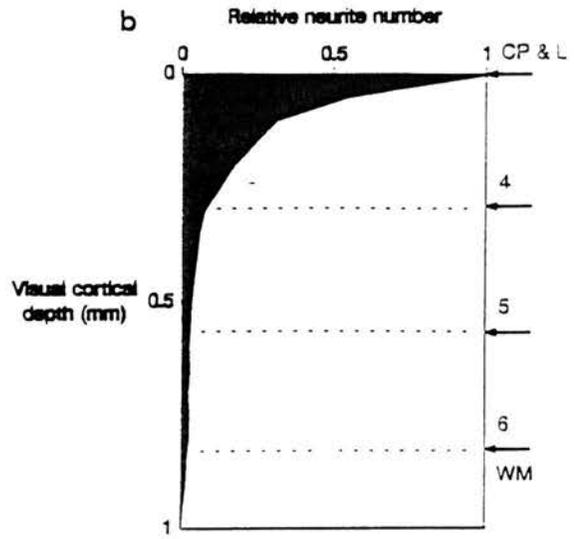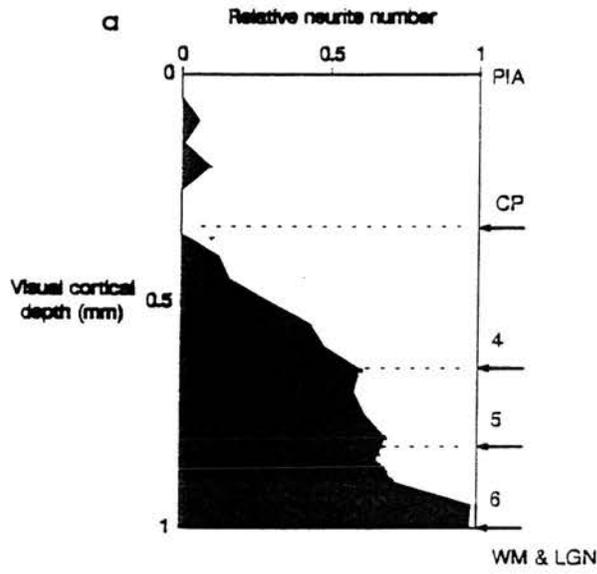
Fig. 3.6 Graphs plot the relative numbers of DiI labelled neurites (averages of three values for each data point) against distance between the edge of the LGN explant and the white matter or pia in slices of (a,b) kitten visual cortex and (c) kitten frontal cortex. LGN fibres entered through the white matter in (a,c) and through the pia in (b). The cortical layers are marked as in Fig. 3(d). In (a) and (b), the slope (rate of change of relative neurite density) is steepest before the 4/5 boarder; note also the sharp decline in layer 6 in (a). In (b), the LGN explant had compressed the pial surface of the kitten visual cortex. CP, cortical plate; WM, white matter.

opposed to the stunting of neurite invasion by premature fixation, for two main reasons. First, most fibres that crossed the pial surface were substantially shorter than those that entered from the white matter, as they encountered layer 4 much sooner (compare the distances travelled by neurites in Figs. 3.3a and 3.5a to those in Figs. 3.3b and 3.5b,c). Thus, although geniculate axons had the potential to grow longer distances, they stopped when they reached layer 4. However, these data suggest that many of the geniculate fibres did not reach layer 4, as the most presipitous drop in outgrowth occured before layer 4. This may have been a result of the pial barrier. Previous studies have shown that the pia can act as a barrier to geniculate ingrowth (Molnar and Blakemore, 1991). Furthermore, growth cones (Fig. 3.5d) were rarely observed in layers 4 and 6, although a few were present on neurites that had grown beyond these layers.

In vivo, layer 6 is the major source of corticogeniculate projections. In culture, reciprocal connections develop from both layers 5 and 6 of the rodent cortex to the rodent LGN (Yamamoto et al., 1992; Novak and Bolz, 1993). Similarly, I found retrogradely labelled corticogeniculate cells only in layers 5 and 6 in kitten frontal and visual cortex when LGN explants were placed against the pia (Fig. 3.5b,c); these were not visible when there was geniculocortical ingrowth from the white matter, but they may have been present and hidden by the profusion of axons. Thus, only the lower layers of the kitten cortex can locate and send reciprocal fibres into the murine LGN, even when it lies in an inappropriate position.

DISCUSSION

I have shown previously that, in the rodent, the cortex has a growth-promoting effect on the embryonic LGN in culture (Lotto and Price, 1994; Rennie et al., 1994). I confirmed this in the present study. Furthermore, I confirmed that, as in the rat (Yamamoto et al., 1989; Yamamoto et al., 1992; Molnar and Blakemore, 1991; Bolz et al., 1992; Gotz et al., 1992), murine geniculate axons stop in layer 4 of co-cultured murine cortical slices when entering through either the white matter or pial side. My new

findings, from the cross-species co-cultures, suggest that the growth promoting effects of the cortex on the LGN and the termination of geniculate axons in cortical layer 4 are mediated by molecules that are conserved between divergent species of mammal. The orders Carnivora and Rodentia diverged from a common ancestor about 100 million years ago (Novacek, 1992). Other mammals, including primates, probably also arose from the same common ancestor (Novacek, 1992) and it is possible that the growth promoting factor(s) and termination signal(s) examined in this study are even more widely conserved. Indeed, my preliminary experiments with co-cultures of mouse and marmoset tissue indicate that the growth-promoting factor(s) may be the same in primates (unpublished observations).

There are many interspecies similarities in cortical organisation and development, but there are also a vast number of differences and specialisations. For example, the organisation of layer 4 is much more complex in higher mammals (e.g. cats and primates) than in rodents. Despite the increased complexity of this structure in higher mammals, my results suggest that the molecules controlling its innervation have been conserved throughout evolution. Therefore, I can now identify the molecules responsible for promoting the growth of geniculate axons and target recognition in layer 4 with the knowledge that results obtained in less advanced species are very likely applicable to higher mammals, including humans. My findings will provide important clues as I study the identity of these molecules. Thus, it is most likely that the promotion of geniculate growth is mediated by secreted diffusible molecules, that may be present throughout the developing cortex and other non-thalamic regions of the central nervous system, and that are sufficiently conserved to be functional in a range of mammals. In the first instance, candidates from among the known neurotrophic factors are worthy of consideration; for example, brain derived neurotrophic factor is an interesting possibility since its mRNA levels in the cortex are affected by visual activity (Castren et al., 1993). Regarding the termination signal for geniculate axons, conserved molecules that are concentrated in specific layers are candidates. For example, neurotransmitter receptors may play a role; they have been shown to influence neurite growth in other systems (Lipton and Kater,

1989). A recent study by Gotz et al. (1992) has indicated that the layer 4 stop-signal is cell membrane-bound.

In my present study of the growth-promoting effect of brain tissues on the LGN, I have concentrated on evaluating the possibility that the in vitro phenomenon is mediated by conserved molecules. In this work I did not set out to assess the in vivo significance of the growth-promotion. Nevertheless, my findings do allow me to speculate on this issue. It is clear from this work and from my previous studies (Lotto and Price, 1994; Rennie et al., 1994) that the diffusible factors involved are not released only from the cortical targets of the LGN but also from other regions of the cortex and subcortical structures. It is worth noting that I obtained different effects with frontal and occipital cortices from the cat. Although this may be evidence for a chemoaffinity gradient across the cortex necessary to establish proper thalamocortical connectivity, this may also be a consequence of the rostrocaudal gradient of cortical development. While it is clear that the stimulation of geniculate outgrowth can not be achieved by co-culture with any tissue (neither liver nor additional LGNs had any effect), there is little evidence of a specific interaction between visual cortex and LGN. This makes it unlikely that the factors play a role in guiding geniculate axons towards visual cortex. In support of this, I have carried out experiments in collagen gels (that maintain gradients of diffusible molecules) and these failed to demonstrate any chemotropic guidance of geniculocortical fibres by their targets (R.B.L. and D.J.P., unpublished). Rather, these factors may be important in enhancing the growth of fibres that are guided by some other mechanism, either before or after they reach their targets.

Previous studies with rodent co-cultures showed that although geniculate axons are capable of growing into any cortical area, they do not grow well into cerebellar explants (Molnar and Blakemore, 1991). Interestingly, axons from murine LGN explants are able to grow freely in P1 kitten cerebellar slices. This suggests that the inhibition of geniculate fibres on cerebellar explants seen in the rodent may be mediated by factors that are not conserved between species; either the feline cerebellum expresses different inhibitory molecules or the expression of such molecules does not occur in the cat.

In my study, I found that only cells from layers 5 and 6 of the kitten's cortex grew into murine LGN explants. These deep layer cells were capable of finding the murine LGN even when it was cultured on the pial side of the cortical slice. Similar findings have been made in culture for the rat's cortex and LGN (Lotto and Price, 1994). It is not yet clear how this organisation is achieved; it has been proposed that the LGN releases tropic factors that guide corticogeniculate fibres to their target (Novak and Bolz, 1993), although my recent findings from cultures in collagen gels failed to support this hypothesis (R.B.L. and D.J.P., submitted). Why layer 5 neurons project to the thalamus in vitro but not in vivo is not clear. It may be that the mechanism diverting layer 5 axons away from the thalamus towards the colliculous and peduncles is not present in culture. Whatever the mechanism that underlies corticothalamic target recognition, my experiments indicate that the factors involved are also conserved between the rodent and the cat.

In the retinotectal and other non-visual systems, previous studies using cross-species culture methods or xenografting have demonstrated that developmentally important cues are conserved in different species (Kamo et al., 1986; Godement and Bonhoeffer, 1989; Kitchigina and Vinogradova, 1989; Hantraye et al., 1992; Bastmeyer and Stuermer, 1993). To my knowledge, this is the first study to address such issues in the development of connections between the thalamus and cortex. I have provided evidence that some, but not all, interactions of the developing LGN with other tissues are directed by conserved molecules.

CHAPTER 4:

THALAMIC NEURONES BECOME DEPENDENT ON CORTICALLY-DERIVED GROWTH FACTORS FOR THEIR SURVIVAL AT THE TIME THEY INNERVATE THE CORTEX.

ABSTRACT

The major afferent input from the thalamus to the cerebral cortex develops during the late stages of embryogenesis and early postnatal life in the mouse; the first of these fibres reach the developing cortex at embryonic day 16. In these experiments I used in vitro techniques to study whether the survival of murine dorsal thalamic cells of different embryonic and postnatal ages is dependent on diffusible trophic factors released from the cortex. I confirmed my previous observation that most cells in isolated organotypic thalamic explants from embryonic day 15 mice survived when cultured in serum-free medium for up to 3 days. Increasing the age of the isolated organotypic thalamic explants from embryonic day 15 to postnatal day 4 increased the incidence of cell death within them. When thalamic cells were dissociated and cultured under similar conditions, they almost all survived provided that plating density was around 60,000 cells cm $^{-2}$; at plating densities of 40,000 or less, very few or none of these cells survived, while very high plating densities impaired the survival of about 50% of the cells. Under identical culture conditions, and at comparable plating densities almost all dissociated embryonic day 17 and postnatal day 2 thalamic cells became apoptotic. These findings indicate a marked reduction in the ability of thalamic cells to survive in isolation as they age.

Dissociated embryonic day 17 and postnatal day 2 thalamic cells were then either co-cultured with 2, 4, or 8 slices of age-matched posterior cortex or cultured in medium pre-conditioned with these slices. These conditions promoted the survival of significant numbers of both embryonic day 17 and postnatal day 2 thalamic neurones (labelled with MAP-2 antibody). This effect increased as the number of co-cultured cortical slices increased. Heat-treating the pre-conditioned medium reduced its survival promoting

effect, suggesting that the effect was mediated at least in part by proteins. Using time-lapse video microscopy, I demonstrated that mitosis was very rare in these cultures, confirming that the cortex stimulated a trophic rather than a mitotic response among the thalamic cells. Based on these results, I propose that, in vivo, the survival of thalamic cells that have yet to innervate their target is ensured by interactions with other thalamic cells. As thalamocortical connections become established, thalamic cells develop a requirement for additional support that is provided to at least some of them by the cortex.

INTRODUCTION

Results from the application of in vitro techniques to the developing peripheral nervous system have suggested that, in vivo, target-derived diffusible factors influence afferent development, including the competitive processes that underlie axonal refinement and organisation. Competition may occur where afferent neurones are dependent, for their survival, on target-derived factors that are produced in limited supply (Purves, 1988). In support of this hypothesis, experiments have demonstrated that many sensory neurones become dependent on neurotrophins produced by their targets for their survival at the time when they are organising their connections within their targets. Before this time, afferent survival is independent from neurotrophins (review by Davies, 1994).

As has been suggested for the peripheral nervous system, it is conceivable that within the central nervous system the specificity and organisation of cerebral cortical afferents also results from a competitive process in which thalamic afferents compete for trophic factors produced by cortical neurones. Consistent with this hypothesis is the finding that many of the identified trophic factors, including neurotrophins such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) are produced by cortical neurones (Maisonpierre et al., 1990; Castren et al., 1993). Furthermore, the infusion of high levels of NGF into the telencephalic vesicles during monocular deprivation blocks the shift of ocular dominance columns that normally result from this procedure (Domenici et al., 1991; Domenici et al., 1992; Maffei et al., 1992). Since the effects of monocular deprivation are thought to result from the competitive advantage of inputs from the normal eye over those from the deprived eye, one explanation for these effects of NGF infusions is that competition is abolished by the abnormal abundance of a vital resource. However, it is unclear if, and when, thalamic neurones develop a dependency on cortically-derived growth factors for their survival.

Previous experiments on postnatal animals have demonstrated that thalamic neurones are dependent on the integrity of their connections with the cortex for their

survival since they rapidly degenerate after axotomy (see review by Finlay and Pallas, 1989). This death may result from the separation of the thalamic neurones from an important source of trophic factors in the cortex (Cunningham et al., 1987; Hisanaga and Sharp, 1990); any trophic factors involved have not been identified. The aims of my current experiments were to seek evidence for a trophic effect of the cortex on embryonic and postnatal thalamic cells in culture, and to determine whether thalamic cells of different ages have different requirements for cortically-derived growth factors (CDGFs).

MATERIALS AND METHODS

Dissections

To obtain prenatal dorsal thalamic and posterior cortical tissue, pregnant female BALB/c mice were anaesthetised with urethane (0.3ml of a 25% solution in normal saline, i.p.) on embryonic day 15 (E15), E16, E17 or E19; the fetal mice were removed by caesarean section and decapitated. Postnatal day 2 (P2) and P4 mice were also decapitated. The brains from all mice were removed in oxygenated Earle's Balanced Salt Solution (EBSS: Sigma) on ice, and the cerebral hemispheres and explants from the dorsal thalamus were dissected as described by Rennie et al., (1994). Thalamic explants were either cultured intact or dissociated (see below); cerebral hemispheres were sliced parasagittally at 350μm and the posterior half of each section was placed in culture.

Culture paradigms

Two types of culture were utilised: organotypic (n=20) and dissociated (n=107). Organotypic cultures consisted of either E15, E16, E17, E19, P2 or P4 thalamic explants cultured alone, or P2 thalamic explants co-cultured either touching or 1mm away from a P2 cortical slice, on collagen-coated membranes (Costar, Transwell-COL) suspended in serum free-medium (Romijn et al., 1984). After three days, the organotypic explants were fixed with 4% paraformaldahyde in phosphate buffer.

For dissociated cultures, thalamic explants from E15, E17 or P2 mice were dissociated with papain for 50, 60, and 70 minutes respectively at a neutral pH (following

the procedure described by the manufacturers: Worthington Biochemical dissociation kit). The densities of the cell suspensions were determined with a hemocytometer. Trypan blue was excluded from all cells, confirming their initial viability on entry to culture. Aliquots of cells, whose volumes were chosen to provide appropriate plating densities when mixed with culture medium (final volume of 300 - 400μl per well), were placed on the bottom of plastic culture wells or glass coverslips that had been pre-coated with poly-L-lysine (15 minutes at 4μg cm^{-2}). Porous collagen-coated membranes (Costar, Transwell-COL) were suspended 1mm above the bottom of each of these wells by means of plastic inserts. The dissociated thalamic cells were then cultured for 2 days either alone (at various densities) or with varying numbers of cortical slices or thalamic explants on the collagen-coated membranes. With this arrangement, the overlying explants were always kept separated from the dissociated thalamic cells by a microporous membrane with a pore size of 3μm. After two days, the cells were fixed in methanol at -20°C for immunocytochemistry (see below).

At no point in any of the experiments were cells or explants exposed to serum. All cultures were conducted at 37°C with 5% CO_2 and 95% humidity. The in vitro development of dissociated thalamic cultures was recorded using time-lapse videomicroscopy on a Leica microscope housed in an incubator whose temperature, humidity and CO_2 level could be regulated at the values stated above.

Heat-treating of conditioned medium

To assess whether CDGFs were likely proteinaceous, E15 or P2 cortical slices (3 per well) were cultured on collagen-coated membranes in serum-free medium for 4 days. This preconditioned medium was either added directly to dissociated P2 thalamic cells, or was first heated to 60°C for 60 minutes. The thalamic cells were then cultured for two days as described above, and fixed in methanol.

Characterisation of cell death in dissociated cultures

In most of the dissociated cultures, at least some cells died and became phase-bright. To characterise these cells, some cultures were fixed after 24 hours in 90%

ethanol and 10% formalin for 10 minutes at 4°C. After washing in phosphate buffer saline (PBS) for five minutes, the cells were incubated at room temperature in acridine orange ($1\mu g\ ml^{-1}$) for up to 10 minutes. After further washes in PBS, the cells were viewed with fluorescence or confocal microscopy.

Immunocytochemistry for microtuble associated protein-2 (MAP2)

Dissociated thalamic cells that had been fixed in methanol at -20°C for 10 minutes were washed PBS and incubated overnight at 4°C in PBS containing 2.0% Triton-X with monoclonal anti-mouse MAP2 antibodies (1:300, Sigma). Cells were then washed several times with PBS, and incubated overnight at 4°C in PBS containing 2.0% Triton-X with monoclonal biotinylated anti-mouse antibodies (1:200, Vector Laboratories). Cells were again washed in PBS and incubated at room temperature for 2 hours in avidin and biotinylated horseradish peroxidase (HRP) solution (*elite* ABC kit: Vector Laboratories). HRP was reacted with diaminobenzidine and H_2O_2. For each experiment, controls were conducted by eliminating the primary antibody from the first incubation.

Electron microscopy (EM) of thalamic explants prior to culture

The postnatal thalamus comprises a heterogeneous population of neuronal and non-neuronal cell types. In this study, I was specifically interested in the survival of thalamic neurones, and so it was important to have estimates of the proportions of the different cell types that entered the dissociated cultures. I found that the most reliable method for distinguishing different cell types in the intact pre- and postnatal thalamus was electron microscopy. I did not rely on immunohistochemistry with antibodies such as MAP2 since I observed that, in the late embryonic brain, many young neurones did not label. This was particularly obvious in the cerebral cortex; for example, at E17 cells in the deep cortical layers were expressing MAP2, while those that had recently entered the superficial layers remained unlabeled, even though most would have been neuronal. This uncertainty over exactly when specific cell types begin to express distinguishing markers at detectable levels in vivo, coupled with the relative ease of distinguishing morphological differences between different cell types at the ultrastructural level, convinced me that electron microscopy would provide more accurate data. E15, E17 and P2 mice were

decapitated, dorsal thalamic explants were carefully obtained and fixed acutely in 2% paraformaldahyde, 2.5% glutaraldehyde and 15 ml/100ml saturated picric acid, and post-fixed with 1% osmic acid. After washing in PBS, these explants were embedded in white acrylic resin (medium grade) and sectioned coronally to 0.03 - 0.05 μm (silver/pale gold in colour). Sections collected on 200 mesh copper grids with a support film of Formvar and were stained in uranyl acetate and lead citrate. Five randomly selected sections from each of 2 thalamic explants of each age were viewed and photographed at low (x2800) and high (x9000) magnifications.

Neurones were easily recognised at all ages by their large, round nuclei containing prominent nucleoli and occupying most of the perikaryon, numerous free ribosomes and prominent granular endoplasmic reticulum. Non-neuronal cells were more frequent in the sections from the older mice, and were either endothelial cells or glial cells; the latter were distinguished by their irregular shaped nuclei, often with clumped nuclear chromatin, and the lack of granular endoplasmic reticulum and free ribosomes (Peters et al., 1970). The total numbers of neurones, glia and endothelial cells in all sections at each age were counted, and the proportions of each types were obtained. Since neurones were larger than glial cells, and glial cells were larger than endothelial cells, the chance of encountering neurones in my sections was greater than that of finding the smaller glial and endothelial cells. Therefore, I corrected the proportions according to the following equation (James, 1977):

$$N_v = N_A / D$$

Where N_v is the number of neurones, glia or endothelial cells per unit test volume of tissue, N_A is the number of each of these cells measured in two dimensions from the test sections and D is the diameter of each cell type. For each cell type, D is proportional to the mean of a population of profile diameters (James, 1977); to obtain this value I measured the diameter of 60 of each cell type at each age. Using this equation, I adjusted the percentages of neurones (downwards) and non-neuronal cells (upwards).

Thalamic cell density in vivo

To interpret the data from organotypic explants, I needed a measure of the changes in thalamic cell density with age in vivo. E15 and P2 brains were fixed in 4% paraformaldehyde in phosphate buffer, allowed to equilibrate in phosphate buffer containing 10% sucrose, and sectioned at 10μm on a freezing microtome. The sections were counterstained with cresyl violet and coversliped. The total cell density was quantified using the methods described below for in vitro thalamic explants.

Quantification

To assess the viability of organotypic thalamic explants, that had been cultured alone for three days, four explants of each age tested (E15, E16, E17, E19, and P2) were fixed, embedded in wax, sectioned at 10μm, and Nissl stained. For each of five equally spaced sections taken from approximately the middle of each explant, the density of pyknotic cells (dead or dying cells) was counted in fifteen 1.1×10^3 μm^3 windows. In addition to this quantitative assessment of viability, I also qualitatively assessed the ratio of pyknotic to non-pyknotic cells within each window. Each window was examined and ranked on a scale from 1 to 4 based on the volume occupied by both pyknotic and non-pyknotic cells. Scale: 1, 0-25% death; 2, 26-50% death; 3, 51-75% death; 4, 76-100% death.

Dissociated cultures: after 2 days in culture, E15, E17 and P2 posterior thalamic cells were fixed in methanol, and reacted with MAP2 as described above. MAP2 labelled neurones were counted in forty 0.11mm^2 windows (laid across the middle of the well from one side to the other) for each culture.

Data for all experiments were analysed and their significance determined with a Student's t-test.

Fig. 4.1 Photomicrographs of (a) E15, (b) E17, and (c) P2 thalamic explants taken with an electron microscope. These figures illustrate that cell density decreased between E15 and P2. Furthermore, the ratio of neurones to non-neurones also decreases between these two ages. Scale bar, 35µm.
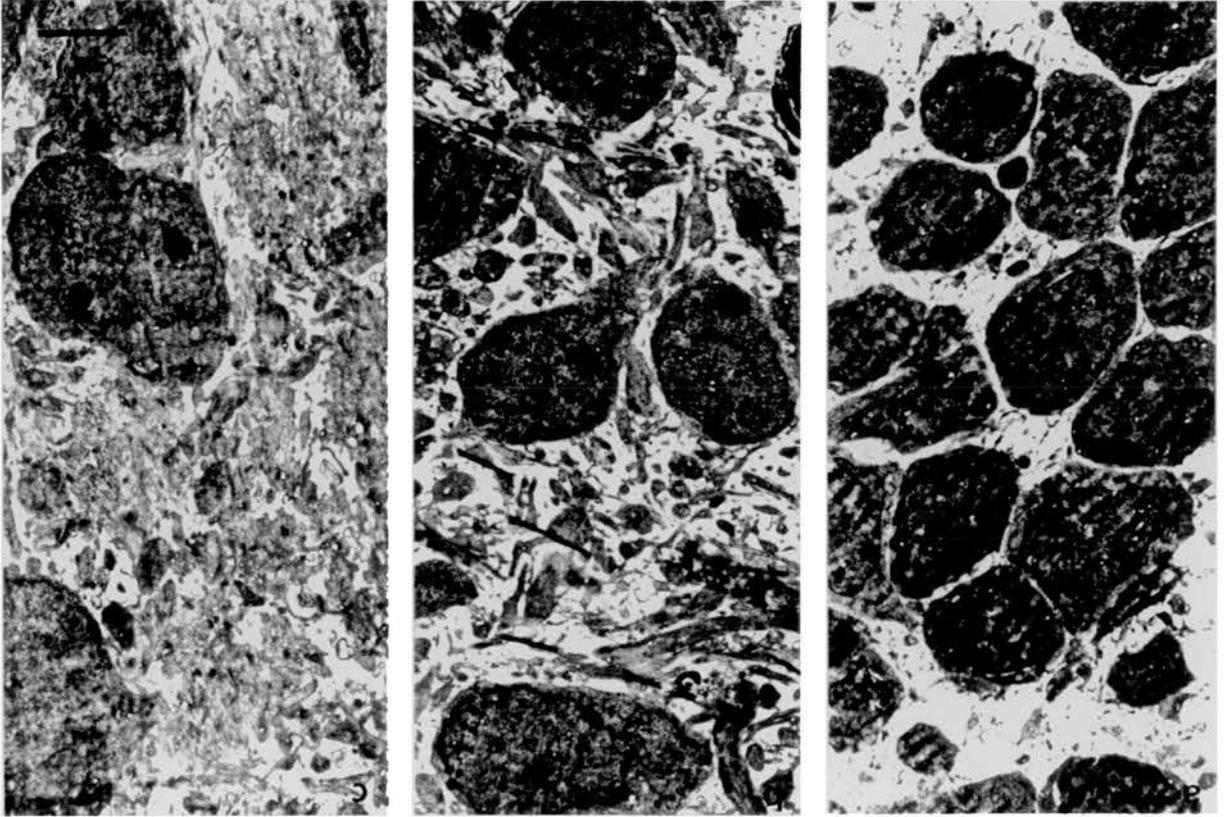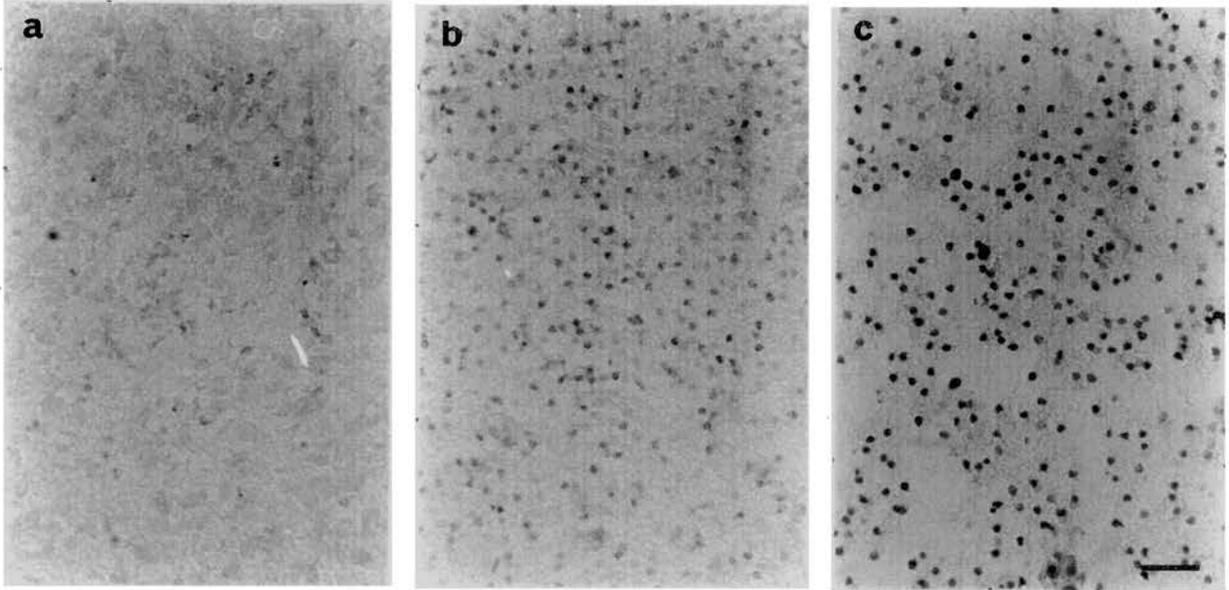
Fig. 4.2 Photomicrographs of Nissl labelled preparations from (a) E15 and (b) P2 cortical explants after 3 days in serum-free culture. While few cells became pyknotic in cultured E15 explants, almost all the cells at P2 did. Scale bar: 35μm

RESULTS

Observations on the developing thalamus in vivo

For the correct interpretation of the in vitro results described in this paper, it was necessary to characterise certain aspects of thalamic tissue at different in vivo ages, before it was placed into culture. In particular, it was essential to know the ratio of neurones to non-neurones at E15, E17, and P2, as well as the overall change in cell density that occurs during this period, the latter of which is illustrated in Fig. 4.1. Using the light microscope, I determined that cell density decreased from 4184 cells per $0.01mm^3$ ($\pm$ 139s.e.m.) at E15 to 2482 cells per $0.01mm^3$ ($\pm$ 127s.e.m.) at P2. In other words, there was a total decrease by 41% of density between these ages. At E17, the total percentage of neurones decreases to 89.7% (Fig. 4.1b), with a further decrease, at P2, to 66.5% from E15 (Fig. 4.1c).

Organotypic explants cultures

I obtained organotypic thalamic explants from pre- and post-natal mice of various ages and then cultured them (n=24) in isolation for three days in serum-free medium to determine how well they would survive without the normal influences via their efferents and afferents. My first measure of survival involved counting densities of pyknotic cells in the explants; this is an easy assessment to make accurately, since pyknotic cells are very obvious in Nissl stained material (Fig. 4.2), and is readily interpreted since, as I have shown before (Rennie et al., 1994), there are very few mitoses and negligible clearance of pyknotic cells from thalamic explants cultured for 3 days. The density of pyknotic cells appearing within the cultured explants increased with the explant's age (Fig. 4.2 and 4.3). The most pronounced increase occurred embryonically, between E15 and E17: pyknotic cell density rose approximately 5 fold from 580 cells mm^{-3} at E15 (Fig. 4.2a and 4.3b) to 3027 cells mm^{-3} at E17 (Fig. 4.2b). At P2, the density of pyknosis again increased by another 281 cells to 3308 cells mm^{-3}.
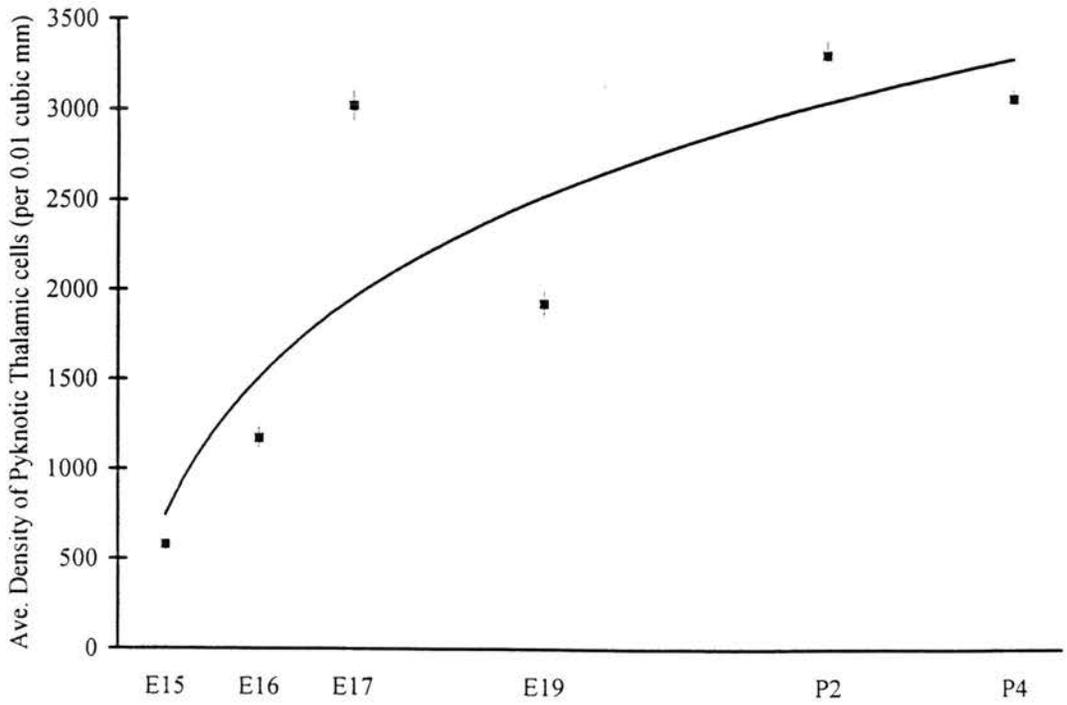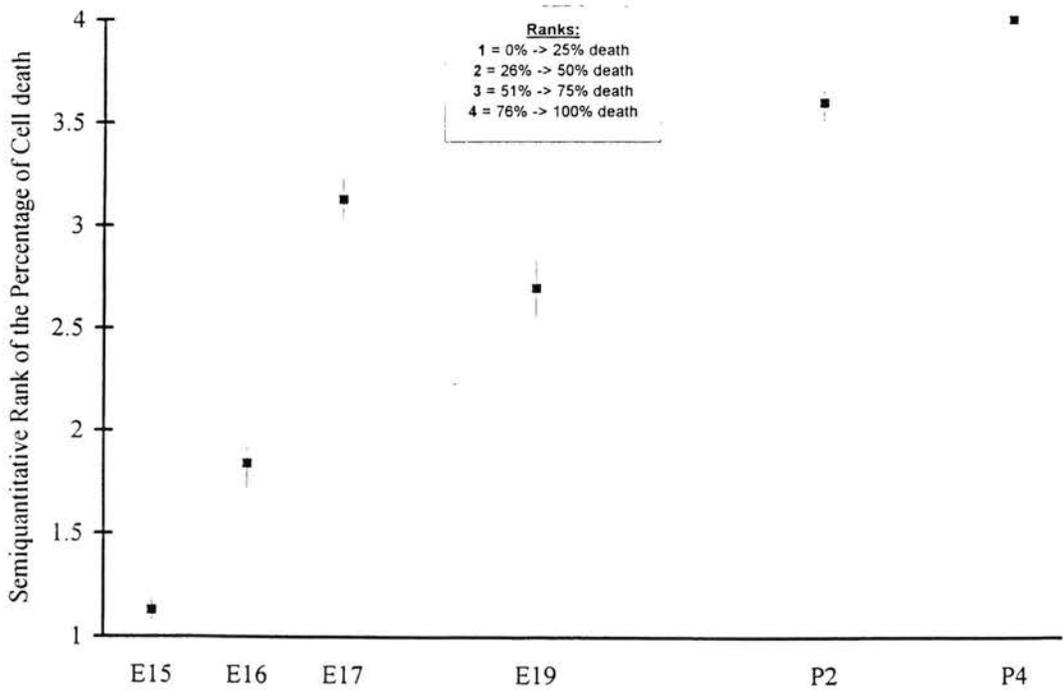
Fig. 4.3 (a) Quantification and (b) semiquantification of the density of pyknotic cells in thalamic explants of different ages after 3 days in culture. The density of pyknosis increased significantly after E15 (p< 0.001).

In these explants, the distribution of pyknotic cells and live cells was not continuous. Rather, at most ages (other than P4), I observed clusters of pyknotic and non-pyknotic cells dispersed throughout the explants. After E15, the volume of the "dead cell clusters" increased in size at the expense of the volume occupied by the "viable cell clusters". Though it was straight forward differentiating between different pyknotic cells, differentiating between different viable cells proved to be much more difficult. Therefore, rather than quantifying the density of viable cells, as I did for pyknotic cells described above, each section was examined and ranked on a scale from 1 to 4 based on the volume occupied by both pyknotic and non-pyknotic cell clusters. Unlike the pure quantification of pyknotic cells, this latter method of analyses takes into consideration the change in cell density that occurs between E15 and P2. As shown in Fig. 4.3b, the percentage of pyknotic cells was less than 25% of the explant. At E17, the percentage increased to 75%, with a further increase to 95% of the cells within P2 cultured explants (Fig. 4.2b & 4.3b). At P4, 100% of the cells became pyknotic.

P2 thalamic explants were then co-cultured either touching (n=8) or 1mm away from (n=8) cortical slices (their major target), to assess whether the presence of the cortex would reduce the incidence of pyknosis within the thalamus. When cultured at a distance, there was no discernible reduction in the density of pyknotic cells as compared to isolated cultures, i.e. approximately 95% of the cells became pyknotic. However, when I plated thalamic explants touching the cortex, a cluster of viable cells remained at the edge of the explant, near to the cortical slice, in 50% of the cases. This observation suggested to me that it was possible for the cortex to rescue cells in the postnatal thalamus, but that factors such as the concentration of trophic molecules, their accessibility to thalamic cells, or direct intercellular contacts, might be important for the effect to manifest itself. Therefore, I pursued this finding with dissociated cell co-cultures.

Dissociated cultures studied in real time

The development of all dissociated cell cultures were recorded with time-lapse videomicroscopy which enabled the following important observations to be made. (i) After 3,586 cell hours of recording, I observed only 4 cases of mitosis: 2 with the cortex