Development Of The Thalamocortical System.

by Gillian Magowan.

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DISCLAIMER

All of the experiments presented in this thesis were performed by me (Gillian Magowan) alone under the direct supervision of Dr. David Price and secondary supervision of Mayank Dutia. Katy Gillies provided some assistance with the histology and Dr. Raimundo Sabater provided assistance with cell counting in Chapter 5.

Gillian Magowan.
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ABSTRACT

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

Development Of The Thalamocortical System.

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The immense complexity of the brain makes questions about its development especially challenging. One fundamental problem is how projections from one part of the brain specifically target and organise in other parts of the brain?

The development of the nervous system is continuous, and neurons that constitute the nervous system proceed through specific developmental stages. These stages include: birth, differentiation, migration, the formation of efferent and afferent connections, and the organisation of these connections based on exogenous and endogenous cues. This thesis focuses on the development of the thalamocortical system during the late embryonic and early postnatal phase, a time when many efferent and afferent connections are forming between these two structures.

Tissue culture was initially created by Ross G. Harrison in 1907, although it has advanced considerably since the pioneering work of Harrison it is still an integral part of neuroscience today. Three different culture techniques are exploited in this thesis in an attempt to investigate thalamocortical development: (i) co-cultures of organotypic explants on a collagen substrate (organotypic cultures have the advantage of maintaining *in vivo* connectivity in culture) (ii) Isolated cultures of embryonic thalamus on a collagen substrate (enables the independent growth and survival of the thalamus to be monitored) (iii) Dissociated cultures of thalamus combined with time-lapse microscopy (enables the growth of individual thalamic neurones to be monitored). These culture techniques were also combined with DiI staining techniques (to label neurite outgrowth), histological methods and immunocytochemistry. Combining these techniques enabled me to examine: the

viability of cultures and to investigate the presence of specific enzymes (e.g. Nitric Oxide Synthase).

These experiments yielded the following results. (i) Several neurotransmitters may be involved in regulating thalamic outgrowth and survival but do not appear to play a crucial role in target selection. A wide range of neurotransmitter antagonists failed to block the ability of E15 thalamic axons to recognise their target cells in cortical layer 4, although several of these antagonists inhibited thalamic outgrowth. (ii) Addition of APV (NMDA antagonist) to thalamocortical co-cultures prevented thalamic axons from terminating within their target layer 4. (iii) An optimal level of activity appears crucial for generating appropriate signals for thalamic ingrowth and termination within layer 4. (iv) Nitric Oxide (NO), a novel neurotransmitter, is present in both the developing thalamus and cortex *in vivo* and is also expressed within the thalamocortical tracts which connect these two structures. (v) Growth cones of dissociated embryonic thalamic neurons are also responsive to added NO *in vitro* providing evidence that NO is involved in regulating thalamocortical development. (vi) The survival and growth of the late embryonic thalamus can occur without external influences and this mechanism becomes increasingly reliant on neural activity for its maintenance as it ages. Although after birth this endogenous control becomes ineffective. (vii) Glutamate can increase survival of the embryonic thalamus in a dose dependent manner although by E19 it has no effect. (viii) Both the NMDA and Kainate/AMPA receptor subtypes appear to be involved in mediating glutamate induced cell survival. I suggest that two different cell populations (i.e. interneurons and projection neurons) may express different glutamate receptor subtypes.

CONTENTS

CHAPTER 1:
GENERAL INTRODUCTION

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The immense complexity of the brain makes questions about its development especially challenging. One fundamental problem is how projections from one part of the brain specifically target and then organise in other parts of the brain.

The first clue as to how such an intricate system develops was provided at the turn of the century by Ramón y Cajal, who discovered neurons, the synapses that allow communication between them and the growth cones that hook them up. Ever since, neurobiologists have struggled to understand how the nervous system gets wired up with such remarkable precision. Experiments over the past decade have generated one general theory which may be a blueprint for all developing systems. Events of growth cone guidance and target recognition appear to rely on molecular mechanisms that generate the initial specificity of synaptic connections. Patterns of neuronal activity then drive the refinement of these initial connections into highly tuned circuits (reviewed by Goodman & Shatz, 1993).

The Study Of The Visual System.

The visual system is composed of many different levels of processing. This thesis is centred on the development of reciprocal connections between the thalamus and the cortex. These connections are primarily involved in transmitting sensory information, such as visual evoked responses, arising from peripheral sensory organs. The retinae project to the lateral geniculate nucleus (LGN), in the thalamus, which sends axons to the visual cortex. Although the development of the geniculocortical system is well characterised in many species including the mouse, it is far from clear how the early development of this system is controlled. The murine system is attractive because of the availability of neurological mutants such as reeler (where the cortical layers are inverted), and the potential for molecular genetic studies in the long term.

Emergence Of The Vertebrate Brain

From the neural tube the future brain develops as three distinct vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon) (Cowan, 1978 & 1979). These swellings are the result of rapid and disproportionate cell proliferation along the neural tube. There is a rostrocaudal progression of maturation in the development of the neural tube, as well as a ventral to dorsal gradient of proliferation (Hamburger, 1948).

The forebrain vesicle ultimately gives rise to the two-part telencephalon and the diencephalon, the midbrain vesicle to the adult midbrain, and the hind brain vesicle to the lower brain stem (pons and medulla) and cerebellum. The paired telencephalic vesicles bulge out laterally from the midline diencephalon. Dramatic expansion of the telencephalic vesicles form the cerebral hemispheres and at the same time compress the diencephalon. Outgrowths from the ventral diencephalon, the optic stalks, develop into the optic cups, which give rise to the retinae and optic tracts.

Development Of The Cortex

Cortical development occurs in a series of stages beginning with neurogenesis and is followed by migration of cells, outgrowth of neurites, connection formation and remodelling of synaptic connections.

Before the cortex is formed, the wall of the telencephalic vesicle is composed of the preplate and the ventricular zone (site of mitosis). The first postmitotic cortical cells from the ventricular zone migrate into the preplate and form a dense layer called the cortical plate. This layer divides the preplate into the marginal zone above (future layer 1), and the subplate below. The cortical plate, an intermediate structure, is the precursor of the cerebral cortex. The cells of the cerebral cortex are generated from stem cells in the ventricular zone. The stem cell divides to produce two daughter cells. One daughter cell migrates along the long processes of radial glial cells to the cerebral cortex (Rakic, 1988; Hatten, 1990) and the other daughter cell remains in the ventricular zone and becomes responsible for the generation of subsequent cells, and thus subsequent layers of the cortex (Berry & Rogers, 1965). As more cells arrive at

the cortical plate, they form the deepest layers of this structure, and eventually the deepest layer of the cortex, namely layer 6 (Angevine & Sidman, 1961; Berry et al , 1964). As mitosis and migration continue, the cortical plate thickens. Cells produced later in development migrate through and settle above the earlier generated cells, forming progressively more superficial layers. Thus the cells of the cortex are formed from inside to outside. Cells of the murine subplate are born on E12-13 (Gillies et al, 1993) and cells of the cortical plate are born between E13-17, with cells of layer 4 born on E14-15 (Caviness et al, 1982).

The mammalian neocortex is composed of 6 major layers, distinguished by differences in cytoarchitecture and by the distributions of efferent projection neurons and afferent connections. Layers 6 and 5 project subcortically (layer 6 projects back to the thalamus and layer 5 to the superior colliculus, pons and spinal cord). Layer 4 is the primary recipient of thalamocortical input and layers 2 and 3 send and receive connections from other cortical areas. The first subcortical projections arise from the subplate neurons.

The subplate is a transient structure which is present in cats (Luskin & Shatz, 1985) and other species (human: Kostovic & Molliver, 1974; monkey: Rakic, 1974; rat: Bayer & Altman, 1990). More recently, Wood et al (1992), demonstrated that the subplate is also present in the mouse. The subplate disappears within the first few postnatal weeks (Wood et al, 1992; Gillies & Price, 1993). It has been demonstrated in kittens that the lateral subplate axons pioneer the pathway between the cortex and the internal capsule, creating a scaffold for axons from both medial subplate and layers 6 and 5 to grow on (M^{c}Connell et al, 1989). Experiments in which the subplate was lesioned support this hypothesis. Following ablation of the subplate with kainic acid thalamocortical axons are prevented from entering the cortex (Allendoerfer & Shatz, 1994) and the targeting of 50% of corticothalamic axons is disrupted (M^{c}Connell et al, 1994). Gosh et al (1990) suggested that the subplate plays a critical role in guiding thalamocortical axons into their correct cortical area. After ablation of the subplate, geniculate axons failed to recognise their target area, even though layer 4 cells were present.

The Development Of The Geniculocortical Pathway *In Vivo*

The thalamus is one of the main relay centres of the brain and is composed of many discrete nuclei, each concerned with a distinct sensory pathway. The lateral geniculate nucleus specifically relays visual information from the retina into area 17 of the striate visual cortex (Simmons et al, 1982). The neurons of the murine thalamus are born between E13 and E14 (Rennie, 1992). Geniculocortical axons start to grow from the LGN between E14 and 15 and reach the subplate by approximately E17 (Blakemore & Molnar, 1990; Catalano et al, 1991; De Carlos & O'Leary, 1992; Ferrer et al, 1992; Lund & Mustari, 1977; Molnar & Blakemore, 1990). They may wait in the subplate (Wood et al, 1992), before entering the cortex shortly after birth and locating their specific targets, mainly in cortical layer 4, during the first postnatal week (Lund & Mustari, 1977). The existence of a waiting period is debated in rodents (Lund & Mustari, 1977; Catalano et al,1991; De Carlos & O'Leary, 1992) although it is accepted in the cat, ferret and the primate. The presence or absence of a waiting period in different species may reflect their different time courses of development. The brain of the rodent is less complex and has a shorter time period to develop and so there may be no need for a waiting period before thalamic fibres enter the cortex (refer to fig 1.1).

It is not known how projections from the thalamus navigate their way into the internal capsule and from there into their correct cortical area. However, different researchers in this field have proposed various hypotheses. De Carlos & O'Leary (1992) and McConnell et al (1989) have provided evidence that subplate axons send the first projections back to the thalamus (see above). These events occur at the same time as the onset of outgrowth from the thalamus. It is possible that these axons fasciculate with each other. However, De Carlos & O'Leary (1992) suggest that this is unlikely since their experiments demonstrate that these two axon populations have distinct pathways in the cortex. Alternatively, Molnar & Blakemore (1990) suggest that thalamic axons may fasciculate with subplate axons using them for guidance to the cortex. Innervation of the correct cortical area may even be determined by tropic or trophic factors. Gotz et al (1992) demonstrated that membrane bound molecules are expressed in the subplate of early postnatal cortex and that these molecules were

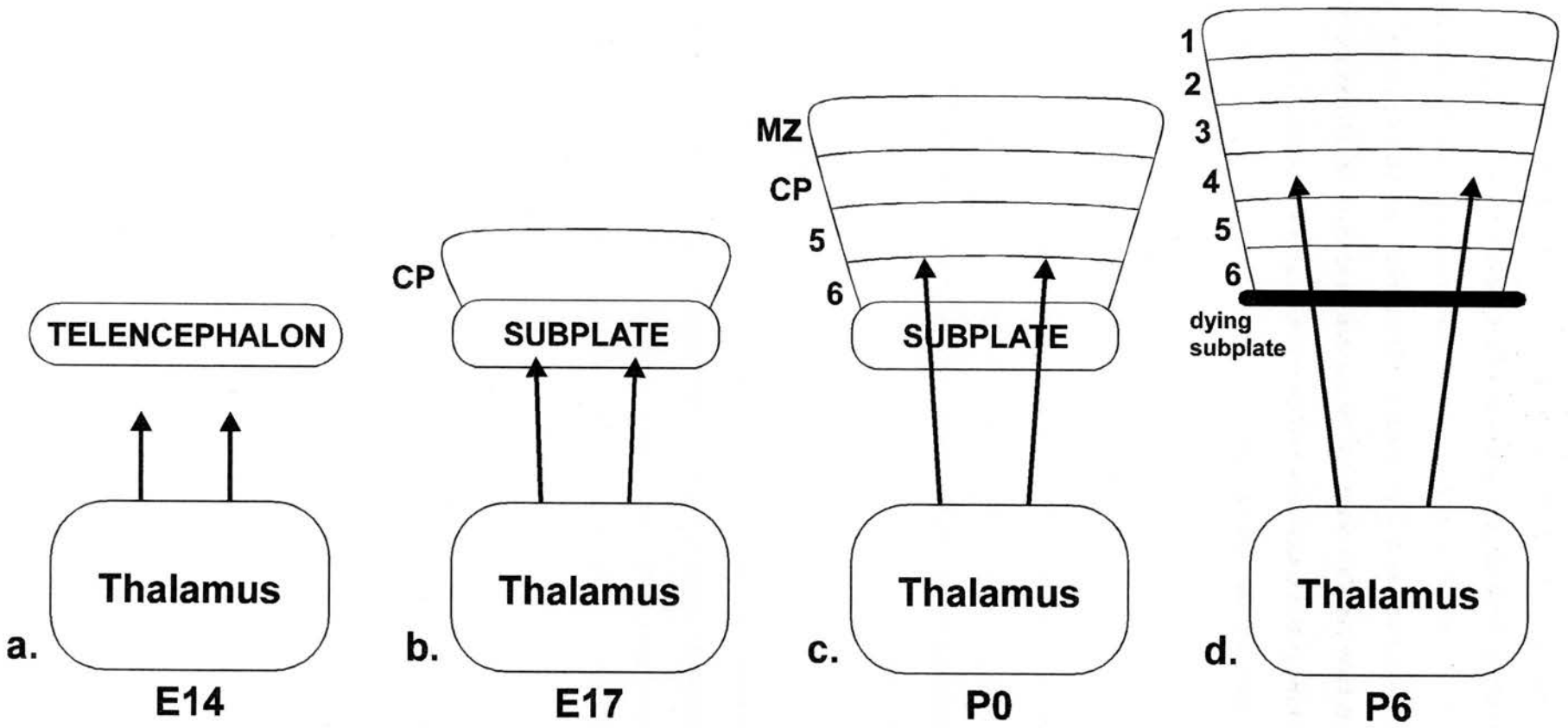

TELENCEPHALON
Thalamus
a.
E14
CP
SUBPLATE
Thalamus
b.
E17
MZ
CP
5
6
SUBPLATE
Thalamus
c.
P0
1
2
3
4
5
6
dying
subplate
Thalamus
d.
P6

Figure 1.1

Schematic diagram displaying the approximate time course of thalamocortical innervation in the mouse. (a) At embryonic day 14 (E14) thalamic neurites sprout and grow towards the developing telencephalon. (b) At embryonic day 17 (E17) thalamic neurites have reached the subplate which is positioned below the developing cortical plate (CP). (c) At postnatal day 0 (P0) thalamic neurites leave the subplate and innervate the deep layers of the cortex. MZ is the marginal zone. (d) By postnatal day 6 (P6) many thalamic neurites have reached their target layer 4. The subplate disappears within the first few postnatal weeks.

upregulated across the developing cortical plate in parallel with thalamic innervation. Furthermore, the temporal and spatial distributions of extracellular matrix molecules, in particular tenascin, suggest that these molecules have a role in guiding axons towards and into the cortex (Shepperd et al, 1991).

After thalamocortical axons reach their target they undergo a stage of refinement; again, whether this occurs in the rodent is debated. 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. Early on in development these connections can adapt to changes in both the level and pattern of activity arising from the eyes. Here the pattern of activity in the terminals from the two eyes plays a decisive role in determining the outcome of competition. This period is termed the 'critical period'. The period of greatest susceptibility in cats and monkeys occurs during the first six weeks of life. Physiological experiments indicate that activity plays a role in synapse elimination, influencing both the rate and outcome of the competition between axon terminals (Betz, 1987; Shatz, 1990a & b).

Competitive Interactions During Development - The Neurotrophic Theory.

An overproduction of neurons and subsequent cell death during the period of synapse formation is a common finding throughout the vertebrate nervous system. Some of the neurons that die may not have established synapses, or may have innervated an inappropriate target. In such cases cell death contributes to the specificity of innervation. Most of the cells that die, however, appeared to have reached and innervated their correct targets . This cell death is primarily a mechanism by which the size of neuronal input is matched to that of its peripheral target. The neurotrophic factor concept states that innervated tissues produce a signal that acts on innervating neurons and selectively limits neuronal death which occurs during development (Purves, 1986; Oppenheim, 1991). This concept arose several decades ago on the basis of the observation that experimental manipulation of the amount of target tissue could modulate the size of neuronal populations.

Experiments by Hamburger and Levi-Montalcini first documented neuronal death in vertebrate embryos and demonstrated that its extent can be influenced by

manipulating the size of the target tissue (Hamburger, 1939). They demonstrated in the developing limb of chick embryos that 40 to 70% of motoneurons that had sent axons into the limb died. Implantation of a supernumerary limb reduced the percentage of motoneurons which died, while removal of the limb bud increased the death of motoneurons (Hollyday & Hamburger, 1976). They concluded that motoneurons were competing for a trophic substance supplied by the target tissue that was necessary for their survival. Recently, two muscle-derived proteins have been identified that can sustain motoneurons in cell culture, cholinergic differentiation factor (CDF) and insulin-like growth factor 1 (IGF1) (Caroni & Grandes, 1990; M^{c}Manaman et al, 1991). Several additional experiments indicate that when the number of competing neurons is reduced a greater proportion of competitors survive. In the ciliary ganglion of the chick, neurons reach their target (the eye) by way of three postganglionic nerves. By cutting some of these postganglionic nerves Pilar and colleagues reduced the number of ciliary ganglion cells before the normal cell death period (Pilar et al, 1980). As a result, a larger fraction of the remaining ciliary ganglion cell population survived to maturity. A third approach used to demonstrate this hypothesis was to produce a mismatch by augmenting the number of neurons that innervate a target before the period of cell death. When both the ismo-optic nuclei are induced to innervate one eye by early enucleation, fewer neurons in these nuclei survive (O'Leary & Cowan, 1984). Until recently these results generated a model of mutually independent retrograde trophic messengers, which are synthesised in distinct target areas and act on restricted neuronal types. This assumption led to a conceptually simple way to arrange and maintain a variety of neuronal subsystems. This has been termed a modular approach to the construction of the nervous system. However, accumulating evidence now suggests that the modular theory is too limited. Neurons can derive trophic support not only from innervated cells (retrograde mechanism), but also from afferent neurons (anterograde mechanism), axon ensheathing glial cells or even themselves (autocrine mechanism). Furthermore, neurotrophic factors interact much less specifically than a modular approach would call for. The pleiotropism of

neurotrophic factors is equalled by their and their receptor's broad distribution. Instead of clear demarcation nature has opted for a fuzzy strategy.

Discovery Of Nerve Growth Factor.

An investigation of the molecular basis of competitive interactions among neurons during development was provided by the work of Levi-Montalcini and colleagues (Levi-Montalcini et al, 1982), who found a factor that supported the growth and survival of the sympathetic and sensory neurons. Initial *in vivo* experiments were made by implanting onto chick embryo's a connective tissue tumor (sarcoma) obtained from mice. On the side where the sarcoma had been implanted there was a profuse outgrowth of sensory and sympathetic nerve fibres from the embryo into the tumor (Levi-Montalcini & Hamburger, 1951, 1953). Further experiments provided evidence that the tumor was secreting a factor. This agent was called nerve growth factor (NGF). Next *in vitro* experiments demonstrated that sarcoma cells produce a similar dramatic effect on tissue-cultured chick ganglia providing a reliable and simple bioassay (Levi-Montalcini, 1964). Using the bioassay, they set about identifying the NGF molecule. Eventually NGF was isolated from the salivary gland of male mice (Cohen, 1960).The purification and detailed characterisation of NGF took an additional decade (Angeletti & Bradshaw, 1971).

Work to date has demonstrated that NGF affects a variety of cell types. However, in the nervous system, its influence is restricted to a few classes of neurons. The apparent role of NGF in the development of the sympathetic and sensory ganglia suggest that this agent ought to be present in the targets of sensitive neurons. Using a two-site enzyme immunoassay, Korsching and Theonen have demonstrated that NGF is indeed found in the targets of sympathetic ganglion cells in amounts proportional to the density of innervation (Korsching & Theonen, 1983). Sympathetically innervated targets were later demonstrated to contain mRNA for NGF (Shelton & Reichardt, 1984). NGF appears to be a requirement for the maturation and survival of both sympathetic chain and dorsal root ganglion cells (Levi-Montalcini & Booker, 1960). In these experiments, new born mice were injected over a period of several days with small doses of antiserum to NGF. The dramatic result was that sympathetic

ganglia nearly disappeared after only a few days (Levi-Montalcini, 1972). Therefore NGF was important in controlling the development of some parts of the mammalian nervous system. Furthermore, systemic treatment of developing mammals with exogenous NGF causes marked hypertrophy of the peripheral sympathetic system (Angeletti et al, 1971; Aloe et al, 1975). *In vitro* studies using dissociated sympathetic ganglion cells confirmed that a major effect of NGF is promotion of cell survival (Levi-Montalcini & Angeletti, 1963; Varon et al, 1973; Greene, 1977a, b; Chen & Patterson, 1977a, b, c; Berg, 1982). Since NGF deprivation enhances the death of sympathetic ganglion cells (and since an excess promotes survival), it seems likely that the amount of NGF normally available regulates the numbers of neurons that survive to maturity in this system.

The Neurotrophic Factors

Neurotrophic factors fall into two broad classifications, the growth factors and a group of distantly related cytokines (Ip & Yancopoulos, 1996). Each category is composed of many different families. The peptide growth factor category includes: the neurotrophins, fibroblast growth factors, insulin-like growth factors and the epidermal growth factor family. In all cases these growth factors function by binding and activating specific tyrosine kinases. This then triggers a cascade of events that culminates in specific programs of gene transcription and particular cellular responses (Heldin et al, 1995). A variety of additional factors have been identified that are unrelated to the growth factors but are capable of producing profound changes in neurons through the activation of cytoplasmic tyrosine kinases. Among these distantly related cytokines are the members of the ciliary neurotrophic factor family and the transforming growth factor (TGF-β) family. Specific examples of some of these neurotrophic factor families are detailed below:

Neurotrophins

The first trophic factor was discovered in 1951 by Levi-Montalcini and Hamburger and was named Nerve Growth Factor (NGF). Since then numerous other neurotrophic factors have been identified. The neurotrophins are a family of closely

related neurotrophic molecules and to date consist of: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and 4/5 (NT-4/5) (Barde, 1989; Korsching, 1993). The production of many of these growth factors during development is correlated with increasing activity in the CNS (Zafra et al, 1990; Isackson et al, 1991; Lu et al, 1991; Castren et al, 1992; Gall et al, 1992; Patterson et al, 1992; Riva et al, 1992). The neurotrophins interact with two types of receptor on the surface of their target neurons (Salter et al, 1979; Ragsdale & Woodgett, 1991). All neurotrophins bind with relatively equal and low affinity (Kd = 10^{-9}M) to a membrane receptor referred to as the low-affinity-fast NGF receptor, or $p75^{NGF}$, which is found in both neurons and non-neuronal cells. There are also high-affinity (Kd = 10^{-11}M) receptors for the neurotrophins. Results of bioassays indicate that the effects of neurotrophins on cell survival and neurite outgrowth are mediated by binding to the high affinity receptors. These receptors consist of an extracellular domain containing the neurotrophin-binding site, a short transmembrane segment, and an intracellular domain encoding a tyrosine kinase. There are at least three members of the TrK family of proto-oncogenes, each acting as the high affinity receptor for one or more neurotrophins.

Fibroblastic Growth Factors

Fibroblastic growth factors (FGF's) which have been characterised by virtue of their mitogenic activities for a variety of cell types (Rifkin & Moscatelli, 1989; Vlodavsky et al, 1991). Currently the FGF family consists of nine members identified on the basis of their amino acid sequence homologies: FGF a and b, FGF 3, 4 and 5 (originally identified as oncogenes), FGF 6 and 7 (keratinocyte growth factor), FGF 8 (androgen inducible growth factor) and FGF 9 have only recently been identified (Baird, 1994). Four distinct FGF receptor genes have recently been identified. These receptors contain three extracellular immunoglobin-like domains and two intracellular tyrosine kinase domains and share 55-70% amino acid identity (Yazaki et al, 1994).

Glial-Derived Neurotrophic Factor

Glial-derived neurotrophic factor (GDNF) is a potent survival factor for central and peripheral neurons (Buj-Bell et al, 1995; Beck et al, 1995; Tomac et al, 1995; Henderson et al, 1995), and is essential for the development of the kidney and the enteric nervous system (Pichel et al, 1996; Sanchez et al, 1996; Moore et al, 1996). GDNF uses a unique receptor system in which a novel glycosyl-phosphatidylinositol (GPI)-linked protein is a ligand-binding component and the tyrosine kinase receptor Ret is a signalling component (Treanor et al, 1996). GDNF might represent an evolutionary transition between the growth factors that use serine threonine kinase receptors (e.g. TGF-β family) and growth factors that use tyrosine kinase receptors (e.g. NGF/PDNF family) (M^{c}Donald & Hendrickson, 1993).

Ciliary Neurotrophic Factor

Peptide sequence and structure comparisons group Leukemia inhibitory factor (LIF) and Ciliary neurotrophic factor (CNTF) with several other cytokines (oncostatin M, interleukin 6, interleukin 11, cardiotrophin-1) into a rather divergent family (Bazan, 1991). CNTF was named for its ability to rescue cultured ciliary neurons (Barbin et al, 1984). However, CNTF acts on a broad range of neuronal and even glial cells. The receptor for CNTF seems to be a heterotrimer of one membrane linked, ligand binding subunit and two transmembrane signal transducing units (Davies et al, 1991; Ip et al, 1992). CNTF induces the jak-STAT transduction pathway which is used by several cytokines. Avian Growth promoting factor is 50% homologous to CNTF and has similar biological activities (Leung et al, 1992).

Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) is a pleiotropic molecule with a multitude of effects for neurons and non-neuronal cells producing blockage or enhancement of differentiation or proliferation depending on the responsive cell population (Smith et al, 1992). The receptor for LIF contains a ligand -binding and signal transducing subunit, both of which belong to the gp130 family of cytokine receptors (Gearing et al, 91,92).

There has also been an increasing realisation that other factors which were identified as proteins (e.g. neuroleukin, glial derived nexin) or as growth factors (e.g. insulin, epidermal growth factor) have also demonstrated neurotrophic activity (Walicke, 1989).

The Role Of Neurotransmitters In Shaping Neuroarchitecture

Other molecules such as neurotransmitters are now increasingly implicated in shaping brain neuroarchitecture (Lipton & Kater, 1989). The available evidence suggests there is, in fact, a continuum of action whereby a given neurotransmitter can stimulate sprouting at low levels (or with localised application), halt neurite outgrowth at only slightly higher levels, and prune dendritic morphology at still higher levels. Zheng et al (1994) have added evidence that an individual neurotransmitter has chemoattractive properties. Their experiments show that a growing fibre can detect and follow a diffusional gradient of neurotransmitter concentration. The response of a cell to a particular neurotransmitter may not be easily identified, as combinational effects of acetylcholine and serotonin have been observed in *Helisoma* neurons (M^{c}Cobb et al, 1988). Acetylcholine can provide protection from the inhibitory effects of serotonin on growth. How a neuron responds to trophic molecules will depend on the expression and regulation of its constituent receptors.

Nitric oxide (NO) and carbon monoxide (CO) are emerging as the first of a novel class of neurotransmitter (see Introduction in Chapter 3 for details). These simple molecules have short half life's of around 5-10 seconds and NO can exert inhibitory effects on growth cones of developing neurites (Hess et al, 1993). Therefore these molecules could mediate short range interactions in developing systems.

Target -Derived Chemoattractants

Chemotropism is the mechanism of axon guidance along gradients of diffusible signal emanating from an axon's target. This model of growth cone navigation was originally proposed by Ramón Y Cajal (1893), and it accounts for directed axon outgrowth when the distance from the nerve cell body to its peripheral target is very

short. This model was supported by work provided by Levi-Montalcini and colleagues. They demonstrated that sympathetic axons grew abnormally into the CNS of the rat after intracerebral injection of NGF (Mesenini-Chen et al, 1978). Lumsden and Davies have also studied the growth of axons from the trigeminal ganglion in the head of the mouse into the adjacent epithelial tissue, a distance less than 1mm (Lumsden & Davies, 1986). If the developing trigeminal ganglion is placed in cell culture near explants from several peripheral tissues, neurites grow from the ganglion toward their appropriate target but ignore other potential targets. Moreover, explants of target epithelium have this effect on axon outgrowth only if they are taken from embryos at the time innervation normally occurs. Thus at the appropriate developmental stage these epithelial cells appear to produce some molecule that attracts the growth cones of trigeminal ganglion cells.

A second set of experiments in which explants of cortex and pons are juxtaposed in tissue culture demonstrate the existence of diffusible factors released from the pons that induce and direct the growth of collaterals from pyramidal cell axons (Heffner et al, 1990). These observations suggest a mechanism by which corticospinal and corticopontine connections are established. Initially all cortical pyramidal cells project to the spinal cord and respond to factors released by the pons; then approximate axon branches are selected from this generic structure and maintained by trophic interactions.

Until recently, the molecular identity of cues that direct axon guidance events were largely unknown. Although NGF can act as a chemoattractant for regenerating sensory axons in culture (Gunderson & Barrett, 1979) it did not appear to be involved in guiding developing axons as they first grew towards their targets (Davies, 1987) or in guiding regenerating axons *in vivo* (Diamond et al, 1992). Tessier-Levigne's team were the first to isolate two related diffusible chemoattractants, namely netrin-1 and netrin-2 (Serafani et al, 1994; Kennedy et al, 1994). They demonstrated that floor plate cells at the ventral midline of the spinal cord secrete a diffusible factor or factors that promote(s) the outgrowth of spinal commissural axons and attracts these axons *in vitro* and that two membrane-associated proteins isolated from chick brain, netrin-1 and netrin-2, possess

commissural axon outgrowth-promoting activity. They also demonstrated that netrin-1 RNA is expressed by floor plate cells, whereas netrin-2 RNA is detected at lower levels in the ventral two thirds of the spinal cord, but not the floor plate.

It is possible that chemotropism may be involved in guiding the development of the thalamocortical system although there is no available evidence suggesting a tropic effect. In other regions of the developing nervous system, it has already been demonstrated that diffusible trophic molecules play important roles in promoting neuronal survival and growth (Lumsden & Davies, 1983 & 1986; Davies, 1988), and results from recent co-culture experiments suggest that postnatal cortex releases diffusible factors that promote thalamic survival and outgrowth (Lotto & Price, 1994, 1995; Rennie et al, 1994). However, the identity of these factors is unknown.

Development Of LGN And Cortex *In Vitro.*

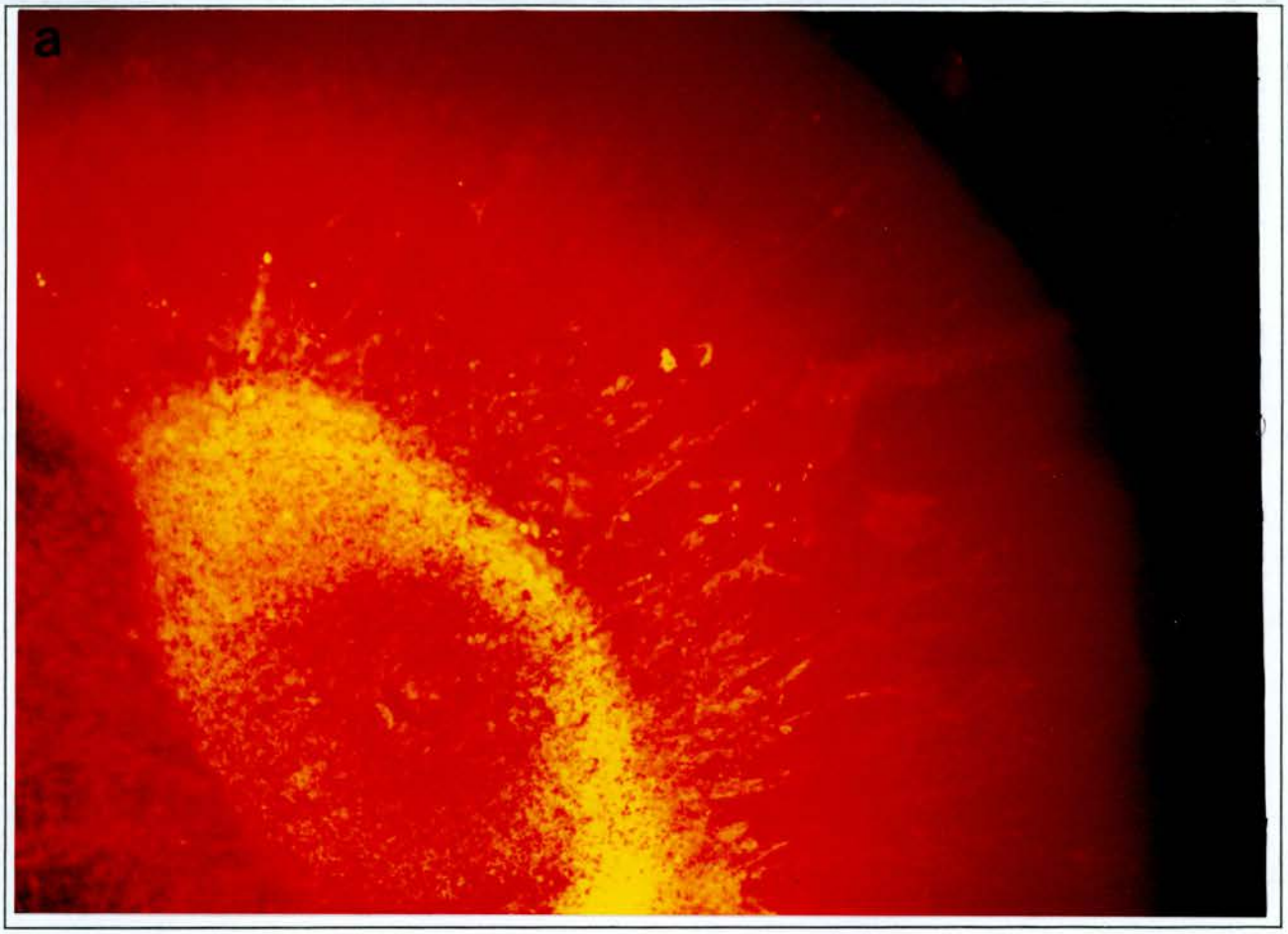
Tissue culture techniques are widely used to study neurodevelopment. They allow easy access to isolated developing systems in controlled environments. The spatial arrangement of co-cultured slices can be re-arranged in an attempt to determine whether local cues present in cortical tissue influence axonal growth or target cell recognition. The age of the animal from which the co-cultures are taken can also be varied. However, *in vivo* pathways and timing cues from extrinsic connections are abolished and although results of *in vitro* experiments allow us to understand developmental mechanisms they cannot always be directly related to the *in vivo* situation.

Yamamoto et al (1989) were the first to perform co-culture experiments consisting of thalamus and cortex. Co-cultures involving thalamic and cortical explants have recapitulated the specificity of the developing thalamocortical system *in vitro* (Yamamoto et al, 1989; Bolz et al, 1990; Molnar & Blakemore, 1991). Axonal outgrowth occurs from explants of rat embryonic LGN into nearby explants of early postnatal visual cortex in culture ("co-culture" of the geniculocortical system *in vitro*). The LGN neurites terminate in layer 4, their normal *in vivo* target. Efferent projections from the cortex to the thalamus also develop *in vitro*, and arise from the same cortical layers as *in vivo* (Bolz et al, 1990). The ultrastructural development of

the cortex continues in the co-cultured system, in a fashion remarkably similar to that *in vivo* (Wolburg & Bolz, 1991).

David J. Price's laboratory has successfully adapted this technique to the murine geniculocortical system: neuronal migration, geniculocortical afferent development, and cortical maturation in organotypic co-cultures of LGN and cortex, continue as *in vivo* (Rennie et al, 1992) (Refer to figure 1.2). To date, work in our laboratory indicates that the cortex and thalamus have an intimate relationship, both affecting each other's development. Co-culture studies demonstrate that the postnatal cortex releases diffusible factors which increase the survival and outgrowth of adjacent thalamic explants. However, evidence from co-cultures of embryonic thalamus with prenatal cortex suggest that, before birth, the cortex has little or no influence on the growth of the thalamus (Price et al, 1995; Rennie et al, 1994). Furthermore, prenatal thalamus can survive when cultured alone for three days (Rennie et al, 1994). Recently, our laboratory has demonstrated that culturing late embryonic thalamus with cortex appears to rescue cells of the cortical sub-plate that would otherwise be destined to die (Price & Lotto, 1996). By synapsing on the sub-plate thalamic afferents may increase the survival of these cells by anterograde transport of a trophic factor.

It seems reasonable to suggest that trophic factors are involved in regulating the development of the thalamocortical connections and that thalamic afferents may regulate the expression of trophic molecules or their receptors and so affect their own development and that of the cortex. However the identity of these trophic factors is unknown although they are conserved across divergent species (Lotto & Price, 1994).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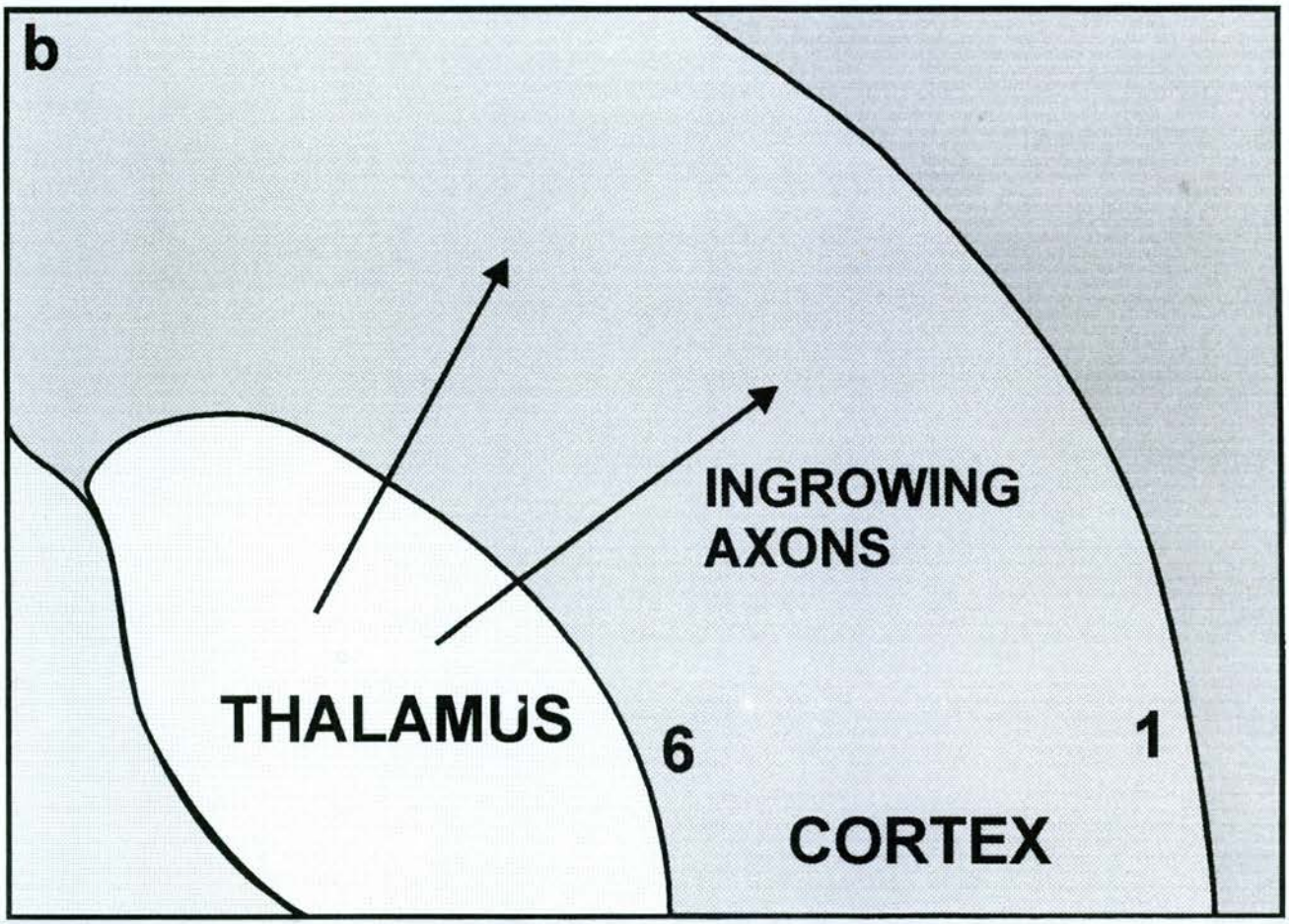

b
INGROWING
AXONS
THALAMUS
6
1
CORTEX

Figure 1.2

(a) Murine organotypic co-culture consisting of embryonic day (E15) thalamus and postnatal day 6 (P6) visual cortex which has been cultured for 10 days on a collagen membrane in serum free medium. Black dotted lines indicate the boundary between the thalamic explant and the cortical explant. Black arrows indicate thalamic fibres which have innervated the cortical explant. After culture the organotypic co-culture was fixed and a crystal of carbocyanine dye was placed within the thalamic explant to label thalamic axons. This dye is lipophilic and is restricted to the membranes of thalamic axons which innervate the adjacent cortical explant. This enables the thalamic innervation of the cortical explant to be viewed under fluorescent microscopy. This co-culture was photographed under fluorescent microscopy (scale =350μm).

(b) Schematic diagram of the above co-culture. Cortical layer 6 is situated next to the thalamic explant. Cortical layer 1 is situated at the outer edge of the co-culture.

Aims Of My Research.

My aim was to examine the role of neurotransmitters in the development of the thalamocortical system *in vitro.*

Chapter Outlines.

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

Chapter 2.

There were four main points to these experiments: (i) To learn and refine the co-culture technique. (ii) To obtain reproducible evidence for thalamic axon termination within layer 4 of co-cultured cortex. (iii) To investigate a role for classical neurotransmitters in the development of the thalamocortical system, in particular, to assess their involvement in target recognition. (iv) To investigate a possible role for spontaneous activity in these co-cultures.

Chapter 3.

The main aim of this chapter was to investigate a possible role for nitric oxide (a novel neurotransmitter) in the development of the thalamocortical system. (i) To investigate the effect of NO on growth cones of dissociated embryonic thalamic neurons. (ii) To examine the distribution of nitric oxide synthase (NOS) during the development of the thalamocortical system both *in vivo* and *in vitro*. (iii) To block NO production *in vitro* and examine any effects on thalamocortical development.

Chapter 4.

In chapter 2, increased activity (added K^+) produced excessive outgrowth from co-cultures consisting of E15 thalamus and P6 cortex and blocking activity with high doses of tetrodotoxin caused these co-cultures to die. In this chapter I attempted to investigate the trophic and outgrowth promoting effects of K^+-induced depolarization on developing thalamic cells in organotypic culture. The aim of this chapter was to

investigate the ability of cultured thalamic explants of different ages from E13 to postnatal day 2 (P2) to survive and grow in isolation with and without increased activity.

Chapter 5.

In chapter 4, I report that the survival and outgrowth of cultured thalamic cells are dependent on a mechanism that becomes increasingly reliant on neural activity for its operation as embryonic development proceeds. I suggest that the increased expression or release of either neurotrophins or neurotransmitters are possible candidates mediating this activity dependent effect. In this chapter, I evaluate the possibility that glutamate, the excitatory neurotransmitter in thalamic cells, mediates this effect. My aim was to investigate the ability of cultured thalamic explants of different ages from E13 to P2 to survive and grow in isolation with and without increased glutamate.

CHAPTER 2:

THE ROLE OF NEUROTRANSMITTERS IN THE DEVELOPMENT OF THE THALAMOCORTICAL SYSTEM IN ORGANOTYPIC CO-CULTURES

CHAPTER 2: THE ROLE OF NEUROTRANSMITTERS IN THE DEVELOPMENT OF THE THALAMOCORTICAL SYSTEM IN ORGANOTYPIC CO-CULTURES.

ABSTRACT

Both the stimulation of thalamic neurite outgrowth and targeting of these neurites to layer 4 were optimised in organotypic co-cultures consisting of embryonic day 15 thalamus and postnatal day 6 cortex. Reproducible quantitative evidence suggesting that these axons recognise their target layer 4 was obtained using Siamcam computer software. Many classical neurotransmitters, which are known to be present during the development of the thalamocortical system *in vivo*, did not appear to play a major role in the initiation of thalamic outgrowth nor the termination of neurites within cortical layer 4 *in vitro*. However, APV (NMDA glutamate receptor antagonist) appeared to disrupt thalamic axons from terminating within their target layer 4 *in vitro*. Results from experiments in which cultures were treated with tetrodotoxin and depolarized with added K^+ revealed that activity levels are important in controlling thalamocortical development *in vitro*. An optimal level of activity appears to be crucial for generating appropriate signals for thalamic ingrowth and termination within target layer 4 of the cortex.

INTRODUCTION

Role Of Neurotransmitters As Growth Regulatory Signals.

Knowledge of the classical electrophysiological events induced in neurons by neurotransmitters dates back to early in this century when Elliot (1904) suggested that epinephrine might be released from sympathetic nerve endings and act as a chemical mediator of interneuronal impulse transmission. Studies by Rakic and Sidman (1973) possibly provided the first clue that neurotransmitters might be involved in shaping brain neuroarchitecture. However, it was not until 1984 that direct effects of neurotransmitters on neuronal outgrowth were established by Haydon et al. They demonstrated that serotonin could inhibit neurite outgrowth from identified *Helisoma* buccal ganglion neurons in culture. Serotonin had a direct effect on the structure of the growth cones of these neurons. It is now recognised that neurotransmitters, in addition to mediating synaptic transmission, play important regulatory roles in the development of the nervous system (Lipton and Kater, 1989). A series of *in vitro* investigations on preparations as diverse as snail ganglia (Haydon et al, 1984), chick (Lankford et al, 1988) and mammalian retina (Lipton et al, 1988), and mammalian hippocampus (Mattson et al, 1988), provides the strongest direct evidence that neurotransmitters influence the morphology of neuronal growth cones and thus affect neuronal architecture. These roles can range from neurite sprouting to dendritic pruning and even cell death. Available evidence suggests that there is in fact a continuum of action whereby a given excitatory neurotransmitter can, at low levels (or with localised application), stimulate sprouting, at only slightly higher levels halt neurite outgrowth, and at still higher levels prune dendritic morphology. Therefore, neurotransmitters could potentially allow a fine control of neural plasticity although they can also be neurotoxic at high doses.

Effects Of Neurotransmitters *In Vitro*

Haydon et al (1984), demonstrated that the growth cones of individual neurons react quite specifically to particular neurotransmitters. However, it is unlikely that normal environments *in situ* are composed of single cues that modulate neuronal outgrowth. The combinatorial effects of substrata, growth factors and neurotransmitters will be integrated to produce a growth cone response. Combinatorial effects of acetylcholine and serotonin have been observed in *Helisoma* (M^{c}cobb et al, 1988), where acetylcholine can provide protection from the inhibitory effect of serotonin on growth. Work on neurotransmitter regulation of neuronal form has been taken one step further to show that neurotransmitters released by afferent axons can alter the morphology of growing neurons. The system for these studies consisted of explants of entorhinal cortex and dissociated hippocampal pyramidal neurons in culture. Glutamate was released from afferent entorhinal cortical axons and had an inhibitory action on the development of both dendritic arbors and presumptive synaptic sites in target hippocampal neurons (Mattson et al, 1988). The actions of glutamate on neurite outgrowth are not only concentration dependent but may depend on the stage of development and the receptor subtype expressed (Mattson et al, 1988). In the above experiment, glutamate release was dependent on activity. However, neurotransmitters are also released spontaneously from growth cones in sufficient quantities to have significant physiological effects (Chow & Poo, 1989). Zheng et al, (1994) have added evidence that an individual neurotransmitter has chemoattractive properties. Their experiments show that a growing fibre can detect and follow a diffusional concentration gradient of neurotransmitter. Furthermore, the classical inhibitory neurotransmitter gamma-aminobutyric acid (GABA) has been shown to play an excitatory role in the regulation of development of hippocampal neurons in early postnatal life (Ben-Ari et al, 1994). Taken together, a wide variety of results from *in vitro* experiments now demonstrate that many different neurons can respond to neurotransmitters in increasingly novel ways. The fact that neurotransmitter receptors develop on neuronal processes before synaptogenesis (Isaacson & Pribram, 1985), strengthens the suggestion that modulation via these receptors may influence growth.

Effects Of Neurotransmitters *In Vivo*

Although evidence exists for the effect of neurotransmitters in cell culture, information is also required from *in situ* or *in vivo* experiments. A temporal correlation between neurotransmitter activity and the outgrowth of neurons in early development is provided by a study conducted on the immature cat telencephalon. Lateral geniculate axons are arrested at the cortical subplate at a time that coincides precisely with the appearance of a large number of cholecystokinin-, neuropeptide Y-, and somatostatin- containing neurons that appear transiently at the cortical subplate (Chun et al, 1987). After these transient neurons die axon elongation resumes. Other examples include transmitter influences on the plasticity associated with long-term potentiation (Collingridge, 1987) and the combinatorial action of neurotransmitters on the expression of functional neuronal connections within ocular dominance columns in the visual cortex (Kleinschmidt et al, 1987). In an elegant *in vivo* experiment on the amphibian optic tectum (characterised by a highly specific and orderly arrangement of inputs from the eyes), an additional eye primordium is implanted resulting in adults with one tectum innervated by two eyes. This manipulation produces segregated inputs from the two eyes, resulting in a striped organisation of eye terrains on the surface of the tectum (Reh & Constantine-Paton, 1985). Application of tetrodotoxin to the optic nerve diminishes the striped organisation and demonstrates that activity is involved in its formation. Addition of 2-amino-5-phosphovalerate (APV), a selective NMDA antagonist, to the tectum after the striped pattern has formed results in the dissolution of the stripes (Cline et al, 1987). These experiments demonstrate that activity dependent neuronal 'neighbour relationships' are communicated between ingrowing axonal processes.

Proposed Second Messenger Signalling Pathways For Neurotransmitter Effects.

Secondary messenger systems which are common to both the mature and immature nervous systems are emerging. There appears to be a strong correlation in many species between the effect of a transmitter on membrane potential and its influence

on outgrowth and survival. The membrane depolarization is believed to induce an increase in intracellular calcium through voltage-dependent calcium channels (Kater et al, 1988; Choi, 1988), or through channels coupled directly to neurotransmitter receptors (MacDermott et al, 1986), such as NMDA receptor-activated channels (fig 2.1). Calcium induced calcium channels which are permeable to calcium and activated by increased intracellular calcium may also serve to amplify calcium signals within the cell (Lipton, 1987). Recently, in Xenopus spinal neurons, calcium transients have been shown to encode information in their frequency, in a way similar to action potentials (Gu & Spitzer, 1995). Two types of spontaneous events, spikes and waves, are expressed in distinct frequencies in early development. Natural spike activity is sufficient to promote normal neurotransmitter expression and channel maturation, whereas wave activity is sufficient to regulate neurite extension and differentiation. Indeed a mechanism linking electrical activity, calcium and cytoarchitecture appears to be highly conserved in evolution even across kingdoms. For instance, fucoid eggs become polarised in a calcium gradient (Robinson & Cone, 1980) and cytoplasmic streaming (a form of plant cell motility) in the algae *Chara*, is inhibited by action potentials (Hepler & Wayne, 1985).

cAMP and products of phosphoinositol hydrolysis (e.g. IP_3 mobilising intracellular calcium and diacylglycerol activating protein kinase C), both appear to play a role in stimulating or inhibiting neuronal outgrowth and/or survival depending on the system investigated (Mattson, 1988). These conflicting results could be explained by these secondary messengers exhibiting a bell-shaped dose-response curve similar to that observed with intracellular Ca. For example, agents that increase intracellular cyclic AMP levels enhanced neurite extension in PC12 cells (Schubert et al, 1978), embryonic rat cortical cells (Shapiro, 1973), and hippocampal pyramidal neurons (Mattson & Kater,1989). *In Helisoma*, elevations in cyclic AMP inhibited neurite elongation and growth cone motility in a subpopulation of buccal neurons (Mattson et al, 1988). This negative effect of cyclic AMP resulted from induced calcium influx through plasma membrane channels.

Figure 1

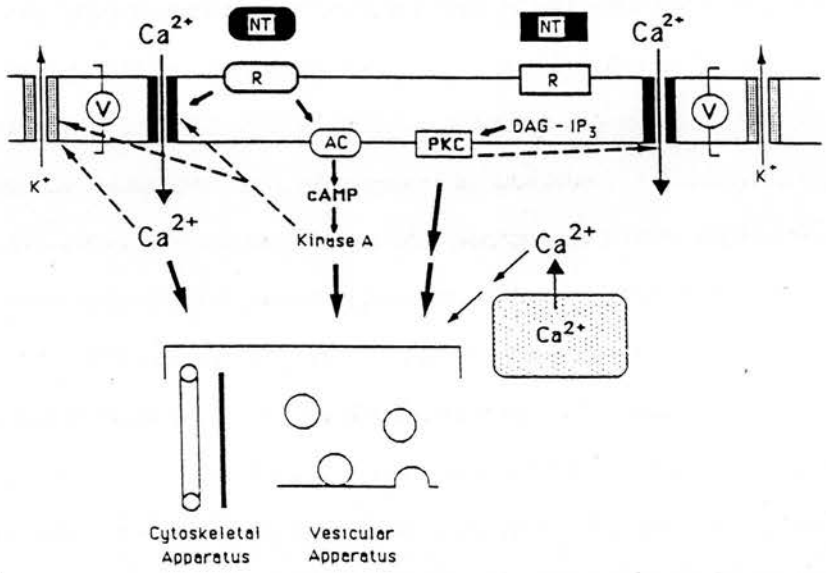

Ca2+
NT
NT
Ca2+
R
R
V
V
AC
PKC
DAG - IP3
K+
K+
cAMP
Ca2+
Kinase A
Ca2+
Ca2+
Cytoskeletal Apparatus
Vesicular Apparatus

Figure 2.1

Schematic diagram outlining how axonal and dendritic outgrowth could be regulated by neurotransmitters. Neurotransmitters bind to and activate specific receptors which are expressed on the cell membrane. Various secondary messenger pathways could be activated depending on the receptor subtype. Intracellular calcium could be elevated by either receptor coupled or voltage sensitive ion channels. Alternatively, cyclic AMP (cAMP) could be increased by receptor mediated activation of adenylate cyclase (AC); cAMP then activates kinase A. PKC is also activated by receptor mediated hydrolysis of inositol phospholipid resulting in the liberation of diacylglcerol (DAG) which activates PKC, and IP_3 which stimulates calcium release from intracellular stores. These diverse secondary messenger systems may even interact. For instance, kinase A can phosphorylate calcium channels and modulate their activity and hence the resultant calcium influx. PKC can also phosphorylate K^+ channels and hence alter the membrane potential producing a change in the state of the voltage sensitive calcium channels. All of these secondary messengers (PKC, kinase A, and calcium) can regulate cellular systems which regulate outgrowth. There are two main systems; cytoskeletal (microtubules, microfilaments, and associated proteins; e.g. MAPs) and vesicular (proteins involved in endocytosis and exocytosis; e.g. synapsin I) systems.

Cyclic AMP can also effect cell death. Cyclic AMP can increase or decrease cell death depending on the system investigated. Cyclic AMP can prevent programmed cell death of cultured rat cerebellar granule cells (Chang et al, 1996). Vasoactive Intestinal Peptide (VIP) mediated increase in cyclic AMP can also prevent tetrodotoxin-induced retinal ganglion cell death *in vitro* (Kaiser & Lipton, 1990). Furthermore, the survival of postnatal rat ganglion cells *in vitro* is not promoted by peptide trophic factors unless their intracellular Cyclic AMP is increased pharmacologically or they are depolarized by K^+ or glutamate agonists. This suggests that both neurotransmitter stimulation and electrical activity enhance the survival of developing rat ganglion cells (Meyer-Franke et al, 1995). Alternatively, stimulation of primary granulosa cells by high levels of cyclic AMP catalyses programmed cell death (Aharoni et al, 1995).

Beyond Second Messengers.

The events beyond second messengers which underlie changes in neuronal morphology initiated by neurotransmitters have not been examined but are likely to focus eventually on ionic channels, cytoskeletal elements (microtubules, microfilaments, and associated proteins) and the vesicular apparatus (fig 2.1). In *Helisoma* neurons, cyclic AMP-dependent protein kinase may act either on plasma membrane calcium channels to enhance influx or directly on the cytoskeletal elements regulating growth cone motility (Mattson et al, 1988).

Development Of Neurotransmitter Systems In The Thalamocortical Pathway.

In the adult, there is considerable evidence that glutamate is released as an excitatory synaptic transmitter from terminals of geniculocortical axons (Tamura et al, 1990; Tsumoto et al, 1990). Recent observations imply that neonatal geniculocortical axons of many animals, including mice, may be initially cholinergic (Robertson et al, 1989; Kageyama et al, 1990). In the neonate, acetylcholinesterase (AChE) is expressed transiently by geniculocortical neurons, and is found in their axons as they grow into the recipient layers in the visual cortex, mainly layer 4 (Kageyama et al, 1990). In the

rat the expression of AChE begins on PD3, is maximal on PD8-10 and tapers to adult levels by PD16 (Robertson et al, 1987). The early increase of expression is coincident with geniculocortical termination and organisation in layer 4 of the cortex and a role for ACh, or AChE itself, in the targeting of geniculocortical axons on layer 4 has been suggested (Kageyama et al, 1990; Robertson, 1987).

Lipton et al (1988), have also provided evidence for the involvement of cholinergic receptors in regulating development, by showing that a blockade of cholinergic receptors on retinal ganglion cells *in vitro,* with the inclusion of (+)-tubocurarine or mecamylamine in the culture medium, initiates sprouting of new neurites.

Expression Of Receptors For Neurotransmitters In The Neocortex *In Vivo*

Information on the developmental expression of receptors in the neocortex is limited. Transitory expression of a given neurotransmitter and its receptor(s) during development before functional synapses are formed is a good indication of possible trophic function for this molecule. However, many receptors for neurotransmitters that are present in the immature brain can enter a latent state, where the binding sites for neurotransmitters are no longer coupled to transduction mechanisms. It is therefore necessary to consider whether receptors detected in the immature brain really correspond to functional receptors.

Glutamate And Acetylcholine

Glutamatergic and cholinergic receptors (AChR) have been demonstrated in the visual cortex of the neonatal rat, and their concentrations increase with age (Coyle and Yamamura, 1976; Kuhar et al, 1980; Sanderson and Murphy, 1981). In the neonatal rat cortex, NMDA glutamatergic receptors are most densely distributed in the superficial layers (Monaghan & Cotman, 1985). However, the laminar distribution of non-NMDA glutamatergic receptor subtypes in neonatal neocortex is not clear (Tsumoto, 1990), though the adult distribution of these receptors is better understood. Levels of NMDA receptors in the visual cortex of kittens are highest during the developmental stage which is considered the critical time for experience-dependent modifications of the cortex (Bode-Greuel and Singer, 1989). In the adult, cholinergic receptors are

expressed at different levels across the cortical layers; in particular the m2 receptor subtype is localised within layer 4 (Levey et al, 1991). The distributions of these receptors in neonates are not well defined.

Noradrenaline

Noradrenergic fibres are present throughout all the layers in the neocortex and arise from neurons of the locus coeruleus, although in the primate the density of innervation varies with cortical region and lamina (Foote et al, 1983). In the rat , cat and primate the cerebral cortex possesses a high density of α_1 and β adrenergic receptors and a moderate density of α_2 adrenoreceptors. Interestingly, the distribution of α_1 adrenoceptors in the rodent displays marked laminar differences with high levels found in layers 4 and 5 (Jones et al, 1985). In the cat primary visual cortex, however, α_1 adrenoceptors appear to be localised most densely in layers 1-3, with a lesser band in layers 5 and 6 (Parkinson et al, 1988).

Serotonin

Serotoninergic fibres appear as at least two distinct types in the cerebral cortex. Axons from the median raphe appear as beaded fibres which form pericellular nests around the cell bodies and proximal dendrites of non-pyramidal cells in layers 1-3, while axons from the dorsal raphe appear as fine and varicose fibres which are found in all the layers of the cortex, although their density varies from lamina to lamina dependent upon the region examined (Berger et al, 1988; Hornung et al, 1990; Kosofsky & Molliver, 1987; Mulligan & Tork, 1988; Tork, 1990). In the rat serotonergic receptors attain peak levels in fetal or early neonatal life and then decrease to adult levels (Uzbekov et al, 1979; Daval et al, 1987).

Dopamine

The primate visual cortex is only poorly innervated by dopaminergic fibres with those that are present being largely restricted to layer 1. In the cat the primary visual cortex displays low levels of binding for both D_1 and D_2 receptors with the lamina of highest

density of D_1 receptors being layer 1 (Richfield et al, 1989). In other regions of the nervous system, activation of D_2 receptors results in inhibition through activation of a potassium conductance (Lacey et al, 1988).

GABA

The distribution of GABA receptors in the pre- and post-natal monkey have been established by Shaw et al (1991). GABAa receptors are detected in the cortical plate, marginal zone and the subplate between E61-72 (35% of gestation). On E119 (70% of gestation) layer 4 can be distinguished by its high density of GABAa receptors; post-natally the density of GABAa receptors increases in all layers but remains highest in layer 4. GABAb receptors first become evident on E126 (75% of gestation); their density is least in layer 4, and this pattern is maintained until adulthood. Although GABA is normally associated with intrinsic connections, Shaw et al (1991) have suggested that, in addition to neurotransmission, GABA and its receptors may subserve modulatory roles during development, including cortical cell differentiation, the guidance of ingrowing axons to correct targets, and the modulation of ocular dominance column formation. Information on the distribution of GABA receptors in other species is limited.

From the above detailed information it becomes clear that there are two broad classes of neurotransmitter action in the primary visual cortex as well as other regions of the neocortex: the fast acting neurotransmitter systems comprising GABAergic and excitatory amino acid releasing neurons, and the modulatory transmitter systems composed of the cholinergic, noradrenergic, dopaminergic, and serotinergic ascending systems.

Evidence for the involvement of neurotransmitter systems in the development of the thalamocortical pathway coupled with the increasing amount of literature on distributions of neurotransmitter receptors in the neocortex, directed me to investigate the possibility that thalamocortical input and termination within layer 4 of the cortex may involve a neurotransmitter receptor mediated mechanism.

Experiments using organotypic co-cultures consisting of embryonic thalamus and postnatal cortex were conducted to examine this hypothesis. Thalamic outgrowth starts at approximately E14-15 *in vivo* (Blakemore & Molnar, 1990; Catalano et al, 1991; De Carlos & O'Leary, 1992; Ferrer et al, 1992; Lund & Mustari, 1977; Molnar & Blakemore, 1990) and older thalamic explants do not survive very well in culture, therefore E15 thalamic explants were studied. Time matched E15 cortical explants would not be any good for studying the termination of axons into target layer 4 since the cortex is more immature at this age. Target layer 4 cells are only born at approximately E14-15 (Caviness et al, 1982), and have not yet migrated through the cortical plate. P6 cortical explants were studied since layer 4 cells are mature at this age.

Thalamocortical Development Is Reproduced In Organotypic Co-cultures

The mammalian cerebral cortex consists of many structurally and functionally specialised areas with characteristic input from particular nuclei of the thalamus. The mechanisms that control the development and differentiation of the neocortex are the subject of intense study. In brief, the mammalian neocortex is composed of six major layers, distinguished by differences in cytoarchitecture and by distributions of efferent projection neurons and afferent connections. In the visual system the retinae project to the lateral geniculate nucleus (LGN) in the thalamus, which sends axons to the visual cortex. Layer 4 of the visual cortex is the main recipient of this thalamocortical input and cells in layer 6 project back to the thalamus. Many cells in layer 5 project to other subcortical targets, while cells in upper layers 2 and 3 give rise to cortical projections. Co-culture studies involving thalamic and cortical explants from the rat have recapitulated this specificity *in vitro*
(Yamamoto et al, 1989; Bolz et al, 1990; Molnar and Blakemore, 1991). The LGN neurites terminate in layer 4, their normal *in vivo* target. Efferent projections from the cortex to the thalamus also develop *in vitro*, and arise from the same cortical layers as *in vivo* (Bolz et al, 1990). The ultrastructural development of the cortex continues in the co-cultured system, in a fashion remarkably similar to that *in vivo* (Wolburg and Bolz, 1991).

In our laboratory this technique has been successfully adapted to the murine geniculocortical system: neuronal migration, geniculocortical afferent development, and cortical maturation in organotypic co-cultures of LGN and cortex, continue as *in vivo* (Lotto & Price, 1994, 1995; Rennie et al, 1994). However, it should be taken into account that the co-culture system is a model of reinnervation and not a model of *de novo* targeting of thalamic neurons.

AIMS

The main aim of the study described in this chapter was to exploit this novel *in vitro* system to investigate the signalling mechanism present *in vitro* which allows thalamic explants to successfully and specifically innervate layer 4 cells of co-cultured cortex. My objectives were to achieve the following:

1. To learn and refine the co-culture technique.

2. To achieve reproducible evidence for thalamic axon termination within layer 4 of the cortex.

Immediately I faced two main problems: I needed to accurately locate and mark layer 4 within the cortical explants and to refine an analytical paradigm which will allow me to accurately but relatively rapidly assess axon termination.

3. To investigate the possibility that neurotransmitters are involved in the development of the thalamocortical system *in vitro*. In particular, to assess their involvement in target recognition and axon termination.

HYPOTHESES

My main hypothesis was that activity dependent or independent neurotransmitter release in the developing thalamocortical system is involved in providing the necessary information to stimulate thalamic outgrowth and instruct axonal growth cones to collapse, synapse and arborise in layer 4 in the cortex.

Possible scenarios included :

1. Axons innervating the cortical explant might spontaneously release neurotransmitter from their growth cones. Layer 4 cells might express a compatible receptor and a receptor mediated event might result in the release of a growth cone collapsing molecule, a molecule inhibitory to growth or a protein involved in synaptogenesis.
2. Receptors specifically located in the superficial layers of the cortex may regulate the release of inhibitory neurotransmitters which prevent innervation beyond layer 4.
3. Spontaneous release of neurotransmitter from ingrowing thalamocortical axons might stimulate cortical receptors that upregulate the expression of adhesion molecules which guide axons to layer 4.

4. Target layer 4 cells may spontaneously release tonic levels of neurotransmitter which act postsynaptically on ingrowing thalamic neurites to inhibit outgrowth at high concentrations (i.e. near source of release) and stimulate growth at low concentrations.

EXPERIMENTAL PROTOCOLS

Experiment 1: Controls

(a) E15 thalamic explants were placed adjacent to the ventricular surface of P6 cortical explants and were cultured for 6 days. These co-cultures were used to obtain reproducible evidence for axon termination in layer 4. A quantitative analytical paradigm was devised to allow rapid assessment of axon termination.

(b) Co-cultures of E15 thalamus and P0 or P2 cortex were investigated as in (a) to examine the timing of target recognition (layer 4 is not mature in younger cortical explants).

(c) Co-cultures of E15 thalamus and P6 cortex were grown for 10 days, and isolated E15 thalamic explants were cultured for 6 days. These experiments were to ensure that even after 10 days thalamic neurites did not grow past layer 4 and to prove that isolated thalamic axons are capable of growing long distances in culture when no cortex is present.

(d) Co-cultures were set up as in (a), except that the thalamic explant was placed adjacent to the pial surface of the cortex, to investigate whether target recognition of layer 4 was maintained when axons were made to grow abnormally through the superficial layers of the cortex.

(e) A few P0 and P2 cortical explants were isolated and cultured for 6 or 4 days respectively, before E15 thalamic explants were placed opposite them and co-cultured, as in (a). This experiment was to determine whether the termination signal in the cortex would develop normally in isolated culture without any previous innervation of layer 4 by the thalamus *in vivo*.

Experiment 2: Effects Of Neurotransmitter Antagonists

(a) Co-cultures were set up as in (1a) and I added a wide variety of neurotransmitter antagonists to the culture medium to investigate the importance of specific neurotransmitters in thalamocortical development *in vitro*. These were :

Pertussis toxin: a G protein inhibitor

Phenoxybenzamine HCl: a non-specific α adrenoreceptor antagonist.

Propanolol HCl: a non-specific β adrenoreceptor antagonist.

Atropine sulphate: a non-specific muscarinic antagonist for cholinergic receptors.

Mecamylamine HCl: a non-specific nicotinic antagonist for cholinergic receptors.

5-Aminopentanoic acid HCl (AP-5): $GABA_B$ antagonist.

Picrotoxin: GABA Cl^- channel blocker.

2-Amino-5-Phosphono-Propionic Acid (APV): Glutamate NMDA antagonist.

L(+)-2-Amino-3-phosphonopropionic acid (AP-3): Glutamate metabotropic receptor antagonist.

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX): Glutamate Kainate/AMPA receptor antagonist.

Experiment 3: Modulation Of Activity

(a) Tetrodotoxin (a sodium channel blocker) was added to co-cultures set up as in (1a), on day 3 and day 5 of culture, and the effects of blocking sodium channel activity in culture were examined.

(b) K^+ was added to co-cultures, set up as in (1a), at the beginning and after day 3 of the culture period, to examine the effects of increased activity in culture.

MATERIALS AND METHOD

Animals And Surgery.

Occipital cortical slices were taken from BALB/c mice between postnatal day 0 (P0, the day of birth) and P6 and these were co-cultured with E15 thalamic explants (see Table 2.1 for full details). To obtain cortical explants, mice were immediately decapitated and their brains were quickly removed and placed in oxygenated Earl's balanced salts (Sigma) on ice. The occipital cortices were isolated and sliced coronally at 350μm with a M^{c}llwain tissue chopper. Slices were placed in pre-incubated (at 37^{o}C) defined serum-free culture medium (Romijn et al, 1988). Serum-free medium contains: 100mls of Dulbecco's Modified Eagles (DME) formulae D5671, 100 mls of Ham formulae F12 (N4888), 1mg Insulin, 2mg apo-Transferrin, 0.24gm $NaHCO_3$, 3mls Hepes buffer, 2mls L-Glutamine, 2mls of Putrescine, 20μl of Na_2SeO_3, 20 μl of Progesterone (all from Sigma chemicals). E15 thalamic explants were obtained from time-mated BALB/c mice deeply anaesthetised with urethane (0.3ml of a 25% solution in normal saline, i.p.) and foetuses were removed by Caesarean section and decapitated. The brains were removed and the posterior thalamus was carefully dissected out as described before (Lotto & Price, 1995; Rennie et al, 1994), and stored in ice-cold oxygenated Earl's balanced salts (Sigma). 1.5mm^3 (about 1.5x 1 x 1mm) thalamic explants from the posterior thalamus were sliced at 350μm with a M^{c}llwain tissue chopper. These explants were centred on the lateral geniculate nucleus (LGN) and they included neighbouring nuclei (Lotto & Price, 1995; Rennie et al, 1994).

Culture Methods

Cortical and thalamic slices were then positioned adjacent to one another (~ 1mm apart) on a collagen-coated filter (Costar UK, Transwell-COL chambers with 3μm

TABLE 2.1. The numbers of cultures in each experimental paradigm.

FIRST EXPLANT	SECOND EXPLANT	DRUGS	n
Effect of cortex age on axon termination in layer 4			
E15 thalamus	P6 cortex		28
E15 thalamus	P2 cortex		4
E15 thalamus	P0 cortex		13
Effect of ingrowth through the pial surface on axon termination in layer 4			
E15 thalamus	P6 cortex		12
Effect of drugs on axon termination in layer 4			
E15 thalamus	P6 cortex	Pertussis Toxin 1μM	6
E15 thalamus	P6 cortex	Phenoxybenzamine HCl 10μM	10
E15 thalamus	P6 cortex	Propanolol HCl 10μM	8
E15 thalamus	P6 cortex	Atropine sulphate 1μM	8
E15 thalamus	P6 cortex	Mecamylamine HCl 10μM	8
E15 thalamus	P6 cortex	AP-5 1μM	4
E15 thalamus	P6 cortex	Picrotoxin1μM	6
E15 thalamus	P6 cortex	AP-3 10μM	8
E15 thalamus	P6 cortex	CNQX 39μM	8
E15 thalamus	P6 cortex	APV 10μM	6
Effect of blocking activity on axon termination			
E15 thalamus	P6 cortex	tetrodotoxin 0.5μM (after 3 days)	4
E15 thalamus	P6 cortex	tetrodotoxin 10μM (after 3 days)	7
E15 thalamus	P6 cortex	tetrodotoxin 0.5μM (after 5 days)	5

TABLE 2.1. The numbers of cultures in each experimental paradigm.

Effect of enhancing activity on axon termination			
E15 thalamus	P6 cortex	K^+ (50mM)	5
E15 thalamus	P6 cortex	K^+ (5mM: after 3 days)	5
Background DiI diffusion in a co-culture fixed after 24 Hrs growth.			
E15 thalamus	P6 cortex		10

pores) suspended in a chemically defined serum-free medium. The filters and medium had been pre-incubated at 37°C with 5% CO_2 for at least 2 hrs. Two millilitres of medium were placed in the lower chamber of each culture-well and 200-250µl of the same culture medium were placed in the upper chamber, ensuring the explants were just covered. Thalamocortical co-cultures were cultured for 6 days at 37^0C with 5% CO_2 with or without various drugs which were dissolved in the culture medium (see Table 2.1). Culture medium was refreshed every three days. Over the culture period, cortical and thalamic explants spread slightly but maintained their original shape. At the end of the culture period, cultures were fixed in 4% paraformaldehyde in 0.01M phosphate buffered saline (PBS) for a minimum of 1 hour to fix the tissue. Small crystals of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (appropriate for the size of the thalamic explant, ~0.25mm in diameter) were placed in the thalamic explants and left for at least 4 weeks to label the growing neurites. Before analysis, I counterstained the co-cultures (washed in PBS) with a fluorescent nuclear dye, bisbenzamide (Hoescht 33342; final concentration 5µg ml^{-1}: Latt & Stetten, 1976). This enabled me to visualise the cortical layers and assess whether the laminated organotypic structure was intact. The co-cultures were stained overnight at room temperature, then washed in PBS and analysed with standard fluorescence microscopy. Hoescht was viewed through a UV filter and DiI through a rhodamine filter.

SMI32 Immunochemistry

To help determine the location of layer 4 within cortical explants, I began to develop a technique for labelling pyramidal neurons with SMI 32, an antibody specific for non-phosphorylated epitopes in neurofilament H. In other species SMI 32 positive neurons exhibit a high degree of regional and laminar specificity that is correlated with the functional and anatomical diversity of the cortical areas. SMI 32 specifically labels pyramidal neurons at the layer 4/5 and 3/4 border in the cat (Peter Kind: personal communication).

Mice were anaesthetised (0.1ml of sodium pentobarbitone administered intramuscularly) and were perfused with 0.05M Tris buffered saline (TBS), followed

by 4% paraformaldehyde. Brains were dissected out and placed in 4% paraformaldehyde for 10 minutes. Brains were wax embedded and sectioned at 10μm. Sections were mounted on poly-$_L$-lysine coated slides and covered in 10% normal rabbit serum (NRS) in 0.05M TBS (1:30) for 30 minutes at room temp and placed in anti-SMI32 (1:1000; Sternberger Monoclonals Inc.), diluted in 10% NRS in 0.05M TBS (NRS/TBS) for 90 minutes at room temp. They were then washed (x3) in TBS, then incubated in biotinylated rabbit anti-mouse IgG (1:100) in NRS/TBS for 30 minutes at room temp, washed (x3) in TBS, followed by 1 hour in avidin and biotinylated horseradish peroxidase (HRP) solution (elite ABC kit: Vector laboratories). HRP was reacted with diaminobenzadine and H_2O_2 for 8 minutes at room temp, then washed (x3) in TBS, dehydrated and mounted with DPX.

DiI Diffusion Experiment

Some co-cultures were fixed in paraformaldeyhde after only 24 hours of culture, and a crystal of DiI was inserted in the thalamus. Background diffusion was assessed in these co-cultures since only a few thalamic axons had penetrated the deeper layers of the co-cultured cortex, during this brief period in culture.

Neurofilament Staining Of Neurite Outgrowth

To ensure that outgrowth was axonal rather than glial (these can appear similar: Torran-Allerand, 1990), some co-cultures were cultured as before and labelled with an antineurofilament antibody. Co-cultures were fixed for 8 minutes in 4% paraformaldehyde in 0.01m PBS (room temperature), incubated in 90 % methanol at -20°C for 10 minutes, washed (x3) in 0.01M PBS with 2% Triton-X-100 for 5 minutes, incubated in 0.01M PBS with horse serum (10 minutes) and placed in anti- MAP2 (1:300: Sigma), diluted in 2% Triton-X-100 in 0.01M PBS, for 24 hours at 4°C. They were then washed (x3) in 0.01M PBS with 2% Triton-X-100 for 5 minutes and incubated in biotinylated horse anti-mouse IgG (1:200) in 2% Triton-X-100 in 0.01M PBS for 24 hours at 4°C. After incubation they were washed (x3) with 0.01M PBS for 5 minutes followed by 1 hour 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.

Analysis

In these experiments thalamic explants produced massive outgrowth which was directed towards and into the co-cultured cortical explant. This made it impossible to count individual thalamic fibres within the cortex. Instead these cultures were analysed using Siamcam software. DiI labelled thalamic neurites were visualised within the cortex using a rhodamine filter and fluorescence microscopy. The image was recorded and stored on the computer. Switching the fluorescence filters on the microscope to a UV filter enabled me to visualise the counterstained bisbenzamide layers. This image was displayed on a VDU and traced onto transparent acetates. The stored image of DiI labelled neurites was re-opened to reveal their position in the cortex relative to the marked layers. Luminance intensity generated by the DiI label was measured in two sections (50μm wide) across each co-cultured cortical explant (see fig 2.3a). Data was formulated as the average percentage decrease of luminance at each layer border using the white matter/layer 6 border as total luminance ie.100% (fig 2.4).

RESULTS

Co-cultures Maintain Their Organotypic Laminar Structure During Culture.

Counterstained co-cultures with bisbenzamide revealed that the laminated organotypic structure was intact after 6 days of culture (fig 2.5b). The size of co-cultured cortical explants were compared to freshly dissected explants prior to culture (fig 2.5a & b) . The percentage spread of the surface area of explants in culture was calculated to be ~ 4 %.

Background DiI Diffusion.

Co-cultures of P6 cortex and E15 thalamus were set up to examine the extent of background DiI diffusion (i.e. the amount of DiI label spreading into the cortex which is not carried in by thalamic axons). These cultures were fixed after 24 hours and labelled with DiI for the same length of time as in 6 day cultures. Fig 2.2 demonstrates that luminance decreases very sharply from 100% to ~15% within layer 6.

Termination Of Thalamic Axons In Layer 4 Of Co-cultured Cortex (P0-P6).

Outgrowth from thalamic explants was extensive. In all experiments most of the outgrowth was directed into the co-cultured cortex. Photomicrographs reveal that thalamic axons terminated sharply within layer 4 (fig 2.3a and b). Fig 2.4, comparing the ingrowth of thalamic axons into P6 and P0 cortex, shows that the sharpest decrease in luminance was observed within layer 4 in P6 cortices. Luminance decreased over 20% in layer 6, and the graph indicates a continuous decrease in luminance across layer 5. Only ~10% luminance remained in layer 2. In P0 cortices the luminance decreased steadily across the cortex (fig 2.4). A large percentage of axons projected through the pia and grew for long distances onto the collagen membrane (fig 2.6 a & b). No thalamic axons terminated in layer 4 of co-cultured P2 cortical explants (fig 2.7 shows that many previously fasiculated axons turned 90^{o} to each other as they exited the cortex and project onto the collagen membrane, perhaps trying to locate a more suitable substrate).

Thalamic axons remained confined to target layer 4 of co-cultured cortex even after 10 days of culture (fig 1.2). Isolated thalamic explants grew for longer distances when cultured alone for 6 days suggesting axons actively recognised layer 4 in co-cultured cortex and did not simply grow for a set distance.

Thalamic Ingrowth Through The Pial Surface (i.e.Layer 1)

Ingrowth through the pial surface and the superficial layers (which would never occur *in vivo*) also resulted in marked termination within layers 3/4 (it was impossible to differentiate these two layers in many of these co-cultures because the bisbenzamide stain was not as clear: fig 2.8a & b). This finding is in agreement with previous work (Bolz et al, 1993). In fact, termination occurred more sharply in layer 3/4 after ingrowth through the superficial layers as opposed to the deeper layers (fig 2.9).

GRAPH OF BACKGROUND DiI DIFFUSION IN A CO-CULTURE AFTER 24 Hrs GROWTH.

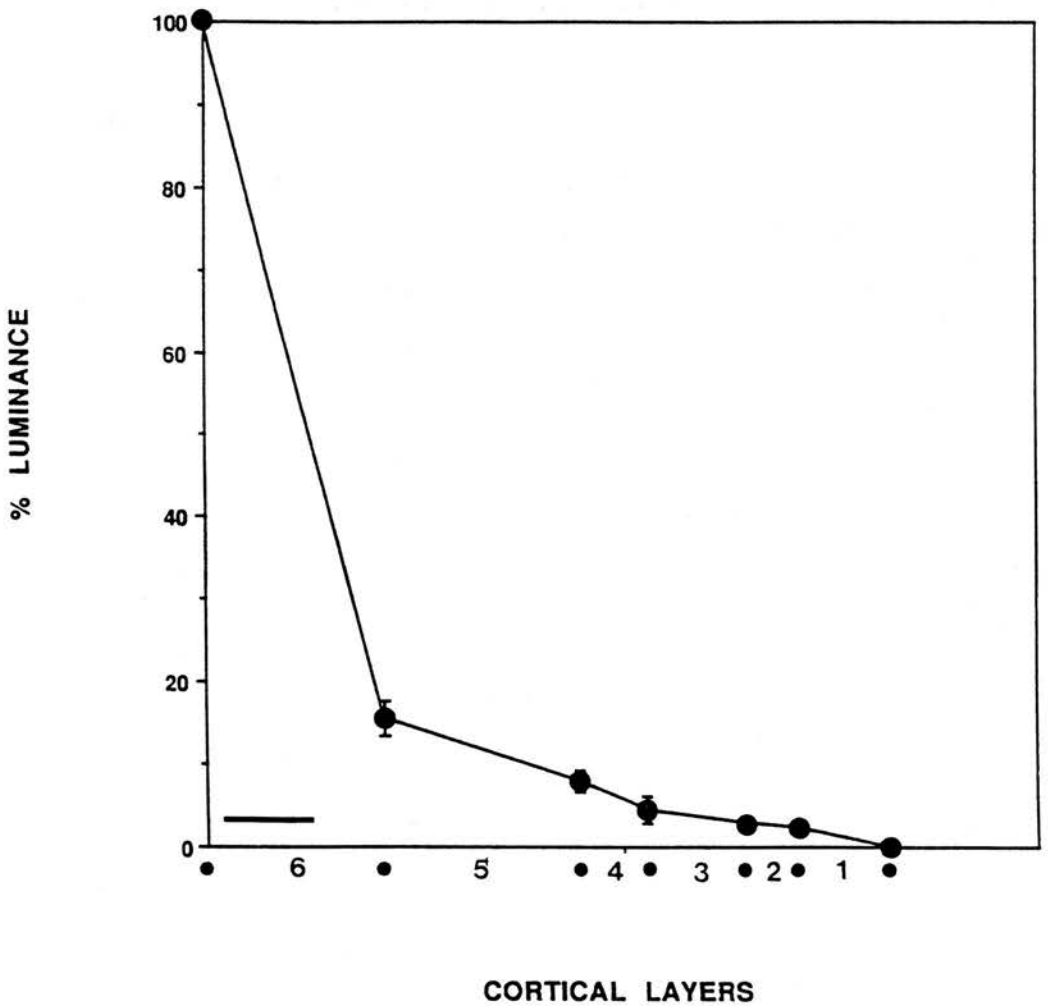


Figure 2.2

This graph compares the ingrowth of embryonic day 15 thalamic axons into postnatal day 6 visual cortex within a 24 hour culture period. This experiment was conducted to examine the amount of background fluoresecent dye diffusion (i.e. dye which is not restricted within thalamic axons) into the co-cultured cortex.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. Filled circles on the X-axis represent the average width of each cortical layer. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 125μm). For all luminance data points n=10.

This graph reveals that luminance decreases very sharply within the cortical explant. Luminance decreases from 100% to 15% within layer 6 of the cortex. Only a few thalamic axons would grow beyond layer 6 after a 24 hour culture period. This experiment indicates that the dye is restricted to thalamic axons.

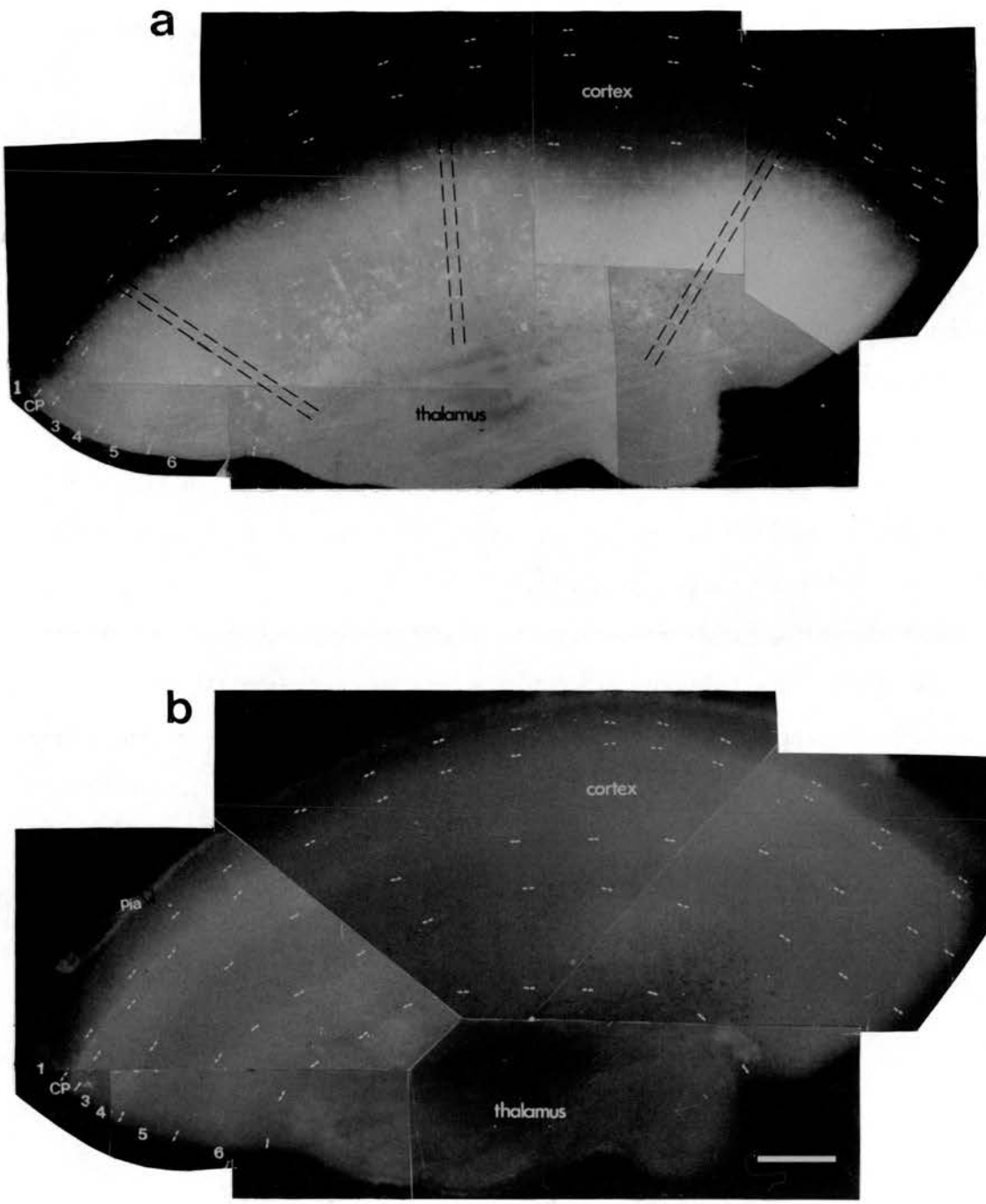

a
cortex
thalamus
1
CP
3
4
5
6
b
cortex
Pia
thalamus
1
CP
3
4
5
6

Figure 2.3

(a) Photomicrograph of a murine co-culture consisting of embryonic day (E15) thalamus and a coronal section of postnatal day 6 (P6) visual cortex.
The co-culture has been cultured for 6 days on a collagen membrane in serum free culture medium. During this period thalamic axons sprout and innervate the adjacent cortex. A crystal carbocyanine dye (see materials and method p38) was placed within the thalamic explant. The dye has diffused along axons which innervate the cortex enabling the co-culture to be photographed under fluorescent microscopy. The growth of many axcns was restricted within the region of layer 4, their *in vivo* target. White dotted lines indicate layer boundaries within the cortex. CP is the cortical plate. Black dotted lines indicate a typical cross section which was analysed using computer software (see analysis p40)

(b) This is a microphotograph of the same co-culture described in (a) except it has been counterstained stained with bisbenzamide and is now viewed under UV fluorescent microscopy to reveal the cortical layers. White dotted lines indicate layer boundaries within the cortex. CP is the cortical plate. The laminated organotypic structure is intact. Layer 1 spreads out in culture probably due to the lack of support from surrounding tissue. Figure 2.2 (a) and (b) can be directly compared to identify the cortical region in which thalamic axons terminate.(scale =350μm).

GRAPH COMPARING THE INGROWTH OF THALAMIC AXONS INTO P6 AND P0 CORTEX.

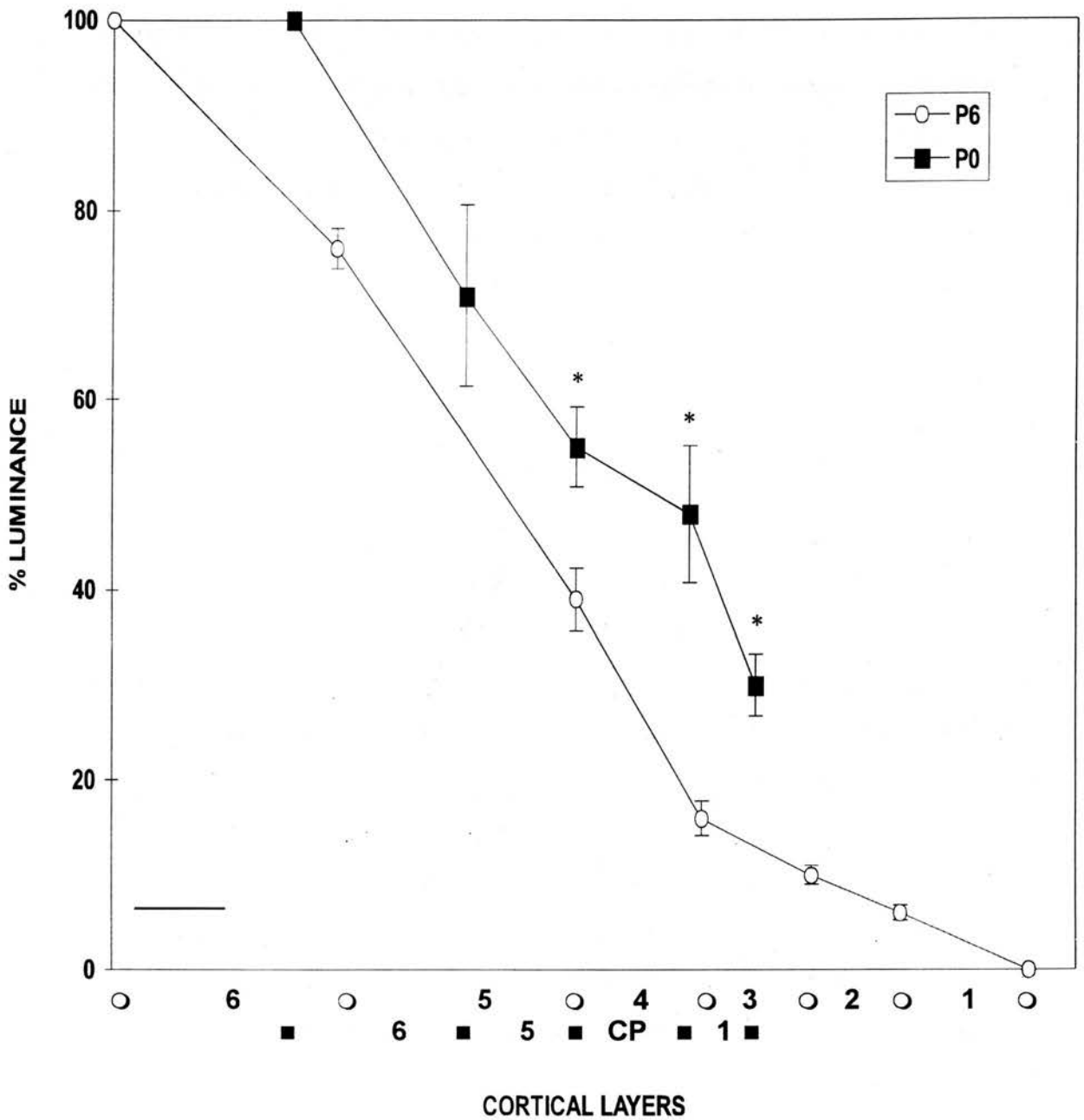


Figure 2.4

This graph compares the ingrowth of embryonic day 15 thalamic axons into postnatal day 6 (P6) and postnatal day 0 (P0) visual cortex. The co-cultures were cultured for 6 days in serum free medium.

Using computer software the intensity of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. White circles and black squares on the X- axis represent the average width of each cortical layer for P6 and P0 cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex. CP is the cortical plate.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 117μm). The graphs have been aligned on the X-axis at the layer 5/4 and 5/CP border. The n values for P6 luminance data ranges from n = 18-28. The n values for P0 luminance data ranges from n = 4-13. The mean luminance values at each cortical layer border were compared statistically between P6 and P0 cortices using a Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/CP, CP/1 and 1/collagen borders within P0 cortices were compared to the 6/5, 5/4, 2/1 and 1/collagen borders within P6 cortices respectively. The 6/5 border was not significantly different but all other borders were significantly different between these two data groups. Gradients from these graphs were also compared statistically (see next page).

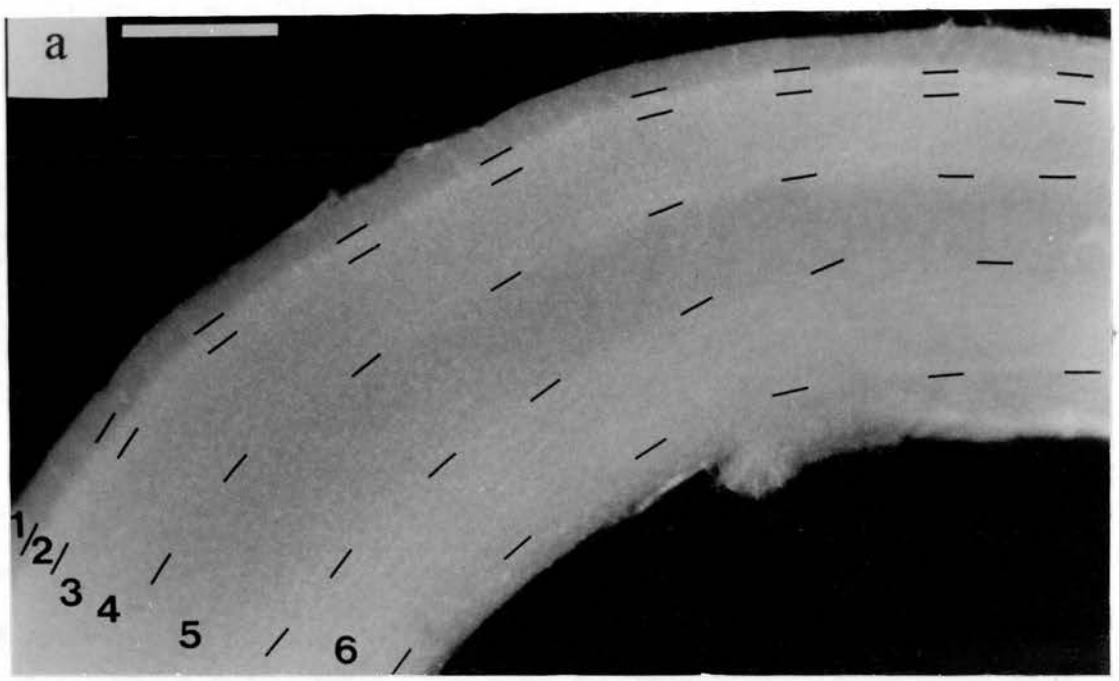
In P6 cortices luminance decreases steadily within layer 6 and 5. However, a sharp decrease in luminance is observed within the narrower layer 4, demonstrating that axons are restricted within this layer. In P0 cortices the intensity of luminance

decreases evenly across the cortical plate. In these cortices layer 4 cells are not mature and the CP will develop into layer 4, 3 and 2 .

Figure 2.4 continued.

Gradients were calculated for the decrease in luminance between the 5/4 and 4/3 borders from graphs displaying the ingrowth of E15 thalamic axons into P6 cortices. Gradients were also calculated for the decrease in luminance between the 5/CP and CP/1 borders from graphs displaying the ingrowth of E15 thalamic axons into P0 cortices. These gradients were compared statistically using a Student's t-test. The gradients were significantly different $p<0.05$.

	P0 cortex gradient	P6 cortex gradient
MEAN	-0.23	-0.37
SEM	0.05	0.06
n	10	19
t-test sig. value	0.031	


a
1/2/3 4
5
6

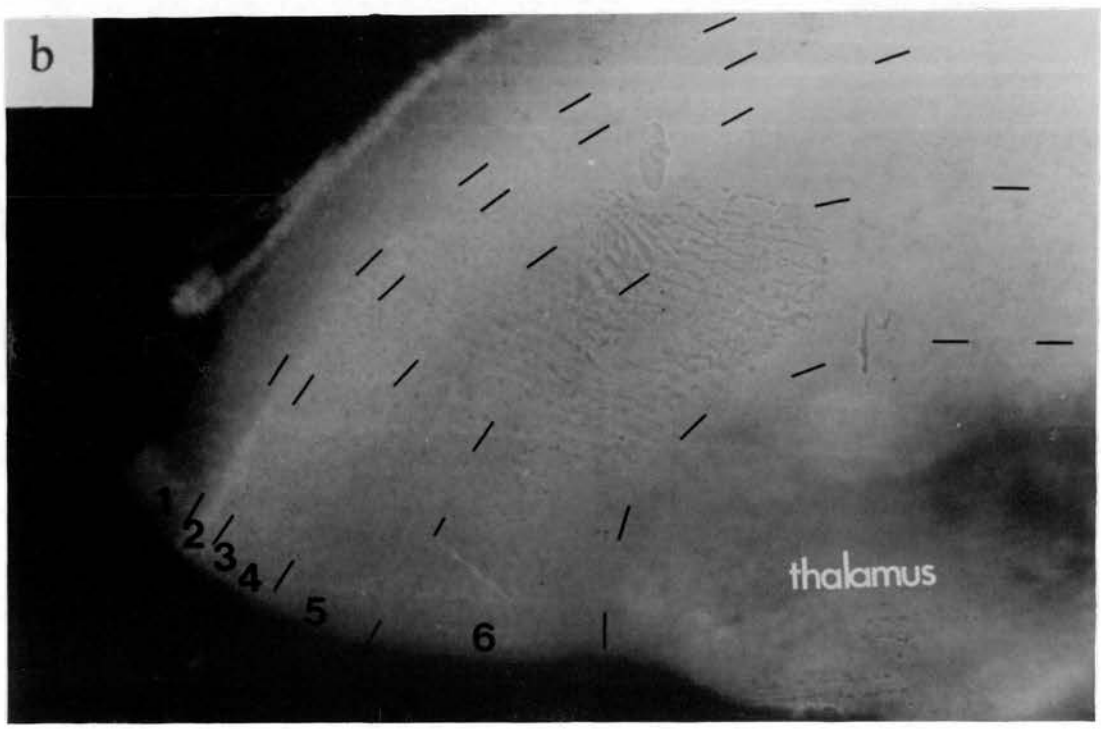

b
1
2
3
4
5
6
thalamus

Figure 2.5

Photomicrographs comparing the amount of spread in cultured cortex. (a) uncultured coronal slice of acute postnatal day 6 (P6) visual cortex stained with bisbenzamide. Black dotted lines indicate the cortical layers. (b) Co-culture consisting of P6 cortex with embryonic day (E15) thalamus which has been cultured for 6 days in serum free medium before staining with bisbenzamide. Layer 1 which is no longer supported with surrounding tissue spreads out in culture, although levels of spread are generally low. (scale =350μm).

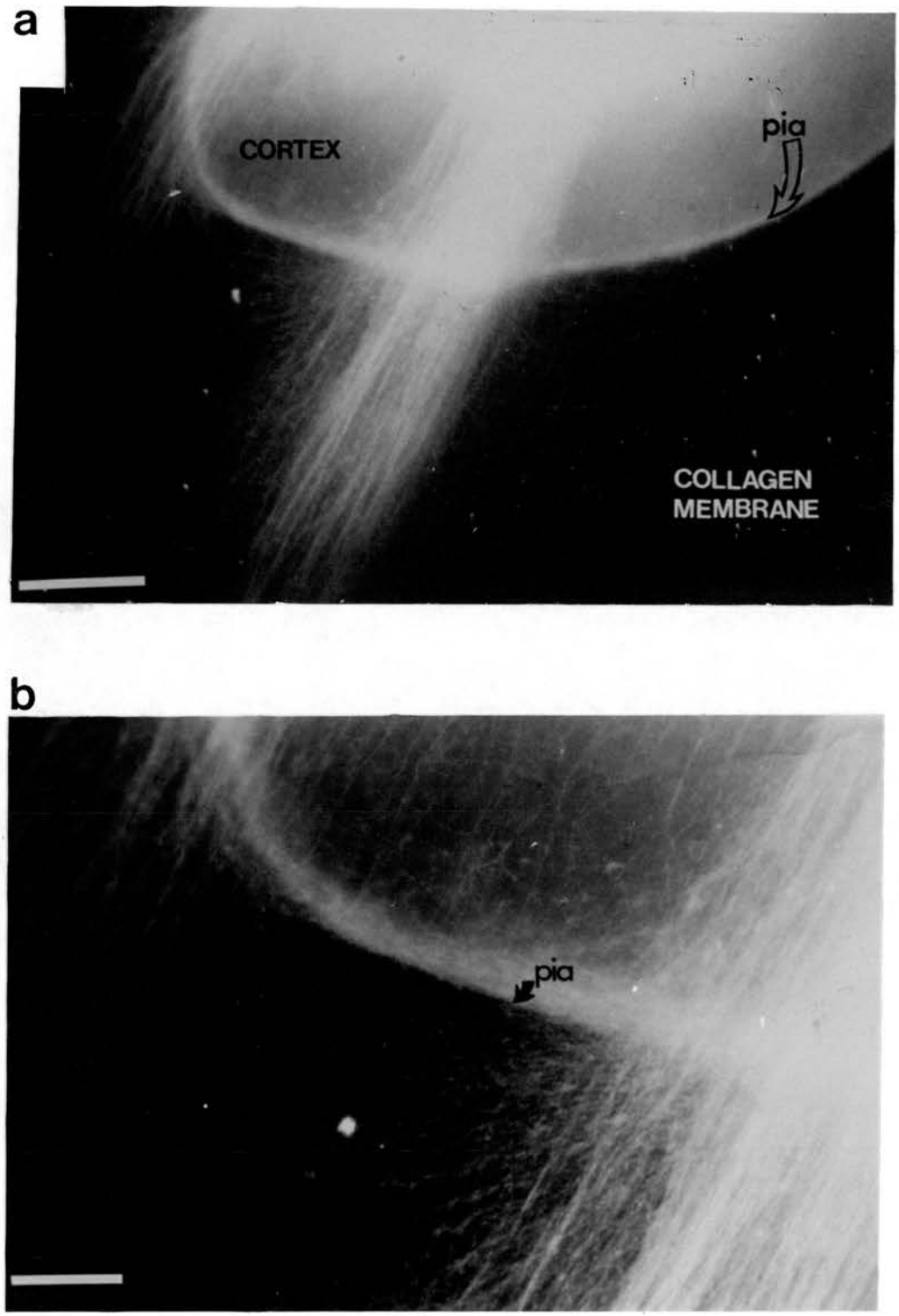

a
CORTEX
pia
COLLAGEN
MEMBRANE
b
pia

Figure 2.6

Photomicrographs of a section of a co-culture consisting of embryonic day (E15) thalamus and postnatal day 0 (P0) visual cortex. This culture was grown for 6 days in serum free medium. In fig (a) and (b) the thalamus is positioned above the cortex but is not included in the photograph. Fig (a) Reveals that axons are very fasiculated and do not terminate within the cortical explant, instead they grow for long distances on the collagen membrane (scale 350μm). (b) as (a) but greater magnification. This photograph demonstrates that many axons become less fasiculated as they leave the cortex, probably turning in an attempt to locate a more suitable substrate (scale = 140μm).

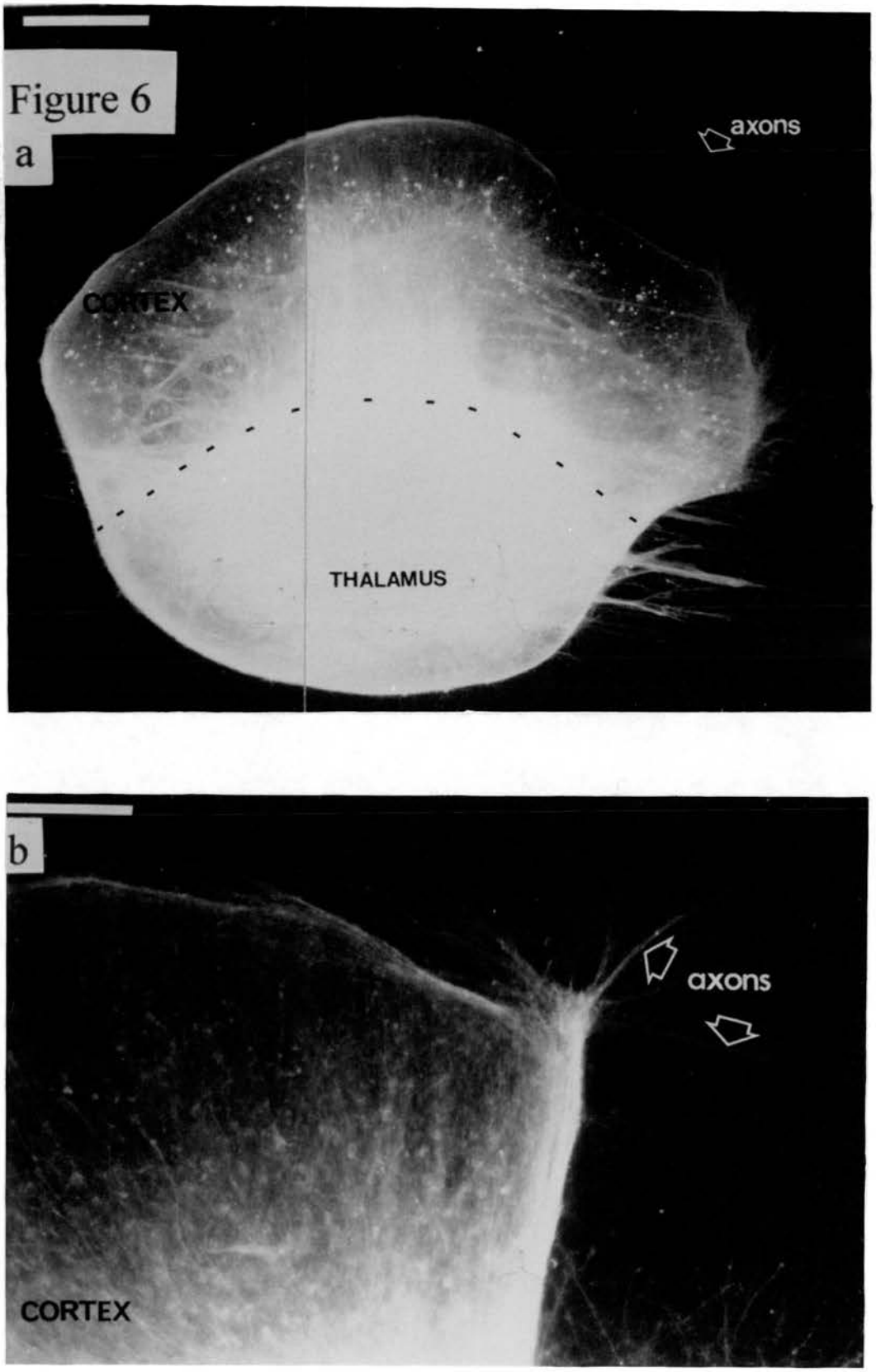


Figure 6
a
axons
CORTEX
THALAMUS
b
axons
CORTEX

Figure 2.7

Photomicrographs of co-cultures consisting of embryonic day 15 (E15) thalamus and postnatal day 2 (P2) visual cortex. This culture was grown for 6 days in serum free medium. (a) Reveals that most axons grow into the co-cultured cortex but do not terminate within it, instead they project onto the collagen membrane. Black dotted lines indicate the boundary between the thalamus and cortex. At P2 the termination signal within layer 4 is still not mature (scale =350μm).

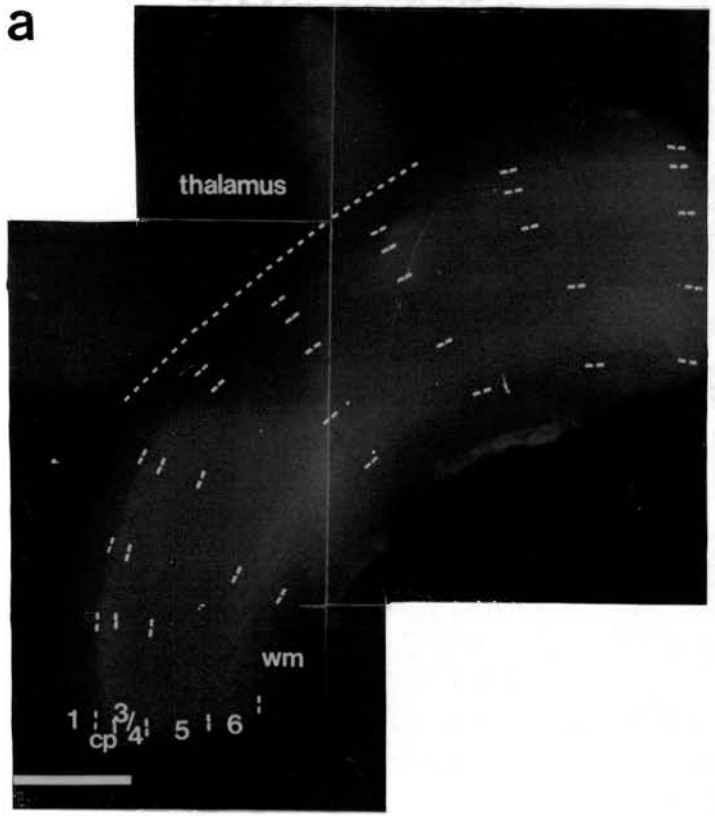
(b) Greater magnification reveals that thalamic axons fan out as they leave the cortex. The thalamic explant is not shown in this photograph but is situated below the cortex. Many axons turn and grow along the edge of the cortex next to the collagen membrane (scale =140μm).

Postnatal Development Of Isolated Cortical Explants.

P0 and P2 cortical explants were cultured for 6 and 4 days respectively, before they were co-cultured next to E15 thalamus for another 6 days. Results from these experiments generated interesting data. In P0 co-cultures, no outgrowth into the cortex was observed, although growth within the thalamic explant was demonstrated by swirling fasiculated axons. Many of these could be seen at the thalamic/cortical explant border (fig 2.11a,b,c). In P2 co-cultures there was excessive fasiculated outgrowth into the cortex and no recognition of layer 4 was observed (fig 2.10 a& b).

Effect Of Neurotransmitter Antagonists On Termination Within Layer 4.

Addition of many neurotransmitter antagonists to the culture medium (see Table 2.1) did not produce any adverse effects on either thalamic axon ingrowth or termination within layer 4 of co-cultured cortex. Fig 2.12 demonstrates that atropine had no effect on luminance within these co-cultures. The majority of drugs produced graphs remarkably like controls. However, AP-5 (GABA B antagonist) and Picrotoxin (GABA Cl^- channel blocker) reduced the amount of thalamic ingrowth into layer 5 and 6 of co-cultured cortical explants (fig 2.13a). More interestingly APV (NMDA antagonist) prevented many thalamic axons from terminating within target layer 4 (fig 2.13b). There was 60% luminance present within layer 2 of these cultures and 30% luminance at the outer edge of layer 1 indicating that many of these axons projected beyond the cortical explant onto the collagen membrane. For photomicrographs of co-cultures exposed to various drug antagonists refer to fig 2.14.


a
thalamus
wm
1
cp
3/4
5
6

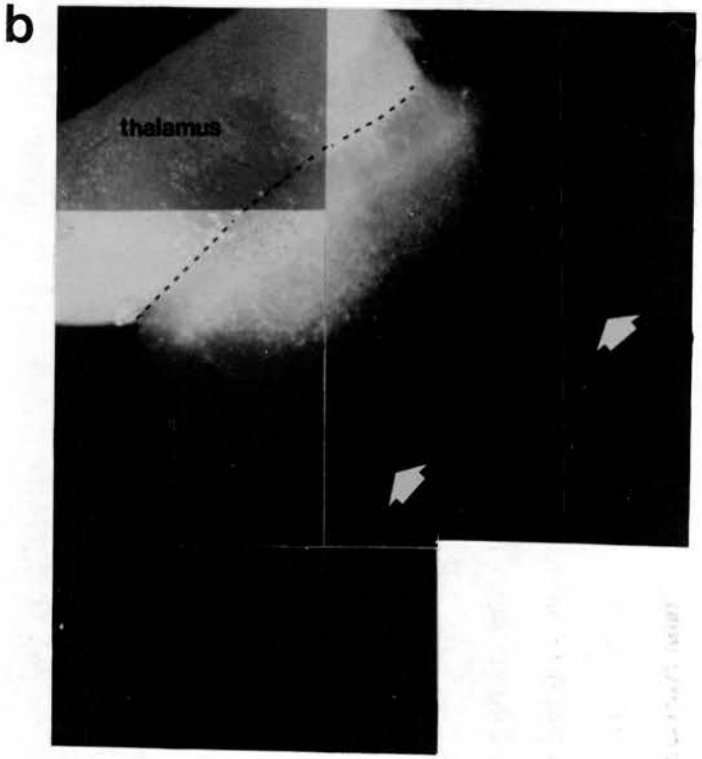

b
thalamus

Figure 2.8

Photomicrographs of identical co-cultures consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) visual cortex which have been cultured for 6 days in serum free medium. The thalamic explant has been placed adjacent to the pia edge (i.e. layer1) instead of layer 6. (a) Co-cultures have been counterstained with bisbenzamide to reveal the different layers. White dotted lines indicate the layer boundaries within the cortex. CP is the cortical plate and WM is white matter. (b) Thalamic axons have been stained with a carbocyanine dye to reveal the ingrowth into the cortical explant. By comparing these photographs it is obvious that thalamic axons do not choose to grow along the superficial layers, instead they grow through them and terminate within the region of layer 4 within the cortical explant. Black dotted line indicates the boundary between the thalamic explant and the cortical explant. White arrows indicate the outer edge of the cortex (i.e. layer 6)(scale =538μm).

GRAPH COMPARING THE INGROWTH OF THALAMIC AXONS THROUGH LAYER 6 OR LAYER 1 OF CO-CULTURED CORTICAL EXPLANTS.

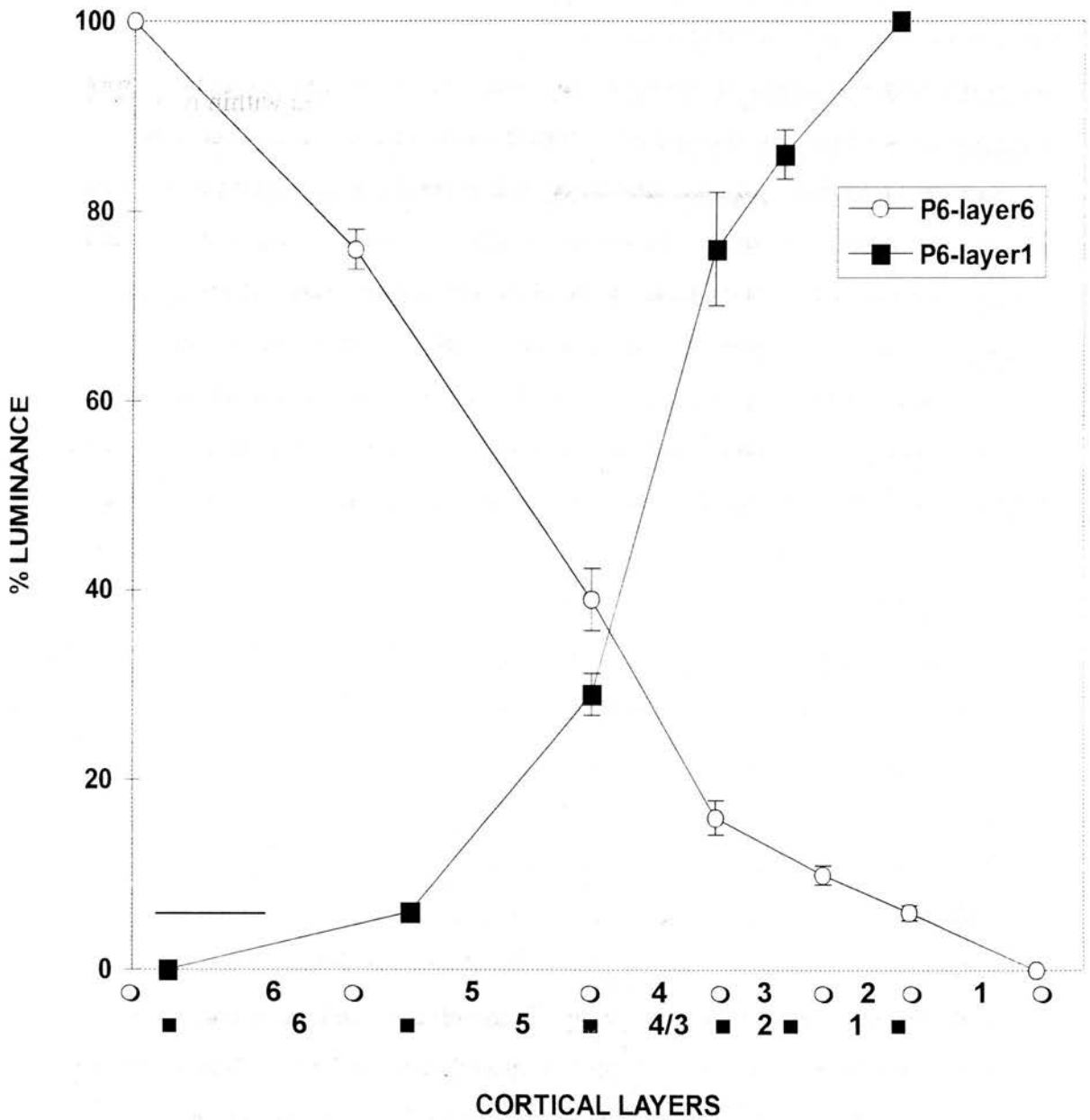


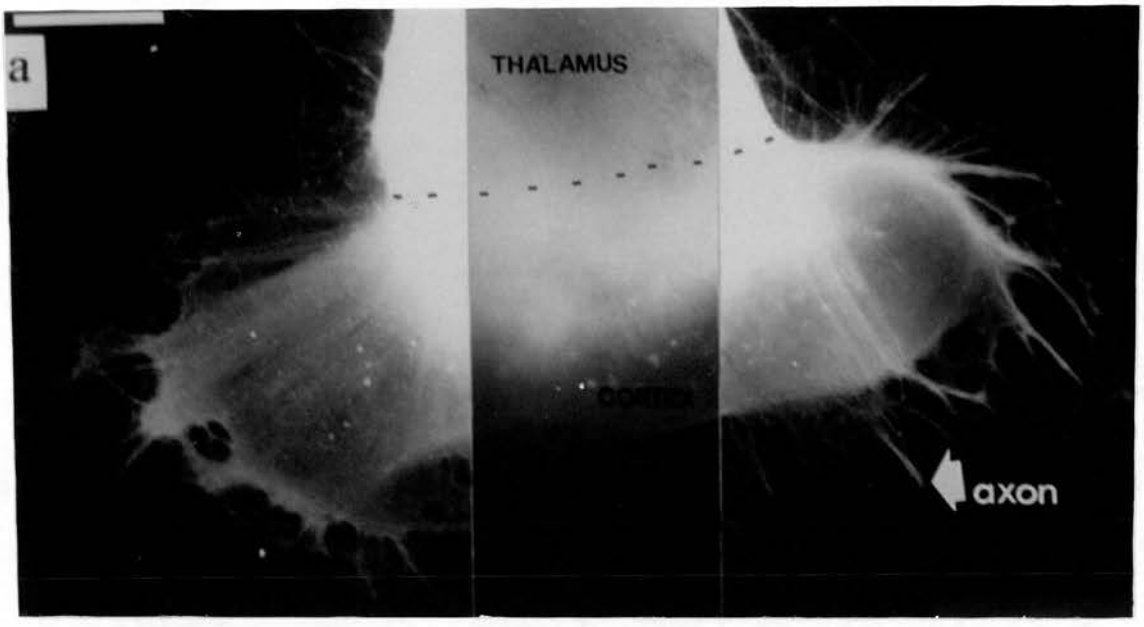
Figure 2.9

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex. Thalamic explants were placed either adjacent to layer 6 or adjacent to the pial edge (layer1) of the cortex. The co-cultures were cultured for 6 days in serum free medium.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance. When the thalamic explant was placed adjacent to layer 6 of the cortex, the luminance intensity at the thalamic explant and cortical layer 6 border was designated to be 100% luminance. When the thalamic explant was placed next to the pial edge (i.e. layer1) of the cortex, the luminance intensity at the thalamic explant and cortical layer 1 border was designated to be 100% luminance. The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. White circles and black squares on the X-axis represent the average width of each cortical layer for P6-layer 6 and P6-layer 1 cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer size values (see scale bar = 149μm). The graphs have been aligned on the X-axis at the layer 5/4 border. The n values for P6-layer 6 luminance data ranges from n = 19-28. The n values for P6-layer 1 luminance data ranges from n = 6-12.

This graph demonstrates the ingrowth of E15 thalamic axons into either layer 6 or layer 1 of P6 cortex. White circles represent ingrowth through layer 6 and black squares represent ingrowth through layer 1. Ingrowth through layer 1 (black squares) displays a very sharp decrease in luminance within the layer 4/3 region. Ingrowth through layer 6 (white circles) displays a less dramatic decrease in luminance within the layer 4/3 region. This graph demonstrates that thalamic axons are restricted to their target region even though they have taken a different route in order to reach it.


a
THALAMUS
axon

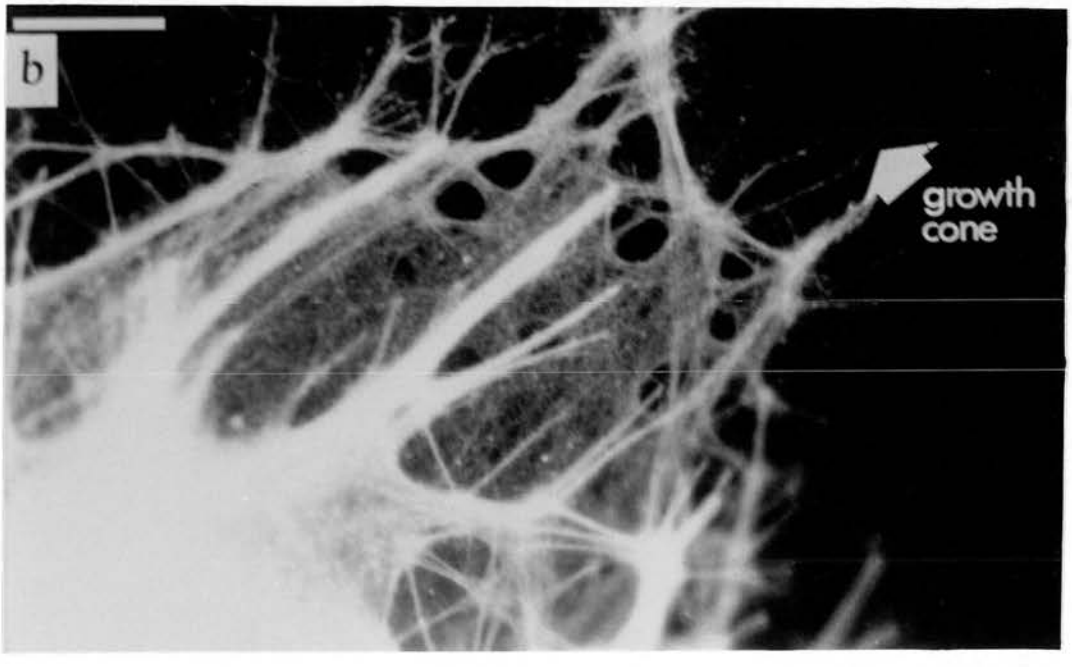
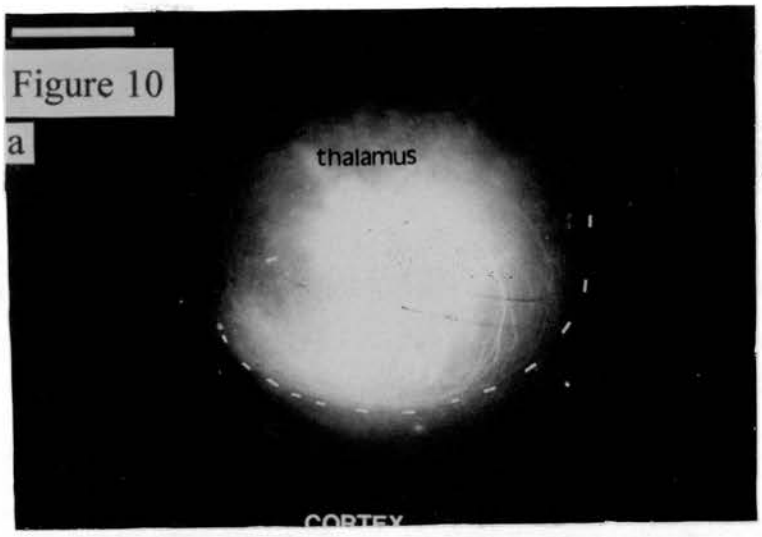

b
growth
cone

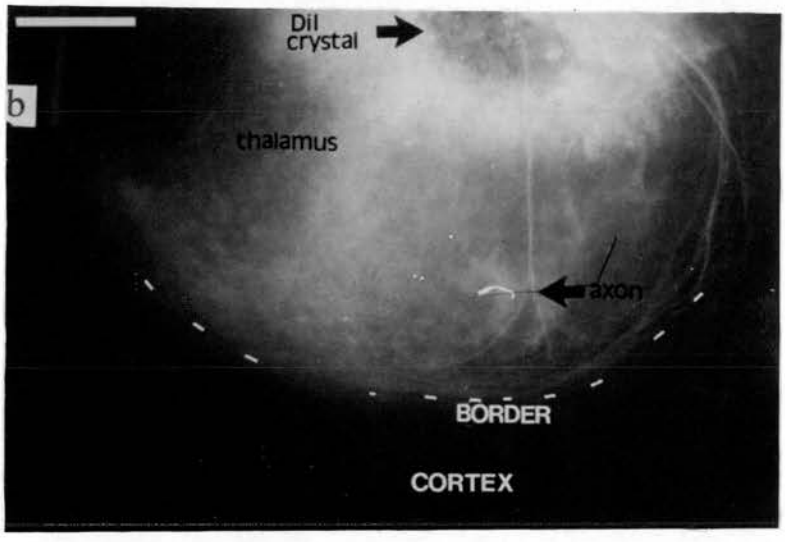
Figure 2.10

(a) Photomicrograph of a co-culture consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) visual cortex which has been cultured for 6 days in serum free culture medium. The cortex from this co-culture was placed in isolated culture at P2 and cultured for 4 days until it was P6, at this point the thalamus was added and the co-culture was then grown for a further 6 days. In these cultures there was excessive fasiculated outgrowth into the cortex and no recognition of layer 4 (scale =538μm). Black dotted line indicates the boundary between the thalamic explant and the cortical explant.

(b) As (a) but greater magnification. DiI labelled thalamic axons which are tightly fasiculated. Growth cones can be identified suggesting that this outgrowth is neuronal (scale = 140μm).

Figure 10


a
thalamus
CORTEX


DiI
crystal
b
thalamus
axon
BORDER
CORTEX

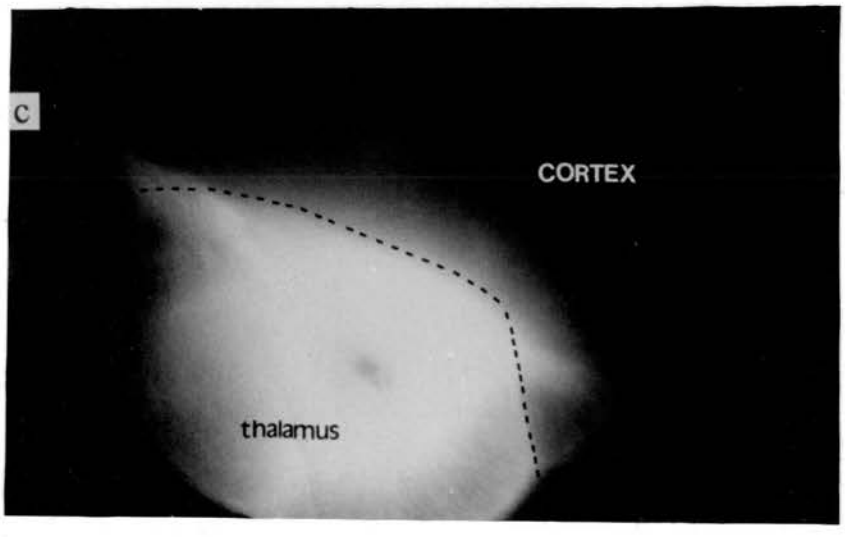

c
CORTEX
thalamus

Figure 2.11

Photomicrographs of a co-cultures consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) visual cortex which has been cultured for 6 days in serum free medium. The cortex from these co-cultures was placed in isolated culture at P0 and cultured for 6 days until it was P6, at this time the thalamus was added and the co-culture was then grown for a further 6 days. (a) & (c), in these cultures the thalamus failed to innervate the cortex even though a lot of growth occurred within the thalamic explant (scale =350μm). Dotted lines indicate the boundary between the thalamus and cortex. (b) This is the same co-culture as depicted in (a) but at greater magnification. Thalamic axons are seen to turn away from the cortex and grow along the thalamus/cortex border (scale =140μm).

GRAPH COMPARING THE INGROWTH OF THALAMIC AXONS INTO P6 CORTEX IN THE PRESENCE OF ATROPINE

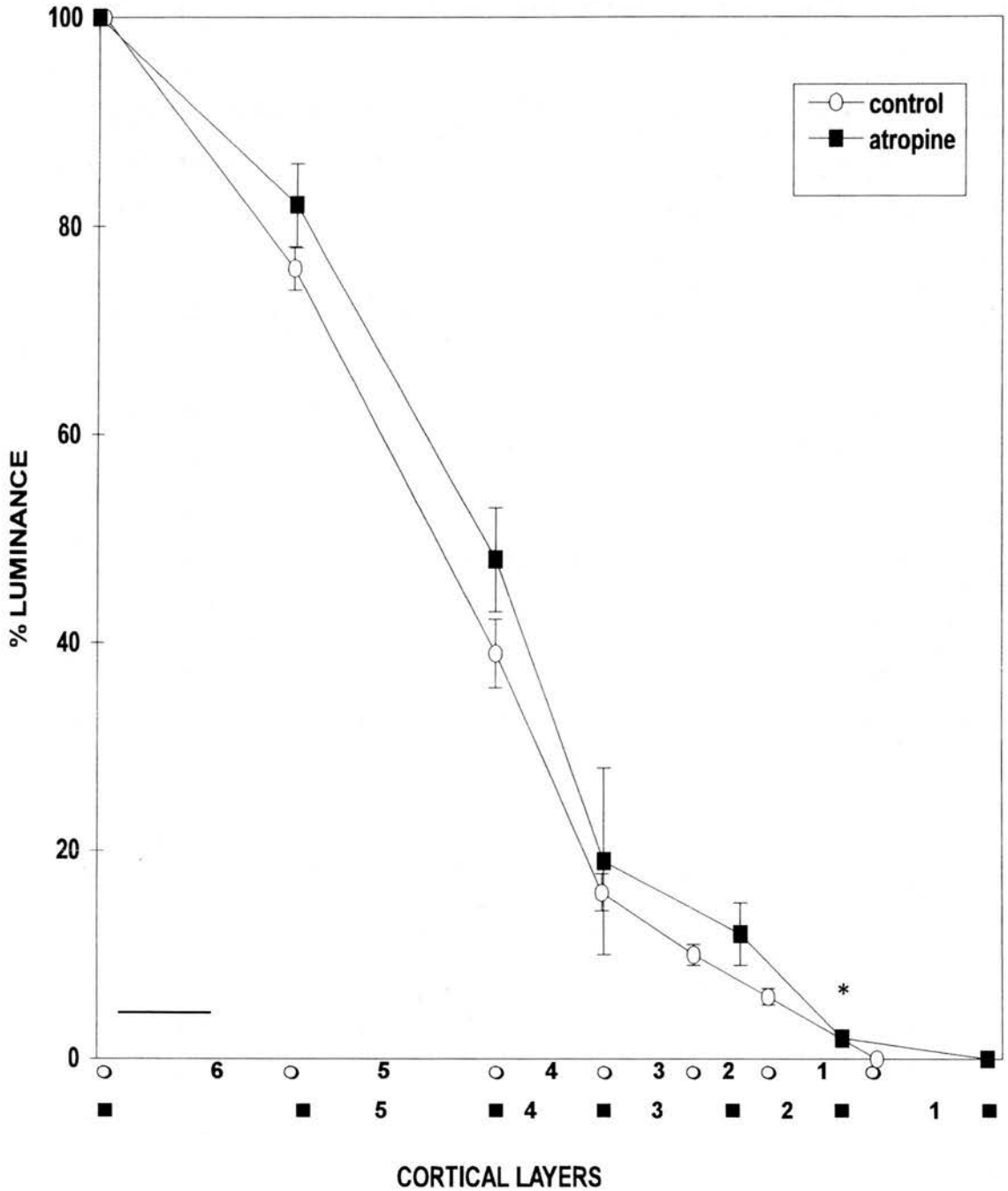


Figure 2.12

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex in the presence and absence of atropine. The co-cultures were cultured for 6 days in serum free medium and atropine was added at culture day 1.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. White circles and black squares on the X-axis represent the average width of each cortical layer for control and atropine treated cortices. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 141μm). The graphs have been aligned on the X-axis at the layer 5/4 border. The n values for control luminance data ranges from n = 18-28. The n values for atropine luminance data ranges from n = 4-8. The mean luminance values at each cortical layer border were compared statistically between control and atropine data using a Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/4, 4/3, 3/2, 2/1 and 1/collagen borders within controls were compared to the 6/5, 5/4, 4/3, 3/2, 2/1 and 1/collagen borders within atropine treated data respectively. Only the 2/1 border was significantly different between these two data groups.

The graph reveals that atropine treated co-cultures were very similar to controls and many axons were restricted within the region of target layer 4.

GRAPH COMPARING THE INGROWTH OF THALAMIC AXONS INTO P6 CORTEX IN THE PRESENCE OF PICROTOXIN AND AP5

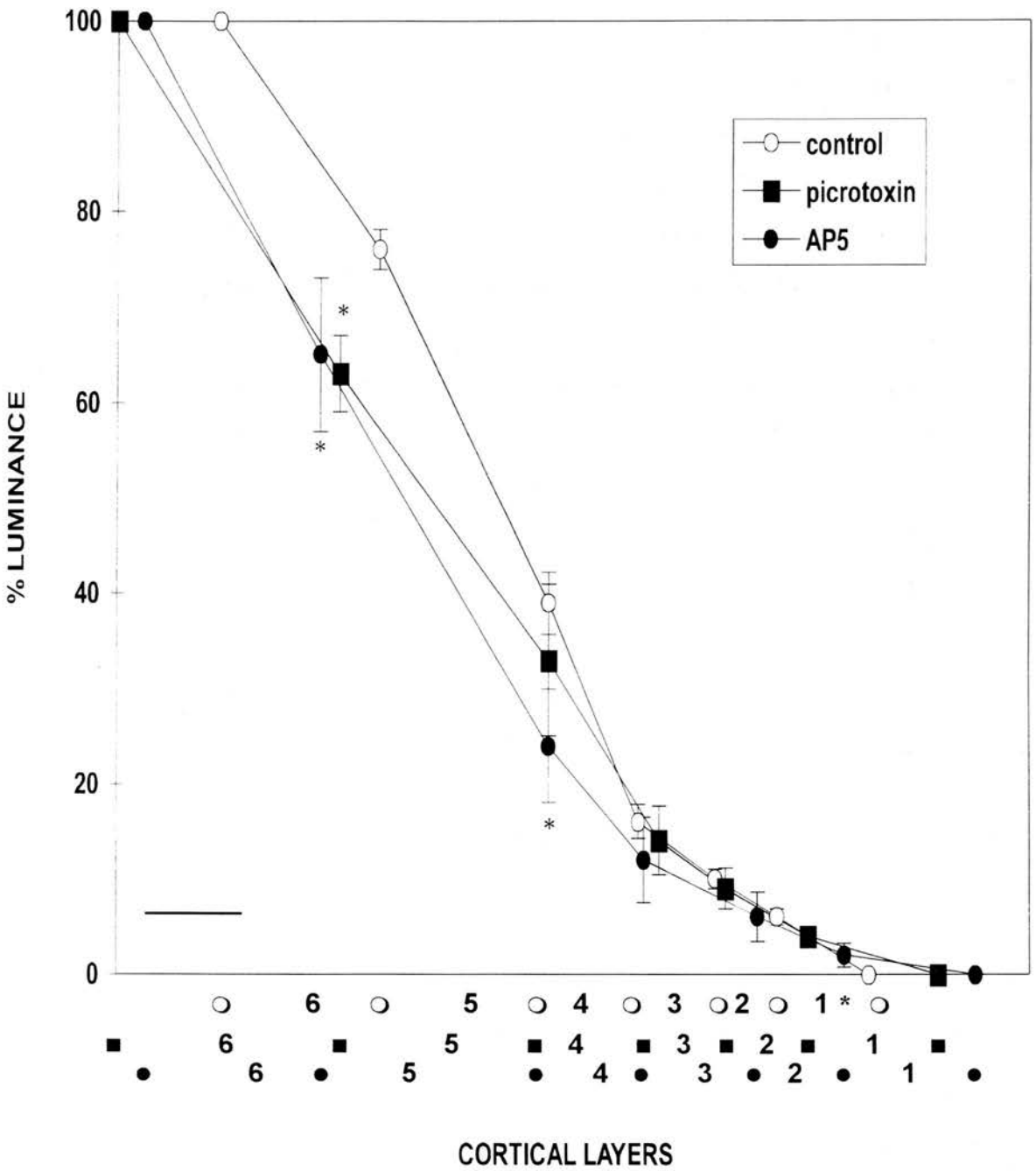


Figure 2.13a

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex in the presence and absence of picrotoxin (GABA Cl^- channel blocker) and AP-5 (GABA B antagonist). The co-cultures were cultured for 6 days in serum free medium and drugs were added at culture day 1.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. White circles, black squares and black circles on the X-axis represent the average width of each cortical layer for control, picrotoxin and AP-5 treated cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 175μm). The graphs have been aligned on the X-axis at the layer 5/4 border. The n values for control data ranges from n = 18-28. The n values for picrotoxin luminance data ranges from n = 3-6. . The n values for AP-5 luminance data ranges from n = 3-4. The mean luminance values at each cortical layer border were compared statistically between control and picrotoxin, and between control and AP-5 data. The statistical test used was the Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/4, 4/3, 3/2, 2/1 and 1/collagen borders within controls were compared to the 6/5, 5/4, 4/3, 3/2, 2/1 and 1/collagen borders within the drug treated data respectively. * are placed above black squares denoting picrotoxin and below black circles denoting AP5. In picrotoxin treated co-cultures only the layer 6/5 border was statistically significant from controls ($p<0.05$). In AP5 treated co-cultures the layer 6/5, 5/4 and 2/1 borders were statistically significant from controls ($p<0.05$).

GRAPH COMPARING THE INGROWTH OF THALAMIC AXONS INTO P6 CORTEX IN THE PRESENCE OF APV

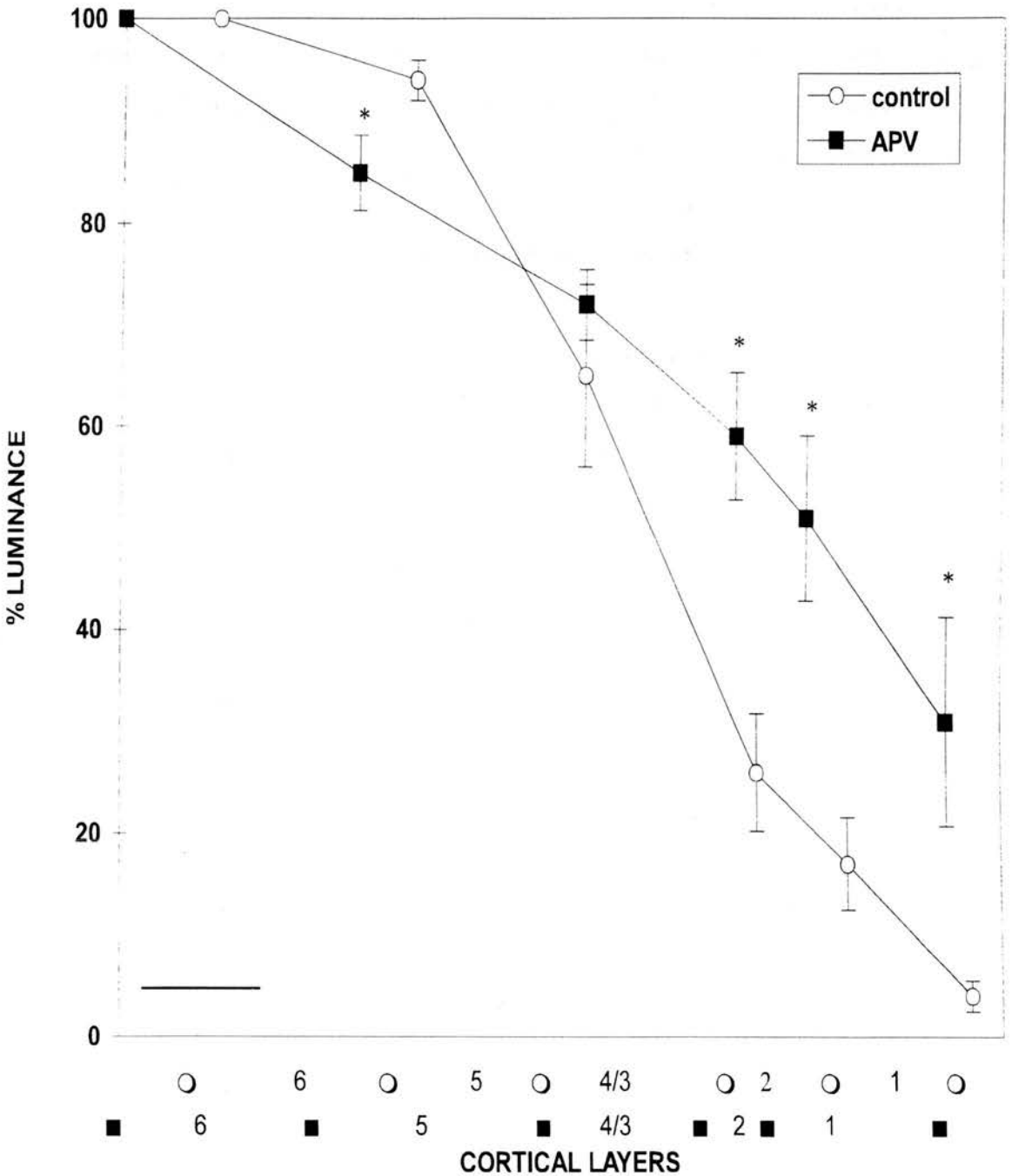


Figure 2.13b

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex in the presence and absence of APV (NMDA antagonist). The co-cultures were cultured for 6 days in serum free medium and APV was added at culture day 1.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. White circles and black squares on the X-axis represent the average width of each cortical layer in control and APV treated cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 146μm). The graphs have been aligned on the X-axis at the layer 5/4 border. n = 5 for control luminance data and n = 6 for APV luminance data. The mean luminance values at each cortical layer border were compared statistically between control and APV data. The statistical test used was the Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/4, 3/2, 2/1 and 1/collagen borders within controls were compared to the 6/5, 5/4, 3/2, 2/1 and 1/collagen borders within the drug treated data respectively. Only the APV 5/4 border was not statistically significantly different from controls ($p >0.05$).

In control co-cultures luminance decreased within layer 5 and 4/3 suggesting that thalamic neurites have been restricted within the region of their target layer 4. Only 25% luminance was present within layer 2 of control co-cultures. In APV treated co-cultures 60% luminance was present within layer 2 suggesting that only a few neurites terminated within target layer 4.

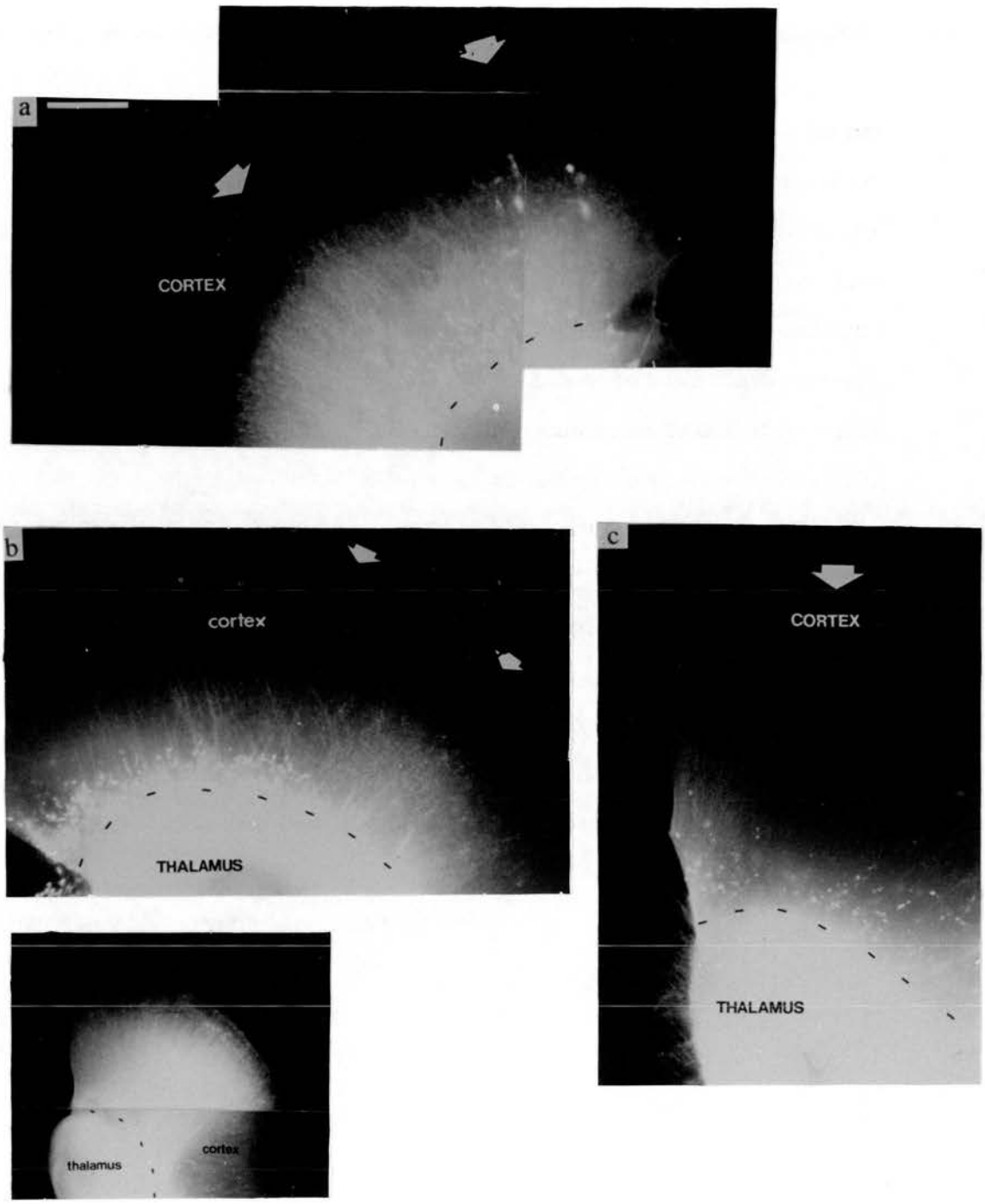

a
CORTEX
b
cortex
THALAMUS
thalamus
cortex
c
CORTEX
THALAMUS

Figure 2.14

Photomicrographs of co-cultures consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) visual cortex which has been cultured for 6 days in serum free medium in the presence of various antagonists. Black dotted lines indicate the boundary between the cortex and the thalamus. White arrows indicate the outer edge of the cortex. A crystal of carbocyanine dye which was placed within the thalamic explant has labelled thalamic axons which innervate the co-cultured cortical explant. These photomicrographs were taken under fluorescent microscopy. (a) Atropine (non-specific muscarinic antagonist for cholinergic receptors). (b) Propanolol (non-specific β adrenoreceptor antagonist). (c) AP-5 (GABA B antagonist). (d) APV(glutamate NMDA antagonist). In (a) the thalamic fraction of the co-culture is not included in the photograph. In both (a) and (b) most axons were restricted within the region of their target layer 4. In (c) few axons innervated the cortex. (d) Axons did not terminate in layer 4. (For a,b,c; scale =350μm: For d; scale =580μm).

Effects of APV On Cell Viability In Thalamocortical Co-Cultures.

Addition of APV to co-cultures consisting of E15 thalamus and P6 cortex produced no adverse effects on cell viability. In control co-cultures thalamic cells and cells found in the deep cortical layers (6 and 5) were mainly viable (refer to fig 2.20a), whereas cells found in the superficial cortical layers were pyknotic. In contrast, thalamic cells and cells found in both the deep and superficial layers of the cortex were viable in APV treated explants (fig 2.20b). Blocking NMDA receptors in these cultures has increased cortical cell viability.

Effect Of Blocking Activity On Termination Within Layer 4

Results from experiments with tetrodotoxin (5×10^{-7}M) (Na channel blocker) added after three days of culture indicated that termination within layer 4 may have been blocked (refer to fig 2.16). 30% luminance was present within the superficial layers and several axons projected onto the collagen membrane (fig 2.15a & b). This is not indicated by the graph because in layer I axons were very dispersed. Only a few axons at the layer 1/collagen border may have been present in the cross sectional area analysed. Photomicrographs also reveal that thalamic axons were generally more dispersed throughout the cortex and depict DiI labelled growth cones, proving that this outgrowth was neuronal. An anti-MAP-2 antibody (specific for neurons) was used to label neurites in tetrodotoxin treated co-cultures to provide further evidence that outgrowth was neuronal (fig 2.15c). Many DiI-labelled cells were present in the cortices of these co-cultures (possibly retrogradely labelled). Interestingly, many were localised in the superficial layers and others were distributed throughout the cortex

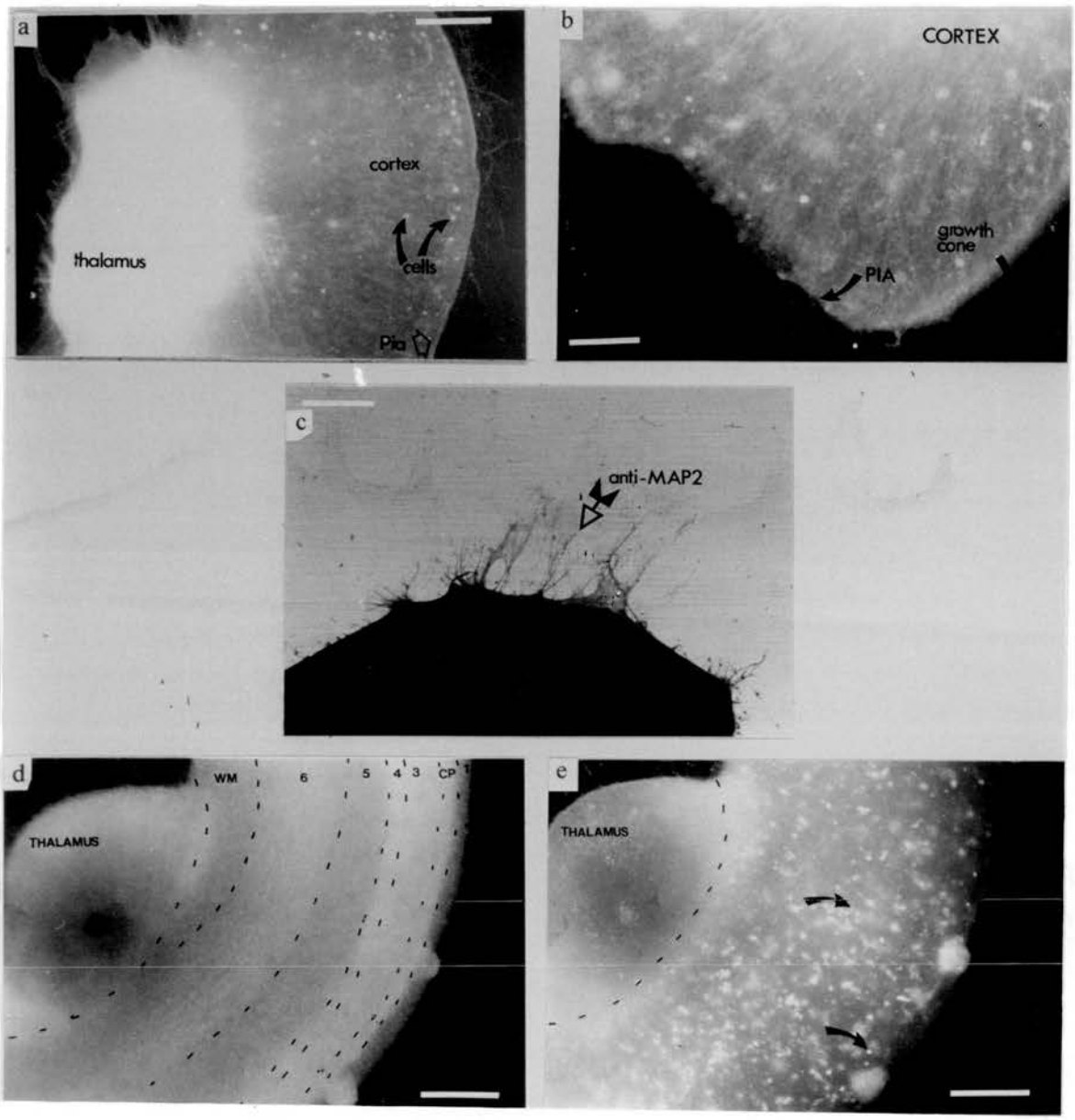

a
cortex
thalamus
cells
Pia
b
CORTEX
growth cone
PIA
c
anti-MAP2
d
WM
6
5
4
3
CP
1
THALAMUS
e
THALAMUS

Figure 2.15

Photomicrographs of co-cultures consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) visual cortex which has been cultured for 6 days in serum free medium in the presence of tetrodotoxin (sodium channel blocker). A crystal of carbocyanine dye which was placed within the thalamic explant has labelled thalamic axons which innervate the co-cultured cortical explant. These photomicrographs were taken under fluorescent microscopy. Black dotted lines indicate the boundary between the cortex and the thalamus and the different layers within the cortex. Black arrows indicate retrogradely labeled cells. WM is the white matter and CP is the cortical plate.

(a) 5 x 10^{-7}M tetrodotoxin added after day 3 of culture prevented thalamic axons from being restricted within their target layer 4 (scale =350μm). (b) Thalamic axons within the cortex viewed at greater magnification. Thalamic axons grew through the pia and onto the collagen membrane. These axons were not fasiculated (scale =140μm). (c) Thalamic explant not included in this photograph. Anti-MAP 2 antibody (specific for neurons) was used to label neurites in tetrodotoxin treated co-cultures to demonstrate that outgrowth was neuronal (scale =350μm). Thalamic axons grew out through layer 1 and onto the collagen membrane. (d) Bisbenzamide counterstaining revealed that the organotypic laminar structure was preserved in these co-cultures (scale =350μm). (e) tetrodotoxin added after day 5 of culture also disrupted termination within layer 4, however axons did not grow onto the collagen membrane (scale =350μm). Many cell bodies in the cortex of these co-cultures were labelled with DiI (a) and (e).

GRAPH COMPARING THE INGROWTH OF THALAMIC AXONS INTO P6 CORTEX IN THE PRESENCE OF TETRODOTOXIN.

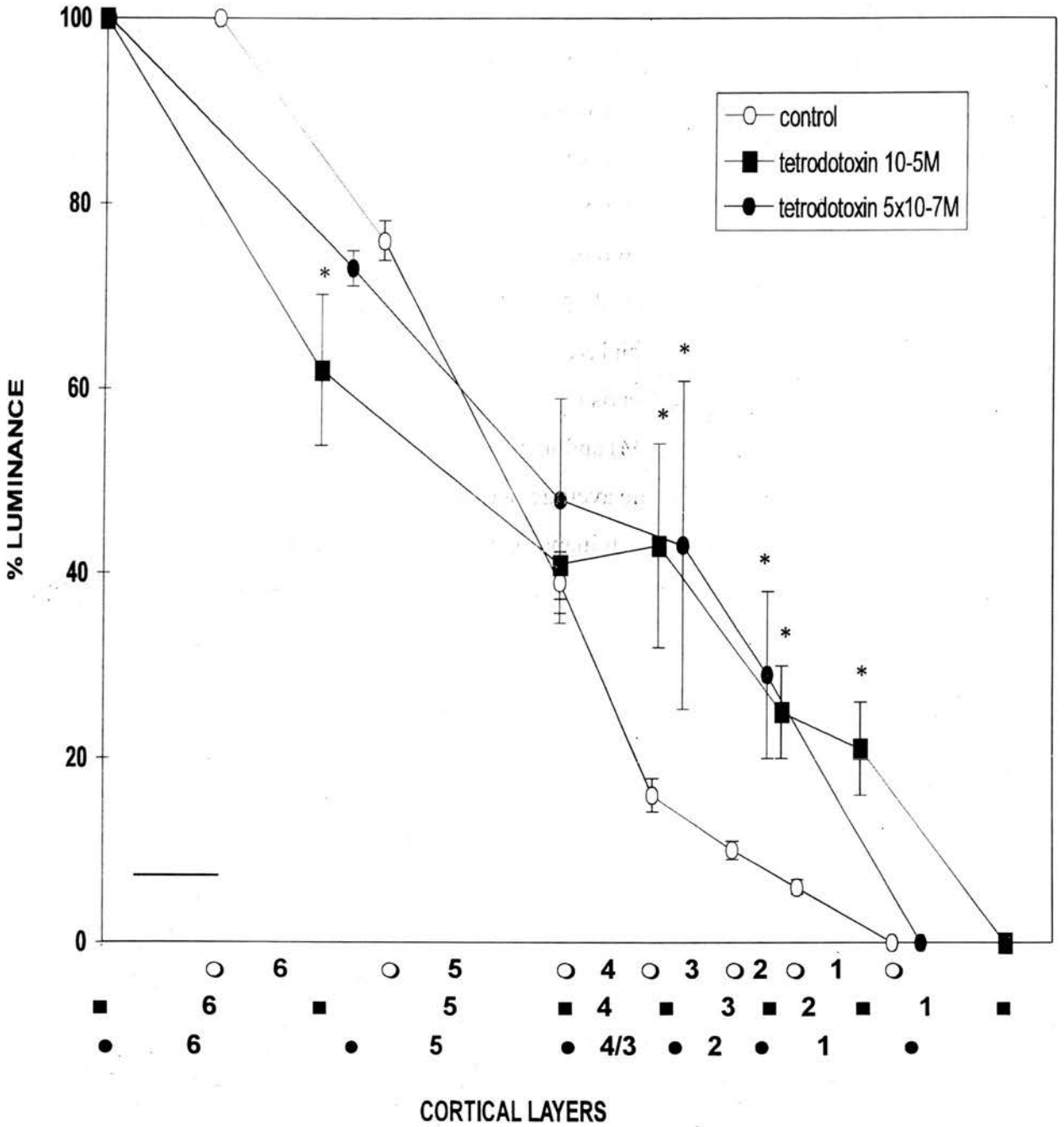


Figure 2.16

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex in the presence and absence of tetrodotoxin (Na channel blocker). The co-cultures were cultured for 6 days in serum free medium and tetrodotoxin (5×10^{-7}M & 10^{-5}M) was added after day 3 of culture.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. White circles, black squares and black circles on the X-axis represent the average width of each cortical layer in control, tetrodotoxin (10^{-5}M) and tetrodotoxin (5×10^{-7}M) treated cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 152μm). The graphs have been aligned on the X-axis at the layer 5/4 border. The n values for control luminance data ranges from n = 18-28. The n values for tetrodotoxin (5×10^{-7}M) luminance data ranges from n = 3-4. The n values for tetrodotoxin (10^{-5}M) luminance data ranges from n = 4-7. The mean luminance values at each cortical layer border were compared statistically between control and tetrodotoxin data. The statistical test used was the Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/4, 3/2, 2/1 and 1/collagen borders within controls were compared to the 6/5, 5/4, 3/2, 2/1 and 1/collagen borders within the drug treated data respectively.

In controls a sharp decrease in luminance is observed within layer 4, indicating that thalamic axons were restricted within the region of their target layer 4. In both

5 x 10^{-7}M and 10^{-5}M tetrodotoxin treated co-cultures there is a gradual decrease in luminance and ~30-50% luminance was present in superficial layers 3 and 2, indicating that thalamic axons were not restricted within the region of their target layer 4.

(fig 2.15a). This label was not observed in control E15-P6 co-cultures (fig 2.3a), although some labelled cell bodies could be seen in layer 5 and 6. Bisbenzamide staining revealed that the organotypic laminar structure was preserved in these co-cultures (fig 2.15d). Tetrodotoxin (5×10^{-7}M) added after 5 days of culture upset termination within layer 4 but did not produce excessive outgrowth or outgrowth onto the collagen membrane (fig 2.15e). However, the laminar structure was preserved and many cell bodies were labelled with DiI . Cortical explants of co-cultures exposed to 10^{-5}M tetrodotoxin were extremely delicate and had a tendency to disintegrate on touch (many were lost whilst trying to insert a crystal of DiI into the thalamus). Many cortical cells probably died upon exposure to this high concentration of tetrodotoxin and none of these co-cultures remained intact after histological processing.

Effect Of Increasing Activity On Termination In Layer 4

50mM K^+ added to co-cultures at the start of the culture period induced massive cell death and caused explants to deteriorate. Fig 2.17 compares the amount of DiI labelled axons entering the cortex to control co-cultures. This graph reveals that some ingrowth into layer 6 occurred. There were no labelled axons in layer 5. However, when 5mM K^+ was added after day three of culture unexpected excessive thalamic and possibly also cortical outgrowth occurred on both the collagen membrane and within the cortical explant (fig 2.18a &b). There was no indication that the thalamic axons preferred the cortex as a substrate and axons failed to recognise their target layer 4. Instead they continued to grow through the pial surface and onto the collagen for long distances. Fig 2.19 compares the amount of DiI labelled axons entering the cortex to control co-cultures. Approximately 60% of DiI labelled thalamic axons entering the cortex exited the explant through the pial surface and continued to grow on the collagen membrane. This outgrowth was neuronal because DiI labelled growth cones were observed (fig 2.18b). In the control experiments illustrated by fig 2.19 and 2.17, many axons appeared to terminate

within layer 5. It should be noted that in these experiments it was difficult to identify the borders of each cortical layer.

SMI32 Label Within The Cortex (P6-30).

To help determine the location of layer 4 within cortical explants, I began to develop a technique for labelling pyramidal neurons with SMI 32, an antibody specific for non-phosphorylated epitopes in neurofilament H. In other species SMI 32 positive neurons exhibit a high degree of regional and laminar specificity that is correlated with the functional and anatomical diversity of the cortical areas. SMI 32 specifically labels pyramidal neurons at the layer 4/5 and 3/4 border in the cat (Peter Kind: personal communication).

SMI32 appeared to label the perikarya and dendrites of a sub-population of large pyramidal neurons at the L5/4 border in P15 and P30 mice (data not shown). Label was not seen in younger mice.

EFFECTS OF K^+ (50mM, ADDED AT 0Hrs) ON TARGET RECOGNITION.

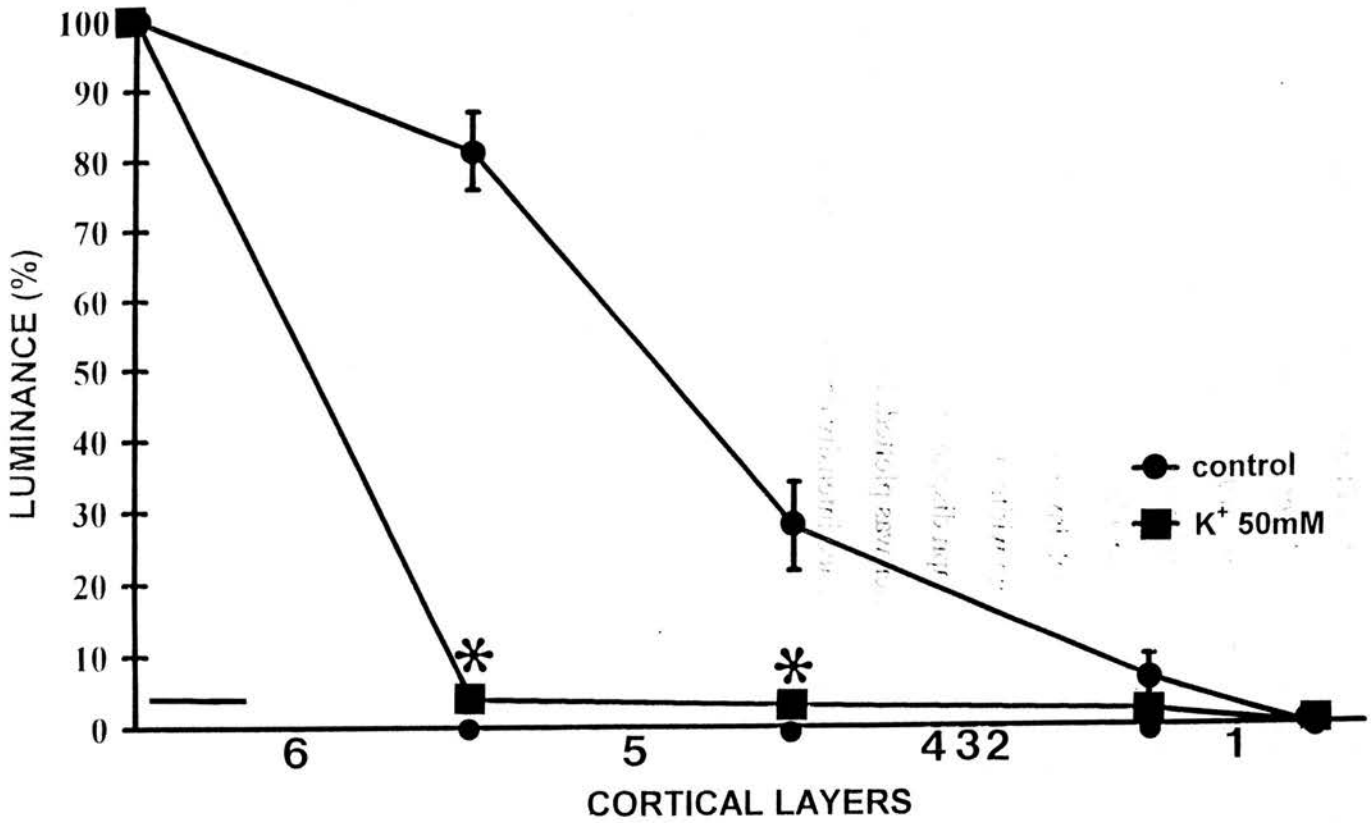


Figure 2.17

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex in the presence and absence of elevated K^+ (50mM K^+ added at the start of the culture period). The co-cultures were cultured for 6 days in serum free medium.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. Black circles and squares on the X-axis represent the average width of each cortical layer in control and K^+-treated cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 62μm). In these experiments the width of control and K^+ treated cortical layers were similar. Data for both K^+ and control cortical layer widths were averaged together. The graphs have been aligned on the X-axis at the layer 5/4 border. The n values for control luminance data is n = 3. The n values for K^+ (50mM) luminance data is n = 5. The mean luminance values at each cortical layer border were compared statistically between control and elevated K^+ data. The statistical test used was the Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/4, 2/1 and 1/collagen borders within controls were compared to the 6/5, 5/4, 2/1 and 1/collagen borders within the elevated K^+ data respectively. Luminance data on the 6/5 and the 5/4 borders were significantly different.

In controls a sharp decrease in luminance is observed within layer 4/5 region, in these experiments the bisbenzamide used was old and I did not achieve optimal staining. It

was difficult to differentiate the cortical layers. In K^+ treated cultures there was very little ingrowth into the co-cultured cortex.

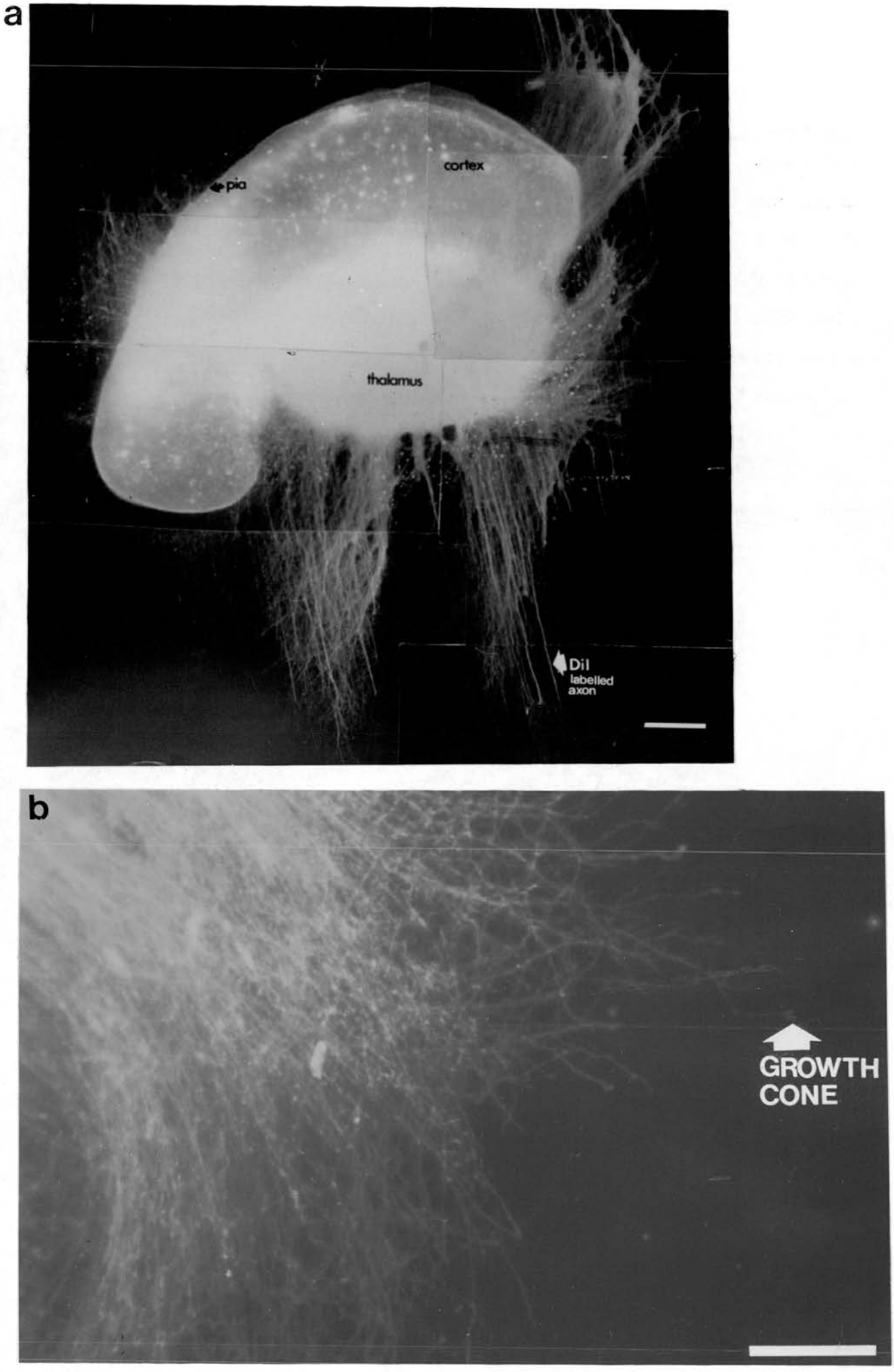

a
cortex
pia
thalamus
DiI
labelled
axon
b
GROWTH
CONE

Figure 2.18

Photomicrographs of co-cultures consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) visual cortex which has been cultured for 6 days in serum free medium in the presence of elevated K^+. 5mM K^+ was added after culture day 3. A crystal of carbocyanine dye which was placed within the thalamic explant has labelled thalamic axons which innervate the co-cultured cortical explant. These photomicrographs were taken under fluorescent microscopy.

(a) Elevated K^+ produced excessive outgrowth possibly from both the thalamic and cortical explant (scale =350μm). (b) This photograph reveals thalamic outgrowth onto the collagen as depicted in (a) except at greater magnification. DiI labelled growth cones are observed indicating that this outgrowth is neuronal (scale =140μm).

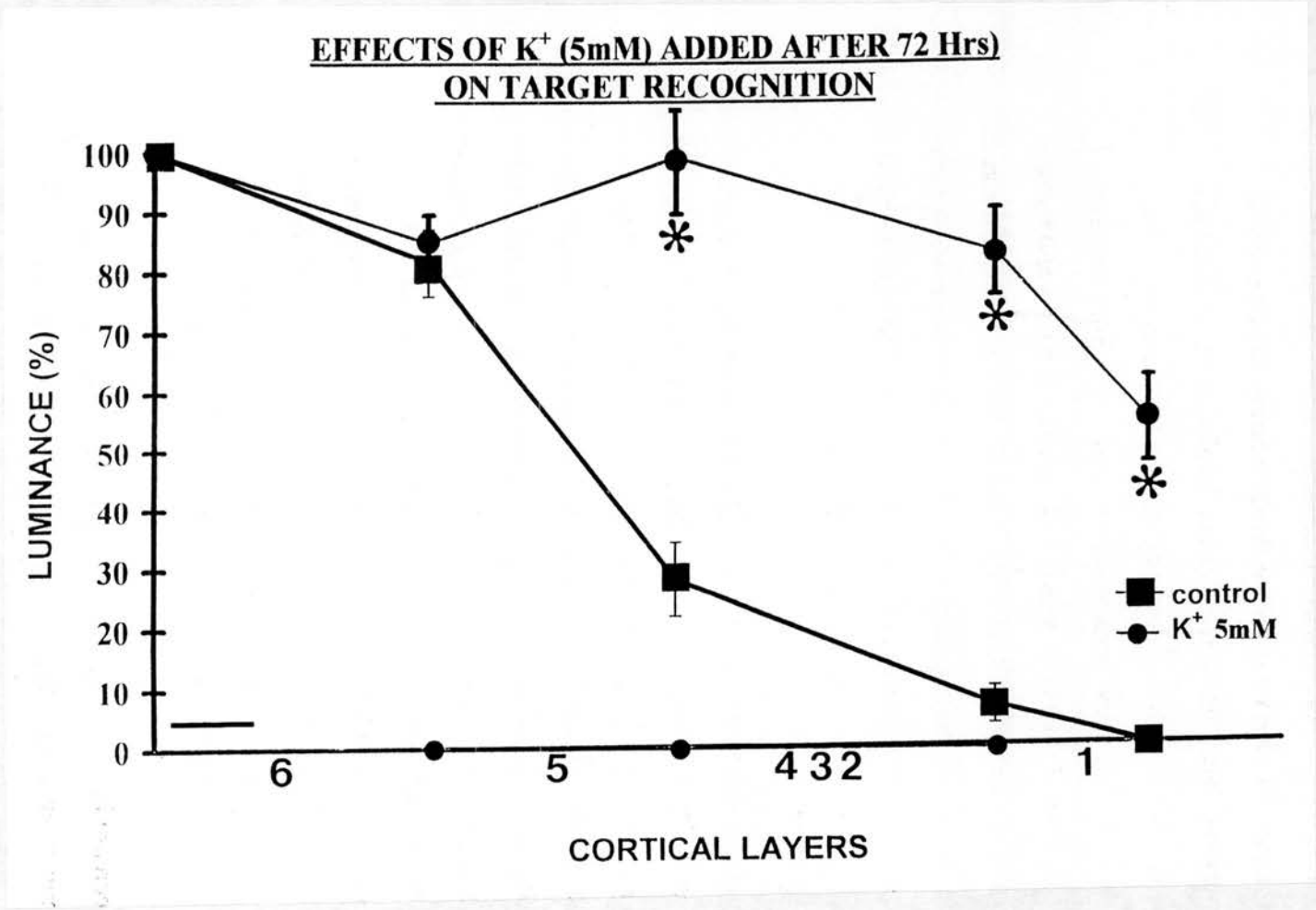

EFFECTS OF K+ (5mM) ADDED AFTER 72 Hrs)
ON TARGET RECOGNITION
LUMINANCE (%)
100
90
80
70
60
50
40
30
20
10
0
6
5
4 3 2
1
CORTICAL LAYERS
control
K+ 5mM

Figure 2.19

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex in the presence and absence of elevated K^+ (5mM K^+ added after day 3 of culture). The co-cultures were cultured for 6 days in serum free medium.

Using computer software the intenisty of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. Black circles and squares on the X-axis represent the average width of cortical layers in control and K^+-treated cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 67μm). In these experiments the width of control and K^+ treated cortical layers were similar. Data for both K^+ and control cortical layer widths were averaged together. The graphs have been aligned on the X-axis at the layer 5/4 border. The n values for control luminance data is n = 3. The n values for K^+ (5mM) luminance data is n = 5. The mean luminance values at each cortical layer border were compared statistically between control and elevated K^+ data. The statistical test used was the Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/4, 2/1 and 1/collagen borders within controls were compared to the 6/5, 5/4, 2/1 and 1/collagen borders within the elevated K^+ data respectively. Luminance data on the 5/4, 2/1 and 1/c borders were significantly different.

In controls a sharp decrease in luminance is observed within layer 4/5 region, indicating that thalamic axons were restricted to their target layer 4. In these experiments the bisbenzamide used was old and I did not achieve optimal staining. It

was difficult to differentiate the cortical layers. In K^+ treated cultures 60-70% luminance was present in superficial layer 1, indicating that most thalamic axons grew through target layer 4.

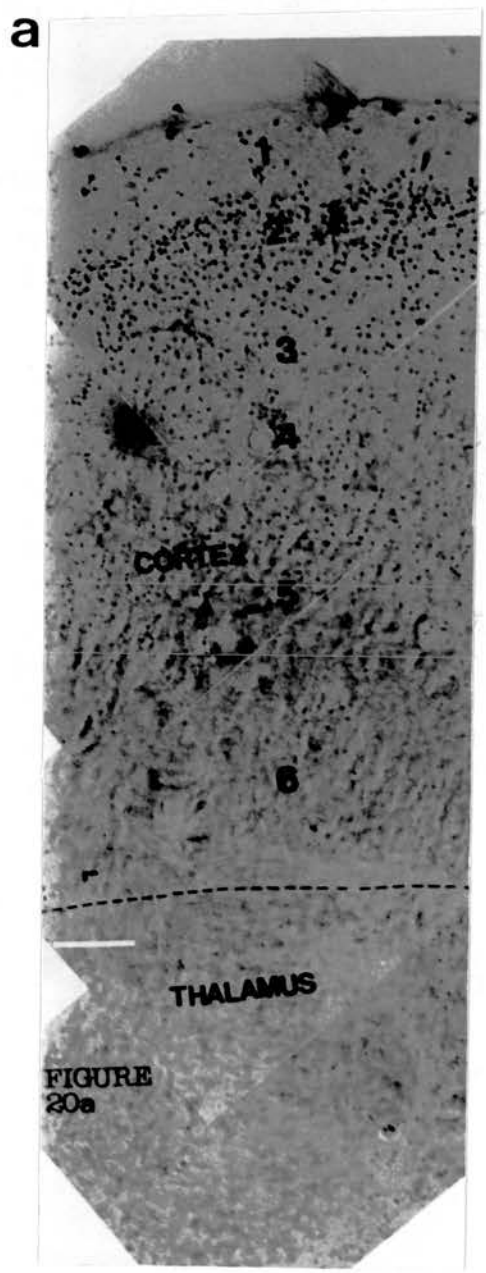


FIGURE 20a

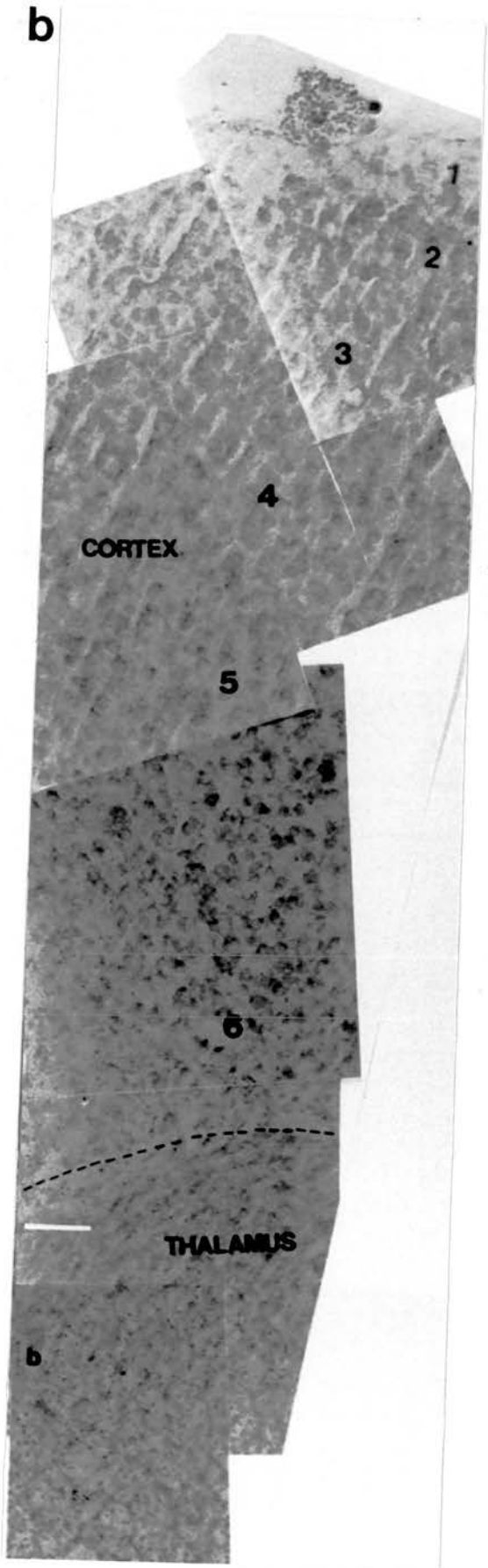


Figure 2.20

Photomicrographs of Nissl stained cross sections of co-cultures consisting of embryonic day (E15) thalamus and postnatal day (P6) cortex which have been cultured for 6 days in serum free culture medium in the presence and absence of 10μM APV (NMDA antagonist). Black dotted lines indicate the boundary between the thalamic explant and the cortical explant. Black numbers indicate the approximate position of the cortical layers. (a) In controls thalamic cells and cells in the deeper layers of the cortex are viable, whereas cells in the superficial layers of he cortex are pyknotic. (b) In APV treated co-cultures both thalamic cells and cells in all of the cortical layers are viable (scale= 70μM).

DISCUSSION

The aim of this chapter was to investigate whether neurotransmitters which are known to be present in the developing thalamocortical system *in vivo* play a role influencing thalamic axon outgrowth or recognition of their target cortical layer 4 *in vitro*. My results revealed that E15 thalamic axons innervated postnatal day 6 co-cultured cortex and these thalamic axons appeared to be restricted within the region of cortical layer 4 (their *in vivo* target). This is a model of reinnervation of the cortex and it is not a model of de novo targeting of thalamic neurons to layer 4.

This development proceeded normally in the presence of a wide range of neurotransmitter antagonists with the exception of APV, an NMDA antagonist, which appeared to disrupt axon termination within target layer 4. The maintenance of optimal activity levels also appeared crucial in controlling normal development. Increasing and decreasing activity levels disrupted the restriction of axons within layer 4. Excessive thalamic axon outgrowth occurred after stimulating activity levels with K^+ (although it is possible that some of this outgrowth may have originated from the cortex possibly by retrograde labelling of cortical cells). Increased activity levels could enhance survival and neurite outgrowth by upregulating a neurotrophic factor in these cultures. In tetrodotoxin treated cultures, levels of thalamic axon outgrowth were comparable to control cultures except that the majority of axons innervating the cortex failed to be restricted within their target layer 4. Many cortical explants died with 10^{-5}M tetrodotoxin; these co-cultures disintegrated whilst I was attempting to insert a crystal of DiI into the thalamic explant. It is possible that low levels of activity are required to keep thalamocortical co-cultures alive and activity dependent glutamate release may be involved in the termination of axons within layer 4.

Co-cultures Maintain their Organotypic Laminar Structure During Culture.

Co-cultures have a tendency to spread *in vitro* because they are no longer supported by surrounding structures, or because the tissue integrity is disrupted. It was

necessary to establish levels of spread in these co-cultures by comparing them to uncultured cortical explants. Results revealed that the percentage spread was low (~5%) and bisbenzamide staining revealed that the integrity of the tissue was well preserved and the six major layers of the cortex were clearly visible. There were no signs of disintegration that appears rapidly in cortical explants that have died due to technical problems (e.g. exhaustion of the gas supply). Evidence from other studies using thalamocortical co-cultures suggests that their development *in vitro* continues as *in vivo* (Bolz et al, 1990; Wolburg & Bolz, 1991, Rennie et al, 1992). Many retrogradely DiI labelled cell bodies were present in the deeper layers of control co-cultures, suggesting that corticothalamic efferents developed in these co-cultures. Although I did not directly assess the viability of many of these co-cultures previous experiments in our lab demonstrate that much older cortical tissue can survive for at least three days *in vitro* (Lotto & Price, 1995).

Background DiI Diffusion

Thalamic axons were visualised in these co-culture experiments by inserting a crystal of DiI into the thalamic explant. DiI is lipophilic and is therefore contained within the lipid membrane of axons. DiI diffused along innervating axons enabling them to be viewed under fluorescence microscopy. Thalamocortical co-cultures were grown for a brief period and fixed to establish levels of background diffusion. Results revealed that background DiI diffusion is minimal in these co-cultures. After 24 hours of growth several thalamic axons had sprouted and entered layer 6 of the co-cultured cortex. DiI label present in layer 6 & 5 is probably mainly due to labelled ingrowing axons. However, very little luminance was detected in layers 4, 3, 2 or 1. Therefore in these graphs it is reasonable to consider the value for luminance to be in direct proportion to the number of DiI labelled thalamic axons.

Termination of Thalamic Axons Within Cortical Layer 4

Layer 4 is the main recipient (~80%) of all projected input from the thalamus. Previous experiments have demonstrated that outgrowth from explants of the embryonic rat LGN terminate selectively in layer 4 of slices taken from both the

visual and frontal cortex of early postnatal rats (Yamamoto et al, 1989, 1992; Bolz et al, 1992; Molnar & Blakemore, 1991; Gotz et al, 1992). Here I provide the first significant evidence that E15 murine thalamic axons innervate and terminate in their target layer 4 of co-cultured murine P6 cortex *in vitro* and these results support those described by Lotto & Price (1994). It was necessary to establish reproducible evidence for axon termination in our co-culture set-up, to enable me to investigate a possible role of neurotransmitters in this signalling mechanism. The co-culture technique was optimised in these experiments resulting in vast outgrowth from the thalamic explants. Previous work with the feline/murine co-culture technique demonstrated thalamic innervation of kitten cortical explants and recognition of target layer 4 by ingrowing thalamic axons (Lotto & Price, 1994). Few axons choose to grow onto the collagen membrane when given the choice of a co-cultured cortical explant suggesting preferential growth into the cortex. The cortex seems to provide a better substratum for growth possibly due to the presence of extracellular matrix molecules. However, it should be noted that the collagen membrane can strongly support thalamic neurite outgrowth (see Chapter 4). Alternatively, the cortex may release molecules which guide thalamic axons towards it. There is no evidence of tropism from previous experiments (Rennie et al, 1994). In these experiments thalamic and cortical explants were cultured at a distance from each other within the same culture well. Thalamic axons displayed no directional outgrowth towards the cortical explant. However, this may be because the two-dimensional culture system cannot maintain concentration gradients which are necessary for chemotropism.

It was necessary to devise a relatively quick but quantitative analysis system to allow a wide spectrum of neurotransmitter antagonists to be screened. Counting individual axons was impossible since outgrowth was prolific. In this study a novel method using Siamcam analytical software was used to quantitatively detect levels of thalamic axons in cortical explants. Results revealed that the density of thalamic axons decreased sharply within layer 4 even though this layer is very small in comparison to thicker layers 5 and 6. This effect was less pronounced in the more immature medial regions of cortical explants. In fig 2.3, a decrease in luminance can be correlated with a decrease in the amount of ingrowing thalamic axons. These data

suggest that the density of thalamic axons decreased by approximately 20% within layer 6. This was not surprising as layer 6 does receive some thalamic innervation *in vivo*. However, the density of thalamic axons decreased a further 30% within layer 5. This layer does not receive afferent innervation *in vivo.* This number may represent axons that were still growing, either because they were stimulated to grow either towards the end of culture or have grown a longer distance to reach the cortex and have still not reached their target layer 4. Alternatively, if this effect was due to a termination signal in layer 4 it may not be specifically restricted to this layer in the culture system (due to cells spreading out in culture). However, data indicates that explants spread very little, limiting the amount of cell intermingling at designated boundaries (this does not exclude the possibility that some cells might migrate). Thalamic axons remained confined within the proximity of their target layer 4 in co-cultured cortex even after 10 days of culture suggesting that synaptogenesis may have occurred. Furthermore, isolated thalamic explants grow for long distances when cultured for 6 days suggesting that axons actively recognise layer 4 in co-cultured cortex.

These data provide further evidence that thalamic axons recognise their target layer 4 in organotypic cultures.

In P0 cortices the ingrowth of thalamic axons decreased evenly across the cortical layers. Thalamic axons were not restricted to any specific layer. Approximately 30% of axons were present at the outer edge of layer 1 and fasiculated axons grew out of the explant and continued to grow on the collagen membrane for long distances. Results with P2 co-cultured cortical explants were comparable to those observed with P0. These results are in agreement with previous work (Molnar and Blakemore 1991) which suggests layer 4 has an innate recognition system which has not yet developed in P0 cultures. My results also demonstrated that maturation of layer 4 is completed between P2 and P6. This is in agreement with previous theories of thalamocortical development which suggest that cortical layer 4 matures as thalamic axons are growing towards their target (Bolz et al, 1992). This may suggest that ingrowing thalamic axons are involved in activating their own termination within layer 4.

Thalamic Ingrowth Through the Pial Surface (i.e. Layer 1)

Co-cultures where the explant from the thalamus is positioned at the pial surface (Bolz et al, 1993) may have yielded clearer termination within layer 4, since this experimental paradigm reduces the distance thalamic axons must grow to reach their target layer 4. A gradual but continuous decrease in the density of thalamic axons occurs in thicker layers 5 and 6, this may be in part attributable to the *in vitro* system. These results provide further evidence that the superficial layers are not inhibitory to thalamic ingrowth and that layer 4 appears to have an innate recognition system. Stopping within layer 4 could be more accurately assessed if layer 4 could be located with more precision, e.g. with an antibody specific for the layer 3/4 and layer 4/5 borders. I conducted some preliminary studies on cortical slices with SMI 32, an antibody specific for neurofilament H in most mammalian species (in other species SMI 32 positive neurons exhibit a high degree of regional and laminar specialisation that is correlated with the functional and anatomical diversity of the cortical areas). The reaction was not successful in P6 cortical slices. P15 was the earliest age at which SMI 32 was observed, possibly because the antigen was not present at younger ages. Label was confined to the layer 4/5 border.

Postnatal Development of Isolated Cortical Explants.

Isolated P0 and P2 cortical explants were placed in culture to develop without extrinsic connections. At P6 (i.e. 6 and 4 days later, respectively), E15 thalamic explants were placed adjacent to the cortex and co-cultured for 6 days. Culture medium was changed at day 3 and day 8. Preliminary data from these experiments generated interesting results. Excessive thalamic axon outgrowth which was stimulated with P2 cortical explants may have been due to the release of diffusible growth factors from cortical explants which had accumulated in the culture medium. It has been shown that early postnatal neocortical explants promote the growth of neurites from thalamic explants cultured in serum-free medium (Lotto & Price, 1994, 1995; Rennie et al, 1994) and conditioned medium and conditioned substrate experiments have shown that this effect is mediated by diffusible cortex-derived growth factors (Rennie et al, 1994). It is possible that some axons were restricted

within layer 4 in these explants but this event could have been masked by numerous axons penetrating the superficial layers. However, P0 cortical explants were not innervated by thalamic axons even though DiI label revealed extensive axon growth within the thalamic explant. It is possible that P0 cortical explants did not develop *in vitro* as they would have *in vivo.* In these experiments it is also possible that the cortical explants no longer presented a suitable substrate for growing thalamic axons. Ghosh & Shatz (1993) demonstrated that ablation of the subplate in the cat with kainic acid *in vivo* prevents thalamic axons from innervating the cortex, despite its close proximity. It is possible that vital subplate interactions which support thalamocortical ingrowth were damaged or deteriorated in these cultures. Results from my experiments described above are in agreement with this finding and suggest that in the mouse critical subplate interactions occur between P0-P2. My experiments reveal that P0 cortex is permissive to thalamic ingrowth when they are immediately co-cultured together, it is likely that growth permissive signals downregulate in P0 cortex when it is isolated for 6 days before it is co-cultured with a thalamic explant. Gotz et al (1992) discovered that membrane bound growth promoting molecules (glycosylated proteins) distributed in the early postnatal cortex are upregulated in the developing cortex, in parallel with thalamic innervation. It is possible that in isolated P0 cortical explants growth promoting molecules were not upregulated due to the absence of thalamic innervation. After 6 days *in vitro*, the developmental window for the upregulation of these molecules had closed and when thalamic explants were placed adjacent to these cortical explants they could no longer upregulate these growth promoting molecules. Thalamic axons would therefore not innervate the unsuitable substrate presented by the cortical explant.

Effect Of Neurotransmitter Antagonists On Termination Within Layer 4.

Many neurotransmitter antagonists which were examined in this study had very little effect on thalamic ingrowth in thalamocortical explants, despite evidence from previous studies demonstrating that many of their receptors are present in this developing system (Kuhar et al, 1980; Sanderson & Murphy, 1981; Monaghan & Cotman, 1985; Tsumoto, 1990; Shaw et al, 1991). Picrotoxin (GABA Cl^- channel

blocker) and AP-5 (GABA B antagonist) reduced the amount of thalamic ingrowth to levels below that normally seen in control cultures. It is possible that GABA regulates the activity levels controlling thalamic neurite outgrowth. This is certainly true in developing hippocampal neurons where GABA plays an excitatory role driving neurite outgrowth (Ben-Ari et al, 1994). 10μM APV (NMDA antagonist) disrupted the restriction of axons within layer 4. This result could suggest that glutamate is involved in a signalling mechanism which inhibits thalamic outgrowth. In other systems glutamate released from entorhinal cortical explants produces an inhibitory action on co-cultured target hippocampal neurons (Mattson et al, 1988). More recently, it has been demonstrated that the arrest of afferent mossy fibres by cerebellar target neurons *in vitro* is regulated by the NMDA receptor (Baird et al, 1996). This is especially interesting as tetrodotoxin (Na channel blocker) also blocked the termination of thalamic axons within target layer 4 (see below), and in the experiment conducted by Mattson et al (1988) glutamate release was dependent on activity. Furthermore, there is considerable evidence that glutamate is released as an excitatory synaptic transmitter from terminals of geniculocortical axons (Tamura et al, 1990; Tsumoto et al, 1990). The evidence outlined above suggesting that glutamate may be involved in controlling thalamic outgrowth or the termination signal in layer 4 is interesting but information on the distribution of NMDA receptors in the developing rat cortex is limited. In the neonatal rat cortex, NMDA receptors are most densely distributed in the superficial layers (Monaghan & Cotman, 1985) but there is little information on the distribution of NMDA receptors in the early postnatal cortex. NMDA receptors are evenly distributed in the adult cat and monkey visual cortex (Rosier et al, 1993).

The above hypothesis is interesting but the survival of thalamocortical co-cultures in the presence of 10 μM APV (NMDA antagonist) produces a discrepancy with results reported in chapter 5. In chapter 5 exposure of isolated E17 thalamic explants to 10μM APV resulted in decreased cell survival (only 19% of the total cell population remained viable). I therefore proceeded to wax embed, section and Nissl stain the APV treated thalamocortical co-cultures to reveal any effects on cell viability. The results were interesting. In control co-cultures cells in the thalamus and the deeper

layers (6 and 5) of the cortex are mainly viable, however the superficial layers (3,2 and 1) of the cortex are not viable. In APV treated co-cultures both the thalamus and all the layers of the cortex are viable therefore the lack of axon restriction in layer 4 of these co-cultures is not symptomatic of a more global problem e.g. cell death. These results also raise the possibility that the cortex could release a factor (e.g. growth factor) which acts on the thalamus to override the toxic effects of glutamate. The reasons why the superficial layers of the cortex survive better in the presence of APV in comparison to controls is probably more complex. In control cultures survival may be reduced due to the lack of corticocortical interactions or other extrinsic inputs which may be necessary to maintain the viability of these cortical cells. If this critical innervation is absent glutamate induced death may occur. Blocking NMDA receptors with APV could help maintain the viability of the superficial layers (3, 2 and 1) in culture. In APV treated co-cultures axons do not stop in the deeper layers (as observed in control cultures) instead axons project through the cortex and onto the collagen membrane. It is also possible that under such circumstances the thalamic axons *in vitro* interact with cortical cells in the superficial layers and help to maintain their survival. Recent evidence suggests that thalamic axons can provide trophic support to the subplate by anterograde transport of neurotrophic factors. This trophic support helps to prolong subplate survival (Price & Lotto, 1996). It is possible that a similar process helps to maintain the survival of the superficial layers. Alternatively, the release of neurotransmitter by thalamic axons may be critical for driving an activity dependent survival mechanism in the superficial layers.

<u>Increased cortical cell survival may provide a better substrate for thalamic neurite growth.</u>

Other alternative explanations for the restriction of thalamic axons within target layer 4 also exist. It is feasible to suggest that thalamic axons do not grow into the superficial layers *in vitro* because these layers do not survive well in culture. It is possible that the effects of APV are non-specific and after treatment the viable superficial layers can now support the growth of thalamic axons Other workers

using the organotypic co-cultures technique who previously described termination within target layer 4 e.g.(Yamamoto et al, 1989, 1992; Bolz et al, 1992; Molnar & Blakemore, 1991; Gotz et al, 1992) did not directly investigate the viability of these co-cultures. Previous experiments in our laboratory (Lotto & Price, 1995) which demonstrated that cortical tissue can survive in culture for 3 days were conducted on feline not murine organotypic co-cultures. If this hypothesis is correct then stopping within layer 4 would simply be an artefact of the co-culture system. However, in experiments in which I placed the thalamic explant next to the pial surface, thalamic axons sprouted and innervated the cortical explant. These thalamic axons also terminated sharply within the region of their target layer 4. This result opposes my previous suggestion that termination within layer 4 is an artefact of the co-culture system. Furthermore, it has been previously demonstrated that neurite outgrowth can occur on cryostat sections of skeletal muscle (Covault et al, 1987) providing evidence that neurite outgrowth can occur on dead cells.

It is possible that the other antagonists did not penetrate the explants to sufficiently block all of the receptors, although this seems unlikely since the molecules are very small and striking effects on ingrowth were observed with APV. I could have investigated the possibility that these antagonists did not penetrate the tissue by performing radio- labelled ligand binding studies. Autoradiography would reveal levels of bound radio-labelled antagonists. The levels of bound radio-labelled antagonists could then be compared before and after culture. Alternatively, neurotransmitters may be important in regulating events earlier in development. Therefore important signals for ingrowth or termination would already be switched on in P6 cortical explants *in vivo*, before they are placed in the culture environment. For instance, critical interactions between the thalamic axons and the subplate would already have occurred. Gosh et al (1990) suggested that the subplate plays a critical role in guiding thalamocortical axons into their correct cortical area. After ablation of the subplate, geniculate axons failed to recognise their target area, even though layer 4 cells were present. It has also been demonstrated in kittens that the lateral subplate axons pioneer the pathway between the cortex and the internal capsule, creating a

scaffold for axons from both medial subplate and layers 6 and 5 to grow on (M^{c}Connell et al, 1989). Experiments in which the subplate was lesioned support this hypothesis. Following ablation of the subplate with kainic acid thalamocortical axons are prevented from entering the cortex (Allendoerfer & Shatz, 1994) and the targeting of 50% of corticothalamic axons is disrupted (M^{c}Connell et al, 1994).

Effect Of Blocking Activity On Termination Within Layer 4.

Tetrodotoxin (5 x 10^{-7}M) added after culture day 3 disrupted the restriction of thalamic axons in layer 4 of co-cultured cortex. In contrast to the overgrowth which occurred in P0 co-cultured cortical explants, growth was less fasiculated with tetrodotoxin. Many growth cones of thalamic neurites were labelled and immunostaining with MAP-2 confirmed that this outgrowth was neuronal. Activity does appear to play a role in segregating thalamic axons to their target layer 4. Wilkemeyer & Angelides (personal communication) observed appropriate target selection within the area of cortical layer 4 but reported an increase in the number of branches and varicosities on thalamocortical axons in the presence of tetrodotoxin. Shatz & Stryker (1988) have reported similar findings where prenatal tetrodotoxin infusion blocks segregation of retinogeniculate afferents. They suggest that spontaneous activity is present in their co-cultures and that it contributes to axon segregation within target layers. It should be noted that target recognition is not the same as axon segregation. Axon segregation often occurs after target recognition, however the involvement of activity is interesting. Types of activity that may be important include receptor mediated activation of ion channels which change the membrane potential as a result of sodium or calcium ion influx. Several tetrodotoxin (10^{-5}M) treated cultures disintegrated due to cell death. An increased cell death in the presence of tetrodotoxin was also observed in other cultured central neurons (Bergey et al, 1981; Lipton, 1986; Ling et al, 1991) and at the ultrastructural level disorganised synaptic terminals have been described after treatment with tetrodotoxin in cultured hippocampal neurons (Mattson et al, 1988).

Increased cortical cell survival could enhance thalamic outgrowth

I cannot directly determine whether the effects of tetrodotoxin were due to a direct disruption of the termination signal or simply due to a non-specific effect on cell survival. If tetrodotoxin did decrease cortical cell survival then the expression of specific inhibitory molecules within cortical layer 4 or within the superficial layers (3,2 and 1) might be suppressed. The absence of termination within layer 4 in the presence of tetrodotoxin could therefore be a non-specific effect of decreased activity. I would not be able to conclude that thalamic axons were involved in an activity dependent mechanism which switched on their own termination signal within layer 4. However, I did begin to section a few tetrodotoxin co-cultures (results not shown) and the survival of cortical cells appeared normal or was enhanced in comparison to controls.

If tetrodotoxin increased cortical cell survival it is possible that increased neurotrophin output from viable cortical cells enhanced thalamic outgrowth beyond target layer 4. However, the fact that thalamic axons recognise layer 4 even when they are placed next to the pial edge of cortical explants *in vitro* argues against the possibility that decreased viability in the superficial layers in culture simply inhibits thalamic outgrowth. Therefore, although increasing cortical cell survival in these explants could increase thalamic outgrowth, I do not believe that this could provide a simple explanation for why thalamic axons do not recognise layer 4 in the presence of tetrodotoxin.

Unusually there appeared to be a lot of retrogradely DiI labelled cells distributed throughout the cortex of tetrodotoxin treated co-cultures. Cortical layer 5 contains cells which project back to the thalamus and these connections have been reported to develop normally *in vitro* (Bolz et al, 1990). This retrograde label was observed within layer 5 in control co-cultures. However, this does not explain the label in the other layers. It is unlikely that by blocking activity with tetrodotoxin the laminar structure of these co-cultures was disrupted, since bisbenzamide staining revealed that lamination in these co-cultures was normal. Alternatively, tetrodotoxin may have stimulated cells to develop abnormal efferent cortical projections.

Tetrodotoxin (5 x 10^{-5}M) added after day 5 of culture also prevented thalamic axons from being restricted within their target layer 4 . However no or very few axons grew onto the collagen membrane. This demonstrates that blocking activity can disrupt axons which I presume may have already terminated in cortical layer 4, providing some further evidence that target selection may be an activity dependent process. Alternatively, the same arguments which are discussed above for tetrodotoxin having a non-specific effect on termination should also be considered.

Effects Of Enhancing Activity Levels In These Cultures.

Initially 50mM K^+ was added to the culture medium at the start of the 6 day culture period since this concentration is considered to induce depolarizing effects in other systems (Lipscombe et al, 1988; Wakade et al, 1983; Acheson et al, 1987; Cohen-Cory et al, 1991). There was no thalamic innervation of the cortex following this dose of K^+ and many co-cultures disintegrated. In the other studies cited above 50mM K^+ was added to culture systems for shorter periods, and in my experiments it is likely that toxic levels of intracellular calcium accumulated in these co-cultures. A lower dose of K^+ (5mM) was then added to the culture medium after day 3 of culture. This resulted in unexpected massive thalamic and possibly cortical neurite outgrowth. No thalamic axons were restricted to target layer 4 and outgrowth was disordered. It is possible that increased activity has enhanced the production of neurotrophic factors from either cortical and/or thalamic cells. It is well established that neurotrophic factors can increase cell survival and neurite outgrowth and more recent evidence has implied that increased neural activity can upregulate production of these factors (Hisanaga & Sharp, 1990 ; Castren et al, 1992; Gall,1992; Lu et al, 1991: Maffei et al, 1992; Patterson et al, 1992; Riva et al, 1992).

Direct effects of $K^{\pm}$ on thalamic neurons

Alternatively, increased K^+ could directly effect thalamic cell survival independently of increased neurotrophin production. The depolarizing effect of K^+ could activate voltage sensitive calcium channels and increase intracellular calcium levels which in turn could activate a wide range of intracellular signalling mechanisms affecting cell

survival and neurite outgrowth (Benari et al, 1994; Collins and Lille, 1989; Collins et al, 1991; Koike et al, 1989; Koike & Tanaka, 1991). For example, increased activity could increase neurotransmitter systems, these are known to play a role in neuronal growth and cell survival (Bessho et al, 1994; Mattson & Kater, 1994).

Conclusion

The main aim of this chapter was to investigate the role of neurotransmitters in thalamocortical development *in vitro* since these molecules are being increasingly implicated in the regulation of neuronal development and axonal pathway formation in the central nervous system. In order to investigate this question I had to develop a suitable analytical system. I refined the organotypic thalamocortical co-culture system to provide reproducible evidence, using a novel analytical method, that the growth of thalamic axons is restricted within cortical layer 4 in co-cultured cortex. This work also added further evidence that the cortical superficial layers are not inhibitory to thalamic growth. Previous studies had provided conflicting evidence on this point (Bolz et al, 1992; Yamamoto et al, 1992). Preliminary studies investigating the development of isolated cortical explants also indicated that early thalamocortical or subplate interactions are important for stimulating thalamic innervation into the murine postnatal cortex, and these signals are downregulated or not produced when isolated P0 cortex is placed in culture and all extrinsic connections are severed.
I investigated neurotransmitters which had previously been demonstrated to have receptors present on geniculocortical afferents or within the developing neocortex. APV (NMDA antagonist) blocked thalamic axons from being restricted within layer 4 suggesting that glutamate may have an inhibitory action on these growing neurites. Results from experiments with tetrodotoxin and K^+ reveal that activity levels are important in controlling thalamocortical development. An optimal level of activity is probably crucial for generating appropriate signals for thalamic ingrowth and termination within layer 4 of the cortex. There is a lot of evidence emerging that supports the hypothesis that some of the elements underlying axonal growth, branching and synaptogenesis are modulated by electrical activity, either in the form of afferent mediated activity, or in early stages, via spontaneous activity. Electrical

activity has been shown to influence a variety of physiological properties in neurons, including axonal sprouting at the neuromuscular junction (Brown & Ironton, 1977; Brown et al, 1981), the expression and organisational pattern of neuronal macromolecules (Hendry & Bhandari, 1992; Hockfield et al, 1990), the extent and pattern of dendritic growth and branching (Dalva et al, 1994; Schilling et al, 1991), and the rate and extent of neurite outgrowth (Cohan & Kater, 1986; Fields et al, 1990).

It is possible that the termination signal may be activated when a critical amount of neurotransmitter, released from ingrowing growth cones, depolarizes layer 4 cell membranes. This criterion would not be reached in poor quality cultures with reduced ingrowth, hence the importance of optimising outgrowth in these co-cultures. Increasing activity with K^+ could enhance basal levels of thalamic outgrowth to such extremes that not all axons could be terminated in layer 4 (non-specific depolarization of the membrane with K^+ could enhance growth factor production etc.). Blocking activity with tetrodotoxin (Na channel blocker), could block the termination signal in the cortex but may not affect ingrowing axons. This hypothesis is especially interesting since APV (NMDA antagonist) also blocked termination within target layer 4. It is possible that glutamate release may be activity dependent. Results from these experiments were interesting and suggest that termination within layer 4 is an activity dependent event. I decided to investigate fully the effects of activity on developing isolated thalamic cells *in vitro*, since results from these experiments are complicated by the presence of co-cultured cortical explants. This study is described in Chapter 4. I also decided to investigate the effects of NO, a novel neurotransmitter which has been shown to inhibit neurite outgrowth by producing direct effects on growth cones (Douglas et al, 1993). Furthermore, NO production has often been described to be regulated by local glutamate receptor stimulation (Bredt & Snyder, 1989; Garthwaite et al, 1989; Johnston & Morris, 1994). This study is described in Chapter 3.

This study has led me to conclude that the regulation of thalamic neurite outgrowth may be delicately controlled by activity levels in these cultures. It has also demonstrated the need for careful interpretation of results from experiments

conducted on the organotypic co-culture system. This is due to problems with decreased cell viability in the cortical explants.

CHAPTER 3:

THE DISTRIBUTION AND ROLE OF NITRIC OXIDE IN THE DEVELOPING THALAMOCORTICAL SYSTEM *IN VITRO*

CHAPTER 3: THE DISTRIBUTION AND ROLE OF NITRIC OXIDE IN THE DEVELOPING THALAMOCORTICAL SYSTEM *IN VITRO*

ABSTRACT

Application of 0.1mM 3-Morpholinosydnonimine (SIN-1) (which releases nitric oxide) to dissociated embryonic thalamic neurons in culture induces growth cones to collapse and retract towards the cell body. The expression of Nitric Oxide Synthase (NOS), the enzyme which synthesises NO from L-arginine is upregulated in the cortex *in vivo* in parallel with thalamocortical innervation. However, although NOS is present in the thalamus it is not present in the cortex of thalamocortical co-cultures which have been cultured for 6 days. Acute cortical slices were compared to slices of cortex which had been cultured alone for 6 days. NOS was present in acute cortical slices but it appeared to have downregulated in culture. NOS was also present in thalamocortical tracts *in vivo*. NOS production was blocked *in vitro* using NOS antagonists (7-nitroindazole) to examine any effects on thalamic outgrowth. Blocking NOS prevented thalamic axons from terminating within their target layer 4 *in vitro*. However, addition of 7-nitroindazole to these co-cultures also decreased the survival of cortical cells, therefore the absence of axon termination within layer 4 may have been symptomatic of increased cortical cell death.

These results suggest that NO is important in regulating thalamocortical development.

INTRODUCTION

Nitric Oxide (NO) is a remarkable regulatory molecule; it acts both as a secondary messenger and neurotransmitter and has been implicated in an extraordinarily diverse range of physiological functions. NO was initially identified as a mediator for macrophages and endothelial cells; it enables white blood cells to kill tumour cells and bacteria, and it allows neurotransmitters to dilate blood vessels. However, since Garthwaite et al,1988, discovered that cultured cerebellar cells release NO after exposure to glutamate analogues, this simple molecule has become the subject of intense research and has recently been recognised as a prominent neuronal messenger. Although there is considerable evidence that NO functions as a neurotransmitter, it is an unusual transmitter, in that it is a labile free-radical gas that is not stored in synaptic vesicles. NO is synthesised by NO synthase (NOS) from L-arginine via a Ca^{2+}/calmodulin-dependent mechanism, probably on demand. Thus increases in intracellular Ca^{2+} resulting from activation of voltage or ligand -gated Ca^{2+} channels could activate the enzyme. NO does not act at a membrane receptor protein, instead it simply diffuses into an adjacent neuron, as opposed to the exocytosis by which conventional neurotransmitters are released. Its receptor target is iron in the active centre of the enzyme that forms cyclic GMP, namely guanylyl cyclase. By binding to iron, NO initiates a 3-dimensional change in the shape of the enzyme, which increases its activity, and consequently the production of cGMP. The action of NO is terminated by diffusion away from its targets, or by forming covalent linkages to the superoxide anion or scavenger proteins.

Localisation Of NOS In The Brain

Immunohistochemical staining with an antibody raised against the purified neuronal NOS antigen (Bredt et al, 1990) revealed that brain neuronal NOS occurs only in neurons and these NOS neurons comprise only about 1-2% of all cells in many areas, such as the cerebral cortex, the hippocampal formation, and the corpus striatum. Recent studies reveal that the distribution of guanylyl cyclase and NOS differ

markedly, indicating that NO influences other targets. However, all NOS neurons stain for NADPH-diaphorase (Dawson et al, 1991; Hope et al, 1991). Anthony Pearse in 1960 observed that when he stained brain slices with a dye called nitro blue tetrazolium, certain neurons turned bright blue when he added an enzyme cofactor called reduced nicotinamide adenine dinulcleotide phosphate (NADPH). Later in 1991, Dawson et al, revealed that NOS is co-expressed with NADPH-diaphorase and that NOS catalytic activity accounts for diaphorase staining. The dye accepts electrons that normally are used to oxidise arginine to nitric oxide, creating a blue colour. In brain homogenates, most of the diaphorase activity is unrelated to NOS (Hope et al, 1991). However, in paraformaldehyde- fixed tissue, NADPH-diaphorase staining is coincident with NOS immunoreactivity in neurons (Dawson et al, 1991). The paraformaldehyde fixation presumably inactivates virtually all NADPH-dependent oxidative enzymes except NOS, enabling the NADPH-diaphorase stain to specifically label NOS neurons. This histochemical method has been widely used to investigate the distribution of NOS in the brain. Its localisation is exclusively neuronal and endothelial.

NO; A Novel Signalling Molecule During Neurodevelopment.

NO was first identified in the nervous system by Takeo Deguchi, 1982, when he noticed that cyclic GMP formation in the brain requires arginine. NO was later postulated to act as a neuromodulator or neurotransmitter because NOS inhibitors block stimulation of cGMP synthesis in brain slices by glutamate acting at NMDA receptors (Bredt & Snyder, 1989; Garthwaite et al, 1989). Other studies support the suggestion that NO influences neurotransmitter release. In several model systems, NOS inhibitors, such as nitroarginine, block the release of neurotransmitters (Dickie et al, 1992; Hanbauer et al, 1992; Lonart et al, 1992; Zhu & Luo, 1992) and NO produced by endogenous NOS can inhibit GABA(A) receptor function in cerebellar granule cells (Zarri et al, 1994). Recent investigations reveal that neuronal and endothelial forms of NOS are inducible in the sense that in the response to certain stimuli, new enzyme protein synthesis occurs. In PC-12 cells, neuronal NOS is

induced after 8 days of exposure to NGF, and the subsequent increase in NO affects activity-dependent neurotransmitter release in these cells (Hirsch et al, 1993). The exact mechanism whereby NO enhances neurotransmitter release is unclear, but may involve phosphorylation of synaptic vesicle proteins through activation of guanylyl cyclase.

NO may be important in activity dependent remodelling of axon terminals during development. NO can through modulation of fatty acylation in growth cones regulate process outgrowth. (Douglas et al, 1993). Evidence with BDNF, NT-3 and -4 suggest that neurotrophin molecules are involved in the developmental regulation of NOS in neuron populations in the spinal cord (Huber et al, 1995). In these experiments numbers of nicotamide adenine dinucleotide phosphate-diaphorase (NADPH-d, a histochemical marker for nitric oxide synthase) positive neurons in embryonic day 16 spinal cord cultures were very low 24 hours after plating. However, treatment with the neurotrophins brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) or neurotrophin-4 (NT-4) significantly increased their numbers after only 24 hours. Treatment with BDNF, NT-3 or NT-4 also augmented numbers of NADPH-d positive neurons when initiated after 3 or 5 days in culture, and became consistently apparent within 24 hours. This suggests that the neurotrophin mediated increase in NADPH-d positive neurons is unlikely to be due to promotion of neuron survival. The neurotrophins are known to play a diverse role in neuronal survival and growth, and levels of neurotrophin production are known to be increased by increased neural activity in other systems (Castren et al, 1992; Gall et al, 1992; Isackson et al, 1991; Lu et al, 1991; Patterson et al, 1992; Riva et al, 1992; Zafra et al, 1990). Therefore, it is possible that increased cell activity which can increase neurotrophin release could in turn increase NOS activity.

Other studies suggest that dendrite outgrowth is regulated by the selective modulation of MAP2 (a cytoskeletal component released during periods of neurite outgrowth). MAP2 mRNA levels in dendrites are regulated by local glutamate receptor stimulation resulting in NO release (Johnston & Morris,1994). This mechanism would allow a sustained stimulation of dendritic outgrowth to be confined to those regions of a neuron's dendritic arbour situated within the diffusion

range of NO. Interestingly, myenteric plexus explants promote striatal neurite elongation in co-culture and this effect is abolished with tetrodotoxin (TTX) but the blocking effect of TTX is partially reversed with a NO donor (sodium nitroprusside). Furthermore a NO blocker (N-nitro-L-arginine methyl ester) significantly reduces the neuritogenic effect of the myenteric plexus (Hopker et al, 1995). This evidence indicates that NO could even have trophic effects on developing neurons.

NO has also been implicated in hippocampal long- term potentiation (LTP), and perhaps NO's enhancement of neurotransmitter release facilitates the neurotransmission that accounts for LTP. Schuman & Madison , 1991, revealed that injection of nitroarginine into the postsynaptic CA1 pyramidal cells of the hippocampus inhibits presynaptic LTP, suggesting that NO might act as a retrograde messenger for LTP (Bohme et al, 1991; O'Dell et al, 1991; Haly et al, 1992). Recently endothelial NOS has been postulated to generate the NO which acts as a retrograde messenger in LTP in the hippocampus (Dinerman et al, 1994). Although evidence discussed so far implies NO mediates normal synaptic transmission, it may be neurotoxic under conditions of excessive formation. Glutamate released in excess acting primarily through NMDA receptors mediates neurotoxicity in focal ischemia since the neurotoxicity is blocked by NMDA antagonists (Choi, 1988; Meldrum & Garthwaite, 1990). Glutamate neurotoxicity may also contribute to neurodegeneration in Alzheimer's and Huntington's diseases. Glutamate neurotoxicity is in part attributable to NOS activation evoked by the increased intracellular calcium concentration. In primary cultures of cerebral cortical neurons, in which a short (5min) exposure to NMDA normally elicits cell death 24 hours later, inhibitors of NOS (arginine analogues, calmodulin antagonists, flavoprotein inhibitors) and reduced haemoglobin which scavenges NO, markedly reduce neurotoxicity (Dawson et al, 1991, 1993a). It is thought that NO and the superoxide anion may interact to form peroxynitrite, which is the ultimate toxic molecule (Beckman et al, 1990; Beckman 1991; Radi et al, 1991 a,b). Recent studies provide evidence that DNA damage which occurs through nucleotide base deamination is the key to NO neurotoxicity (Zhang et al, 1994). NO has been implicated in glutamate neurotoxicity in striatal slices (Kolleger et al, 1993), hippocampal slices (Izumi et al,

1992; Moncada et al, 1992; Wallis et al, 1992) and different culture conditions (Corasaniti et al, 1992; Lustig et al, 1992; Tamura et al, 1992; Cazevielle et al, 1993). However, other investigators have failed to confirm that NO plays a role in glutamate neurotoxicity (Demerle-Pallardy et al, 1991; Pauwels & Leysen 1992; Regan et al, 1993) and even suggested that NO may be neuroprotective (Lei et al, 1992). These discrepancies have been resolved by the finding that NO may possess either neurodestructive or neuroprotective properties, depending upon its oxidation - reduction status, with NO being neurodestructive and NO^+ being neuroprotective (Lipton et al, 1993).

Homologous genetic recombination techniques have recently been used to disrupt the murine gene encoding neuronal NOS-1 and produce homozygous neuronal "knockout" mice (Huang et al, 1993). The animals are normal in most respects despite their lack of NOS-1 catalytic activity in the brain and loss of NOS-1 immunostaining in central and peripheral neurons. Recently, studies on the nervous system of these mice (especially the forebrain) reveal that some calcium-dependent nitric oxide synthesis is present, presumably reflecting other isoforms of NOS (Kharazia et al, 1994). These new isoforms could adopt the developmental role of NOS-1 in "knockout mice".

NADPH-Diaphorase Positive Cells In The Developing Visual System.

Information in the developing murine embryonic visual system is limited, although some information on the distribution and expression of NOS is available on the early rat visual cortex (Luth et al, 1995). NOS positive nerve cells are present on postnatal day 1 in the intermediate (white matter) and subplate (layers 5 and 6) region as small undifferentiated neurons. At P14, neurons reach their typical morphology and appear in all layers. Some NOS-positive neurons appear only transiently although the majority of these cells are reported to survive to adulthood and also stain positively for the neurotransmitter GABA. However, there does not appear to be a specific pattern of neurotransmitter co-localisation for NOS. Other studies reveal that NOS neurons in the cerebral cortex contain, GABA, somatostatin, and neuropeptide Y (Vincent et al, 1983; Dawson et al, 1991). Studies on the

expression of NADPH-diaphorase in the rat telencephalon and diencephalon (Samama et al, 1995) and the ferret lateral geniculate nucleus (Cramer et al, 1995) suggest a role for NO in both the development of the early brain and in the formation of mature connections. Gabbott & Bacon, 1994, identified a subpopulation of inhibitory interneurons within the dorsal lateral geniculate nucleus of the rat which are diaphorase labelled. These interneurons via NO production could affect intrinsic dLGN operations and thalamocortical interactions. Do et al, 1994, suggest that the excitatory neurotransmitter released from ascending sensory afferents which stimulates thalamic relay neurons is NO. They revealed that high levels of the amino acid arginine were released in this brain area following physiological stimulation of afferents and the application of L-arginine on to thalamic relay neurons facilitated sensory synaptic transmission. The evidence cited above strongly suggests that NO could play a role in thalamocortical development and further studies investigating the nature of its action are required.

AIM

The main aim of this chapter is to investigate a possible role for nitric oxide (NO), a novel neurotransmitter, in the development of the thalamocortical system.

1. To investigate the effect of NO on growth cones of embryonic thalamic neurons.
2.To examine the distribution of nitric oxide synthase (NOS) during the development of the thalamocortical system both *in vivo* and *in vitro*.
3. To block NO production *in vitro* and examine any effects on thalamocortical development.

HYPOTHESES

A few examples of possible modes of NO action:
1. NO could be involved in many different regulatory processes which shape thalamocortical development. NO which is released from cortical cells could stimulate or inhibit the ingrowth of thalamic neurons by directly influencing growth cone morphology and structure.
2. NO released from thalamic cells could act as a positive or negative retrograde signalling molecule, which would increase or decrease thalamic outgrowth and/or survival.
3. NO may be a secondary messenger which is regulated in thalamic cells in response to a cortically derived factor and regulates thalamic outgrowth.
4. Activity dependent NO release might be involved in strengthening synaptic contacts or destroying inappropriate connections in the cortex.

EXPERIMENTAL PROTOCOL

Experiment 1

To examine whether NO could directly affect growth cones, I examined E15 dissociated thalamic neurites using time lapse microscopy. Growth rates of individual growth cones were calculated before and after exposure to NO. SIN-1, a compound which spontaneously releases NO was used as the NO source in these experiments. This compound was not commercially available when these

experiments were conducted and SIN-1 was kindly donated by Dr Henning, Cassella AG). Effects of NO on numbers of growth cone filapodia and dendritic branches were also examined.

Experiment 2.

The time course of NOS expression was studied in the developing thalamocortical system both *in vitro* and *in vivo* to determine whether this enzyme, and hence NO, is a possible candidate for regulating thalamocortical development. E15, P0, P4 and P6 cortical slices were examined for NADPH-diaphorase staining (an enzyme co-localised specifically with NOS). To determine whether NO was expressed in a similar manner *in vitro*, thalamocortical co-cultures consisting of E15 thalamus and P6 cortex were grown for 6 days before staining for NADPH-diaphorase. Isolated E15 thalamus or P6 cortex were cultured for 6 days prior to NADPH-diaphorase staining, to determine if NOS expression changed *in vitro* after thalamocortical connections were severed.

Experiment 3.

To investigate the role of NO in thalamocortical development *in vitro,* a specific neuronal nitric oxide synthase inhibitor (7-nitroindazole) was added to the culture medium, and co-cultures consisting of E15 thalamus and P6 cortex were cultured for 6 days as before. Thalamic neurites were labelled with DiI to examine any effects on thalamocortical ingrowth and co-cultures were then counterstained with bisbenzimide to reveal any layer specific effects.

MATERIALS AND METHOD.

Dissections.

E15 thalamic explants were obtained from time-mated BALB/c mice deeply anaesthetised with urethane (0.3ml of a 25% solution in normal saline, i.p.) and foetuses were removed by Caesarean section and decapitated. The brains were removed and the posterior thalamus was carefully dissected out as described before Lotto & Price,1995; Rennie et al, 1994, and stored in ice-cold oxygenated Earl's balanced salts (Sigma). $1.5mm^3$ (about 1.5x 1 x 1mm) thalamic explants from the posterior thalamus were sliced at 100μm with a M^{c}llwain tissue chopper. The thalamus was then rotated through 90^0 and sliced again at 100μm.

Dissociated Cultures.

E15 thalamic explants were prepared, as described above before dissociating with papain for 50 minutes at a neutral pH (following the method decribed in the papain dissociation system: Worthington Biochemical Corporation). The density of the cell suspension was determined with a haemocytometer. Trypan blue was excluded from most cells, confirming their initial viability on entry to culture. Aliquots of cells whose volumes were chosen to provide appropriate plating densities (~40,000 cells cm.sq.) when mixed with the culture medium (final volume 300-400μl per well) were placed on the bottom of plastic culture wells that had been pre-coated in laminin. 1-2 $\mu g/cm^2$ laminin/EBSS solution was placed in each well and incubated at 37^0C for 2 hours. Wells were washed (x2) in culture media before use. The *in vitro* development of dissociated thalamic cultures were recorded using time-lapse videomicroscopy on a Leica microscope housed in an incubator whose temperature, humidity and CO_2 could be regulated at 37^0C with 5% CO_2 and 95% humidity. Dissociated thalamic neurites were recorded within 24 hours of plating. Cultures were observed for a few hours prior to drug addition to enable their growth to stabilize following the trauma of surgery. SIN-1 (donated by Dr. Henning, Cassella AG) was dissolved in water and always freshly prepared and stored on ice shortly

before being added to the culture medium. Refer to Table 3.2 for the numbers of culture in each experimental paradigm.

Time Lapse Microscopy.

Thalamic neurites were recorded for 30 minutes before during and after SIN-1 (1mM- 0.01mM) addition. From a stock solution of 200mM SIN-1 further dilutions were made and stored on ice as required. SIN-1 was carefully added to the culture medium using the most concentrated stock solution possible, hence the volume of ice cold solution added to the dissociated thalamic cells was minimised (~1-2µl). Calculations based on the rate constants for the two step hydrolysis of SIN-1 that yields NO (Bohme et al, 1984) and on the half life of NO (Ignarro , 1990; Bohme et al, 1984) indicate the concentration of NO would rise to about 0.75µM within 1 minute and 3.5µM within 5 minutes of adding 1mM SIN-1, reaching a plateau of about 5µM after 10 minutes (Hess et al, 1993). SIN-1 was removed from the culture wells by gently pipetting off the culture media and replacing it with fresh pre-incubated media (x3). Recording was maintained throughout this procedure, although a lot of data were lost at this point because the culture well was displaced and the location of the recording station lost. When this happened the experiment was then abandoned. The experimental set-up and culture wells could not be easily modified to enable a perfusion system to be fitted. Control experiments were conducted to ensure that NO was responsible for these effects instead of other SIN-1 breakdown products. In these experiments SIN-1 was stored at room temperature for 24 hours before use, by which time NO is no longer generated but other breakdown products remain (Bohme et al, 1984; Manzoni et al, 1992).

NADPH-Diaphorase Histochemistry.

E15, P0, P4, and P6 mice were anaesthetised with 0.1ml of Sagattal administered intra- muscularly. Brains were then perfused with 0.1M saline injected slowly into the left ventricle, followed by 4% paraformaldehyde to fix the brain tissue. The brains were then carefully dissected from the animals and placed in 4% paraformaldehyde until they sank (maximum time in paraformaldehyde was 30

Table 3.2. The numbers of culture in each experimental paradigm.

DISSOCIATED THALAMIC CULTURES	DRUGS [SIN-1]	n
Effect of SIN-1 on dissociated thalamic neurones		
E15	1mM	**3**
E15	0.1mM	**5**
E15	0.01mM	**4**

FIRST EXPLANT	SECOND EXPLANT	DRUGS	n
Effect of NOS inhibitors on thalamocortical development.			
E15	P6	control	**5**
E15	P6	7-Nitroindazole	**5**

minutes). Brains were incubated overnight in a 10 % sucrose solution to prevent ice crystals forming when brains were sliced on the cryostat. 60μm parasagittal cortical slices were collected and floated onto slides in a 1g/100ml gelatin solution, before being left to dry. Slides were incubated in 1% Triton-X 100 for 6 hours at 4^0C, washed in 0.1M PBS (X3) for 10 minutes, before being incubated in the NADPH-diaphorase reaction mixture (Scherer-Singler et al, 1983) for 20 minutes at 37^0C. The incubation mixture consisted of 15mM sodium malate, 1mM NADP, and 0.2mM nitro blue tetrazolium in 0.1M Tris-HCl (pH 8.0). Slides were washed in 0.1 M PBS (X3) before they were mounted with PBS:glycerol (3:1) solution.

To examine NADPH-diaphorase staining *in vitro*, co-cultures consisting of E15 thalamus and P6 cortex were cultured as described previously in Chapter 2. Tissue was fixed for 10 minutes after 6 days of culture, before it was carefully removed from the collagen filter using a scapel blade. Free floating co-cultures were then incubated in 1% Triton-X 100 for 6 hours at 4^0C, before staining for NADPH-diaphorase as described above.

NO Synthase Inhibitors

7-Nitroindazole (Alexis Trading Corporation) is a potent inhibitor of neuronal NO synthase. 10mM stock solution of this compound was obtained by dissolving it in DMSO. Final concentrations of 10 μM were obtained by diluting stock concentrations in pre-incubated culture media. Co-cultures consisting of E15 thalamus and P6 cortex were set up as described previously in Chapter 2. 7-Nitroindazole treated co-cultures had their culture medium refreshed every second day, because the potency of 7-nitroindazole is known to decrease *in vivo* and I could not obtain any information on its potency *in vitro*. After a 6 day culture period co-cultures were fixed in 4% paraformaldehyde and a crystal of DiI was inserted into the thalamus to enable thalamic neurites to be viewed under fluorescent microscopy. Control experiments were conducted by adding a similar volume of DMSO to these co-cultures to ensure that it did not produce any adverse toxic effects.

Control Viability Experiments

Following fixation, the paraformaldehyde in co-cultures treated with 7-nitroindazole was replaced with a similar volume of 0.1M PBS for at least 1hr. Explants were then wax-embedded and sectioned (10μM). Sections were mounted on Poly-$_L$-lysine coated slides and Nissl stained to reveal live and pyknotic cells

RESULTS

Effects Of SIN-1 On Growth Cones Of Thalamic Neurites.

Addition of SIN-1 (which causes release of nitric oxide) to the culture medium of dissociated thalamic neurites produced interesting data. In most neurites addition of SIN-1 induced growth cone collapse and removed small axonal processes. These effects were observed within minutes of SIN-1 addition (fig 3.1a,b,c,d.). The axon also often retracted back towards the cell body. In some cases this retraction was extremely rapid, much faster than previous growth rates (fig 3.1d). The response of each individual growth cone was unique demonstrating that effects produced by nitric oxide may depend on the status of the growth cone. All growth cones were pre-recorded for one hour before SIN-1 addition. Initially experiments were conducted using 1mM SIN-1. However this concentration had a toxic effect on the growth cone, axon and the cell body of neurons. Immediately after addition of 1mM SIN-1, growth cones collapsed and within 15 minutes axons demonstrated signs of disintegration. These effects were not reversible upon wash-out and these experiments were not analysed. The effects of lower doses of SIN-1 were investigated. In these experiments it was clear from the data and the size of error bars that many of the control growth cones studied were advancing at different rates. The fastest growing neurite retracted the longest distance back towards the cell body (fig 3.1d). Fig 3.2 displays the growth rates of neurites before, during and after SIN-1 addition. It is clear from the averaged data that SIN-1 induces growth cones to retract. 0.1mM SIN-1 has a greater effect on growth rates than 0.01mM SIN-1. Fig 3.3 compares the number of small axonal processes and filapodia present on thalamic neurites before and after SIN-1 addition. Small axonal processes and filapodia are often reduced on these neurites after exposure to 0.1mM SIN-1 but are nearly restored to control levels after wash-out.

Control experiments revealed that NO was responsible for these effects instead of other SIN-1 breakdown products. SIN-1 was stored at room temperature for 24 hours before use, by which time NO is no longer generated but other breakdown products remain (Bohme et al, 1984; Manzoni et al, 1992).

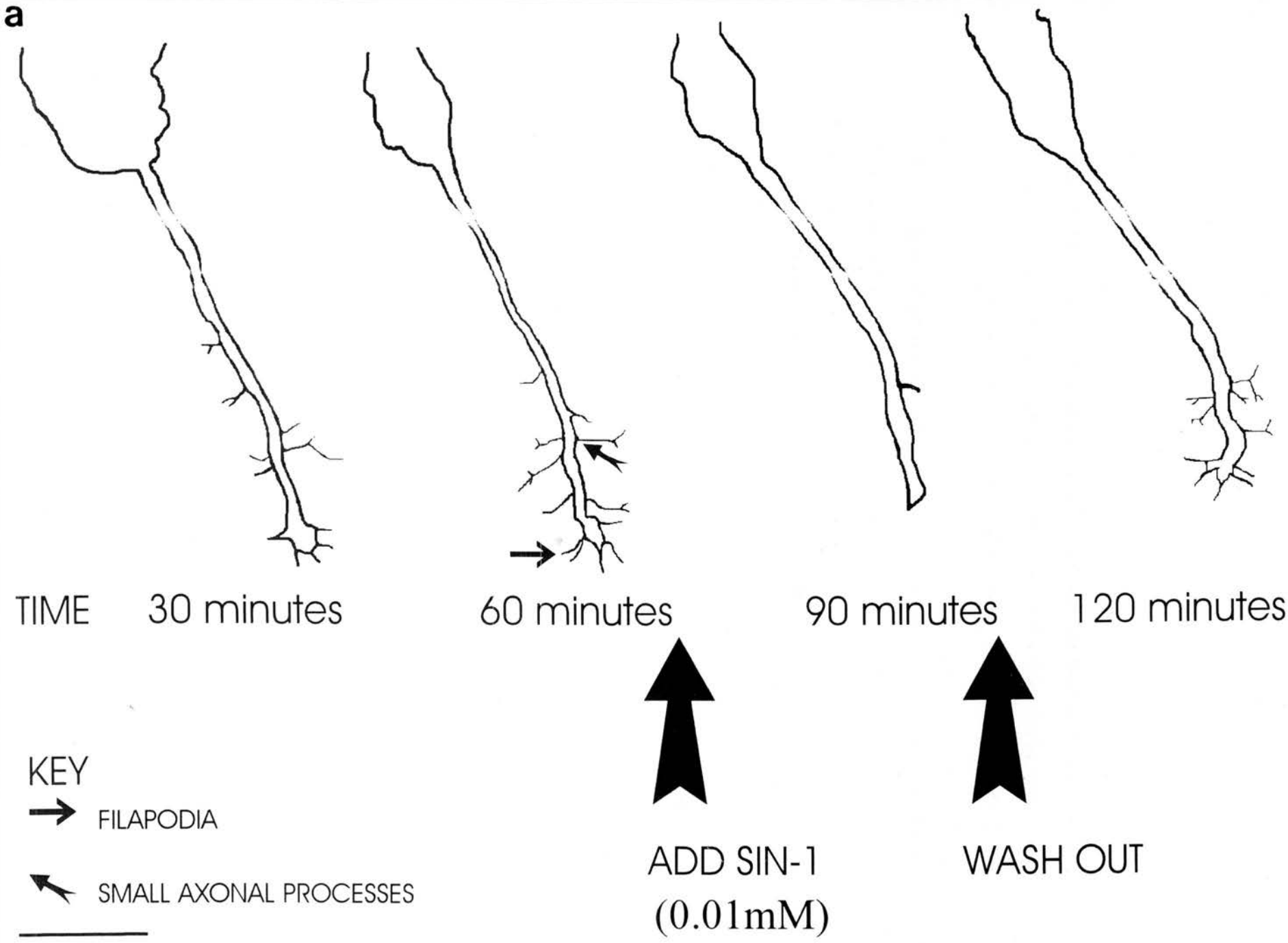

a
TIME
30 minutes
60 minutes
90 minutes
120 minutes
KEY
FILAPODIA
SMALL AXONAL PROCESSES
ADD SIN-1
(0.01mM)
WASH OUT

Figure 3.1(a)

(a-d) Diagram of dissociated embryonic day 15 (E15) thalamic neurons recorded under time lapse videomicroscopy before and after the addition of SIN-1. These diagrams were traced from a visual display unit which was linked to a recording microscope.

(a) This diagram depicts one neurite with several smaller processes extending from the main axon trunk. These processes are not likely to be filapodia because they are located at a distance from the main axonal growth cone. They are also not permanent collateral branches. In this example, the growth cone collapsed and axonal processes retracted after 0.01mM SIN-1 was added to the culture media. The main axon only retracted a short distance. After SIN-1 was washed out the growth cone extended filopodia and some axonal processes regrew. (scale=70μm)

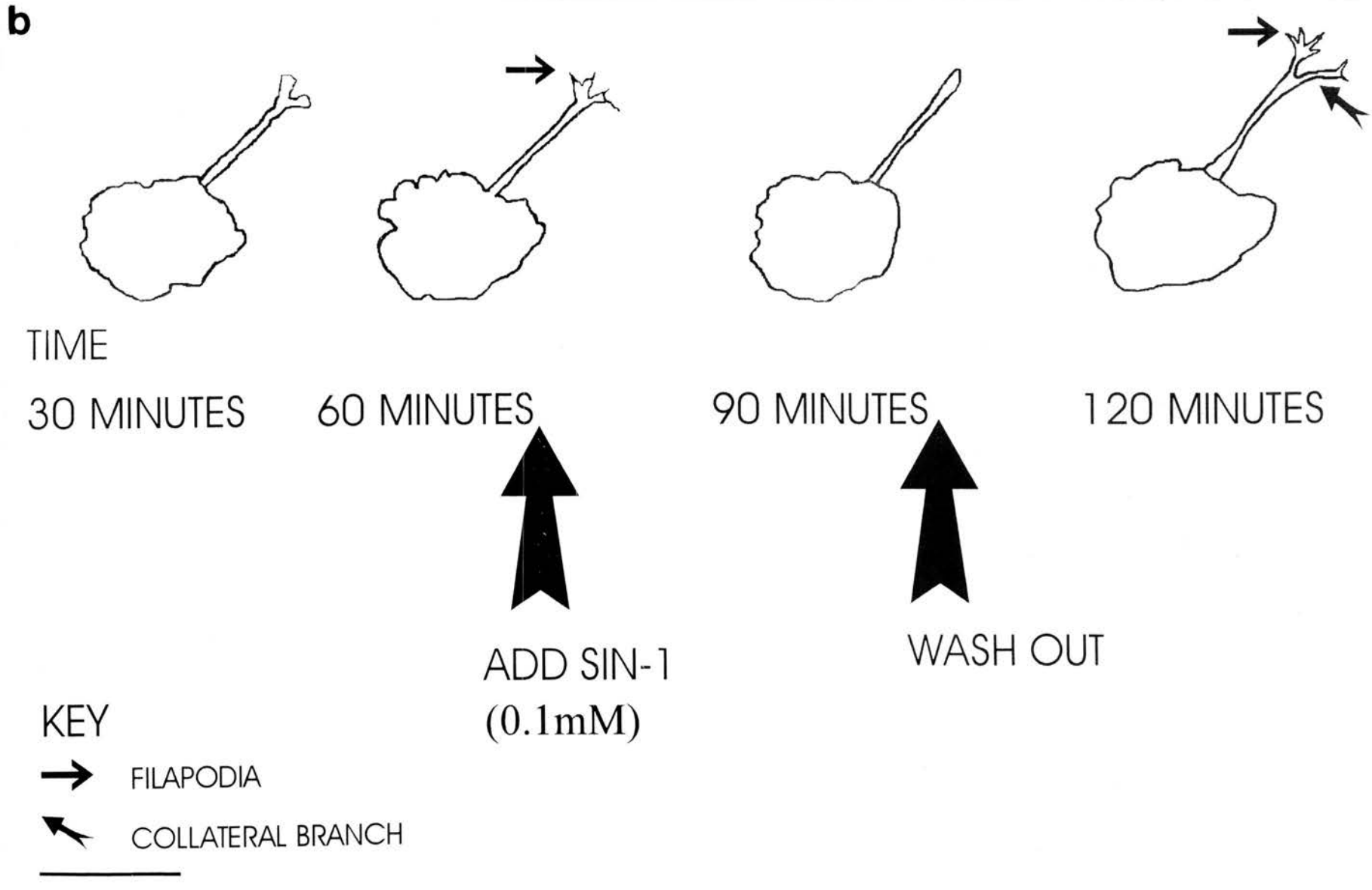

b
TIME
30 MINUTES
60 MINUTES
90 MINUTES
120 MINUTES
ADD SIN-1
(0.1mM)
WASH OUT
KEY
FILAPODIA
COLLATERAL BRANCH

Figure 3.1(b)

This diagram depicts one neurite which has grown from a cluster of cells. The main axon has divided at the end into two collateral branches. Both growth cones collapsed after the addition of 0.1mM SIN-1 to the culture media. After SIN-1 was washed out the filapodia regrew. (scale =70μm)

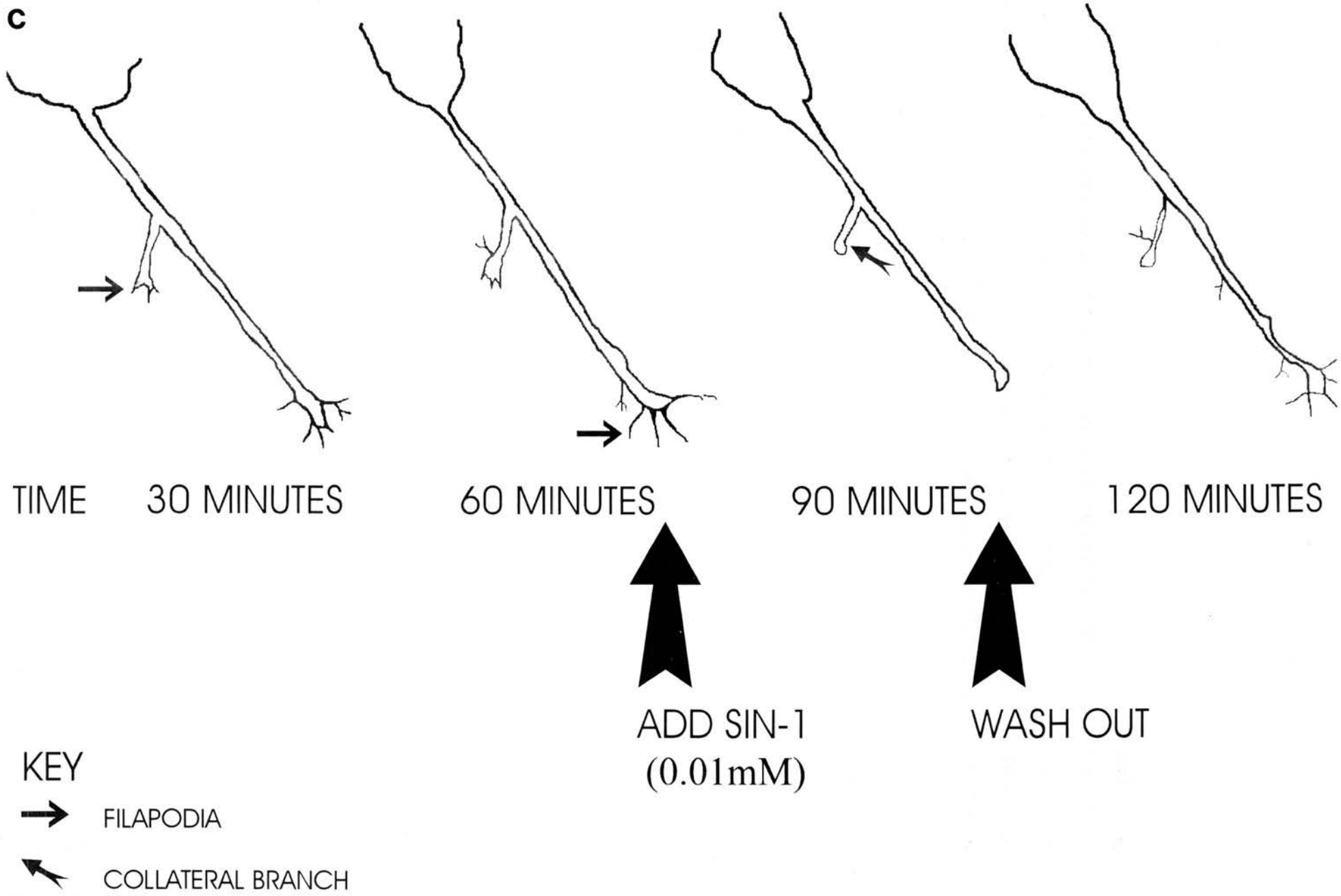

c
TIME
30 MINUTES
60 MINUTES
90 MINUTES
120 MINUTES
ADD SIN-1
(0.01mM)
WASH OUT
KEY
FILAPODIA
COLLATERAL BRANCH

Figure 3.1(c)

This diagram depicts one neurite with one collateral branch extending from the main axon trunk. A few smaller axonal processes are also attached to the main axon trunk. Both growth cones collapsed after the addition of 0.01mM SIN-1 to the culture media. After SIN-1 was washed out the filapodia regrew. (scale 70μM).

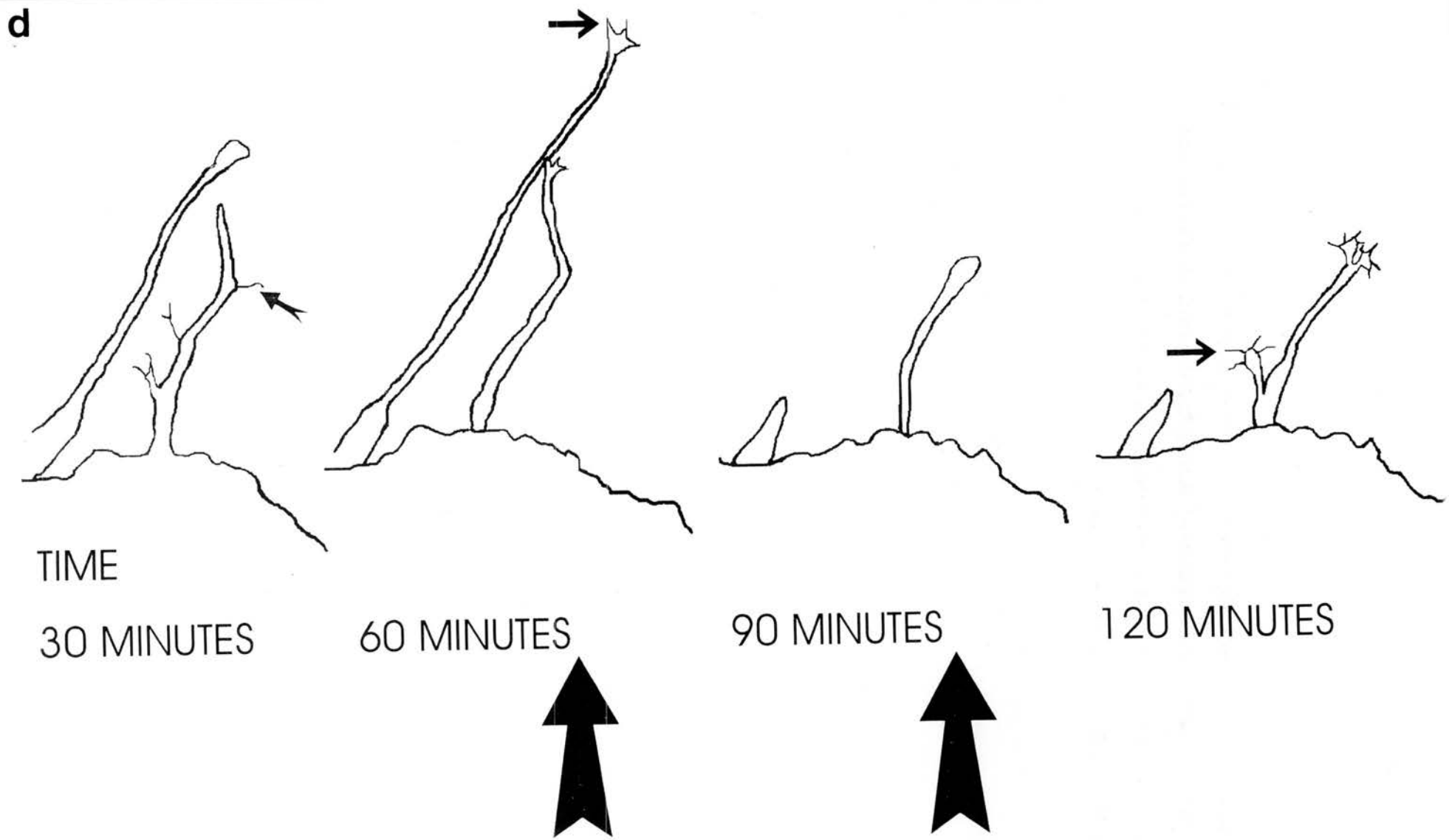

d
TIME
30 MINUTES
60 MINUTES
90 MINUTES
120 MINUTES
ADD SIN-1
(0.1mM)
WASH OUT
KEY
FILAPODIA
SMALL AXONAL PROCESSES

Figure 3.1(d) This diagram depicts two axons with rounded growth cones which were growing very rapidly. After the addition of 0.1mM SIN-1 growth cones collapsed and filapodia retracted. After wash out only one axon recovered and extended filapodia at the growth cones. (scale = 70μm).

THE EFFECTS OF SIN-1 ON THE GROWTH RATES OF REPLICATING E15 THALAMIC NEURITES.

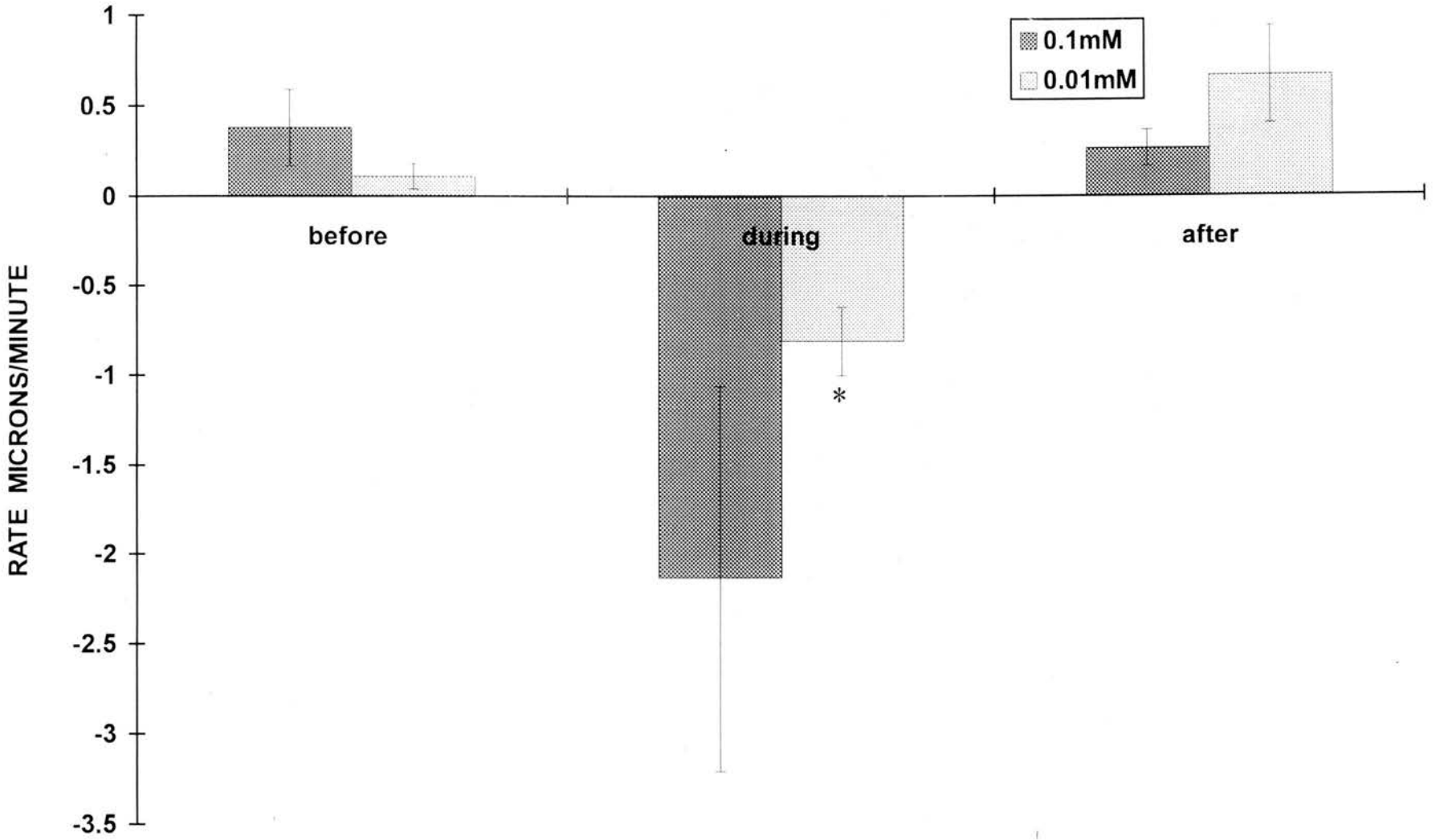


Figure 3.2

Histogram comparing the mean growth rates of dissociated embryonic day 15 (E15) thalamic neurites before, during and after the addition of SIN-1. SIN-1 was added after 60 minutes and washed out after 90 minutes (time of exposure to SIN-1 was 30 minutes). n=5 for 0.1mM SIN-1 treated neurons and n=4 for 0.01mM SIN-1 treated neurons. The effects of SIN-1 on growth rates were compared statistically (using a Student's t-test) to control growth rate data. The growth rates after washout were also compared statistically to control growth rate data. * on the histogram indicates that data is statistically different from controls ($p < 0.05$).

Growth rates range from about 0.1-0.4 microns /minute before the addition of SIN-1. After addition of SIN-1 growth rates drop and some neurites retract. Higher concentrations of SIN-1 (0.1mM) cause neurites to retract rapidly. After wash out, most axons start to regrow at rates similar to those recorded before the addition of SIN-1.

HISTOGRAM COMPARING THE NUMBERS OF BRANCHES AND FILAPODIA ON E15 THALAMIC NEURITES BEFORE AND AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF SIN-1.

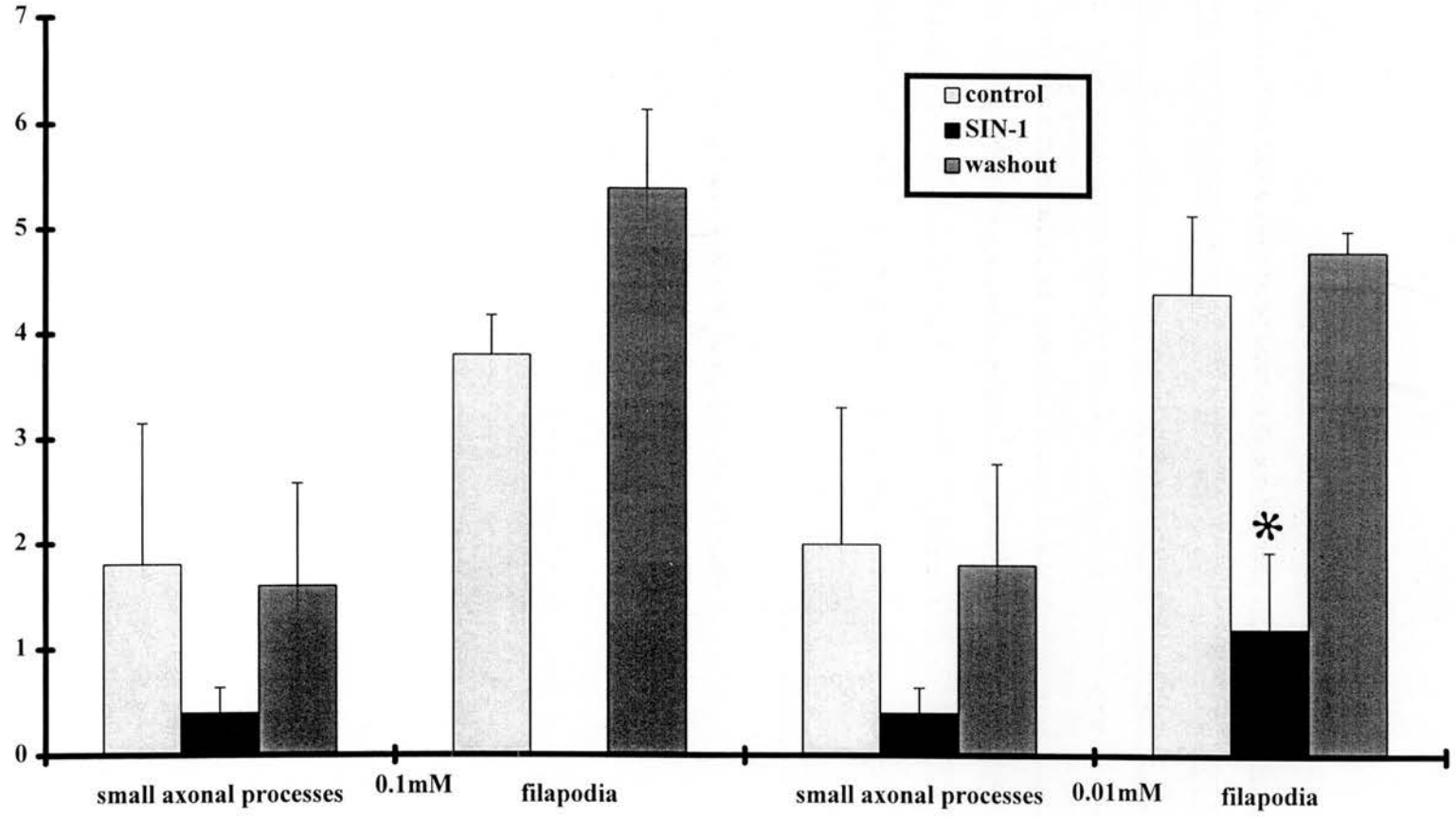


Figure 3.3

Histogram comparing the numbers of small axonal processes and filapodia on embryonic day 15 (E15) thalamic neurites before and after exposure to different concentrations of SIN-1. SIN-1 was added after 60 minutes and washed out after 90 minutes (time of exposure to SIN-1 was 30 minutes). n=5 for 0.1mM SIN-1 treated neurons and n=5 for 0.01mM SIN-1 treated neurons. The effects of SIN-1 on numbers of filapodia and small axonal processes were compared statistically (using Student's t-test) to control data. The numbers of filapodia and small axonal processes after washout were also compared statistically to control data. * on the histogram indicates that the data is statistically different from controls ($p < 0.05$).

0.1mM SIN-1 reduces the number of small axonal processes and filapodia on axons, although many return after wash out. On average 0.01mM SIN-1 had little effect on the numbers of small axonal processes but reduced the numbers of filapodia. After wash out, filapodia returned to control levels.

Distribution Of NOS Within The Cortex *In Vivo.*

The expression of nitric oxide synthase (NOS) was investigated using a staining procedure for NADPH-diaphorase (see Introduction). NADPH-diaphorase expression increased during thalamocortical development. Two patterns of staining were observed in the cortex, a Golgi-like cell body stain which occurred in distinct cells, probably interneurons, and a diffuse background stain possibly due to a lower density of NOS in the neuropil (e.g. synapses, soma, dendrites) (fig 3.7a).

At embryonic day 15 (E15), the expression of NOS was generally low (fig 3.4). No distinct cell body label was apparent. However, a dark diffuse label was expressed in the lower marginal zone, and possibly the subplate. The ventricular zone (VZ) and the cortical plate (CP) contained some label whilst the upper marginal zone (MZ) and the intermediate zone (IZ) were the lightest.

At postnatal day 0 (P0), the diffuse label was quite uniform (fig 3.5). The label is hard to interpret because migration is not complete until ~P4. The cortex was more heavily labelled than at E15. Lower layer 1, cortical plate and an area most likely to be either the subplate or white matter were heavily labelled in comparison to the lighter upper layer 1. No Golgi-like cell body label was present.

At P4, the first distinct laminar differences in labelling intensity were observed (fig 3.6 a & b). Layer 1 was labelled with a dark diffuse stain as well as migrating layers 2/3. Layers 4- 6 are lightly labelled, although there are some signs of labelling in the barrel fields in layer 4. Golgi-like cell bodies are now distinct. They are found mainly in the deeper layers.

At P6, layers 6, 4 and 2 were most heavily stained with a dark diffuse label (fig 3.7 a & b). Layers 5 and 2/3 were weakly labelled. The barrel fields were distinct within layer 4. The Golgi-like label was also upregulated and many cells were heavily labelled within the deeper layers although some cells within the superficial layers were also labelled. P6 parasaggital slices which were stained for one hour reveal heavy staining within the thalamocortical tracts. These could be traced back down to the thalamus, where distinct thalamic nuclei were labelled (fig 3.8 a & b).

FIGURE 4

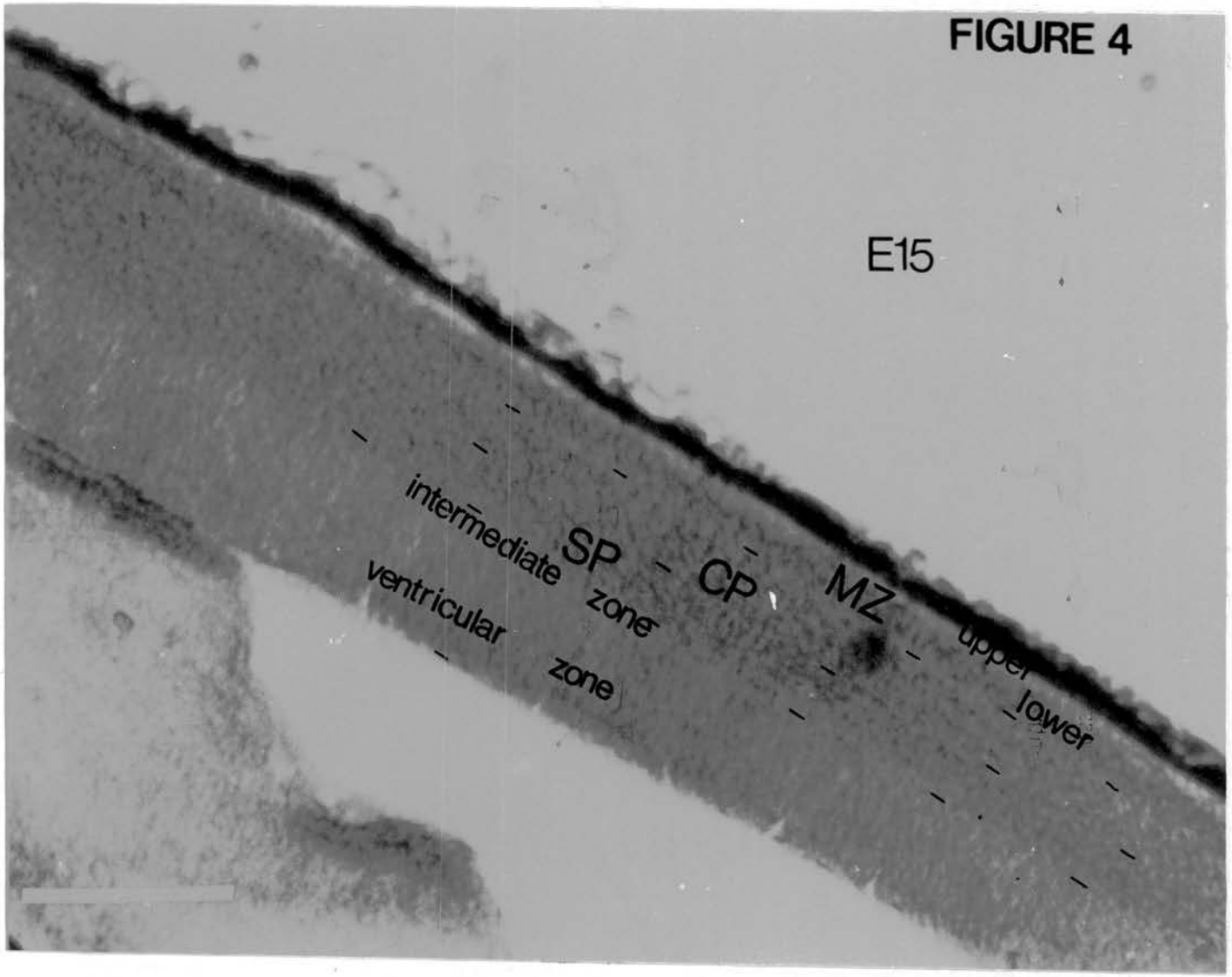

E15
intermediate zone
SP
CP
MZ
upper
lower
ventricular zone

Figure 3.4

Acute *in vivo* parasaggital slice of embryonic day 15 (E15) cortex stained with NADPH-diaphorase. No distinct Golgi-like cell body label was apparent. A dark diffuse label was expressed in the lower marginal zone (MZ) and possibly the subplate (SP). The ventricular zone (VZ) and the cortical plate (CP) contain some label whilst the upper marginal zone (MZ) and the intermediate zone (IZ) were the lightest.(scale =144μm)

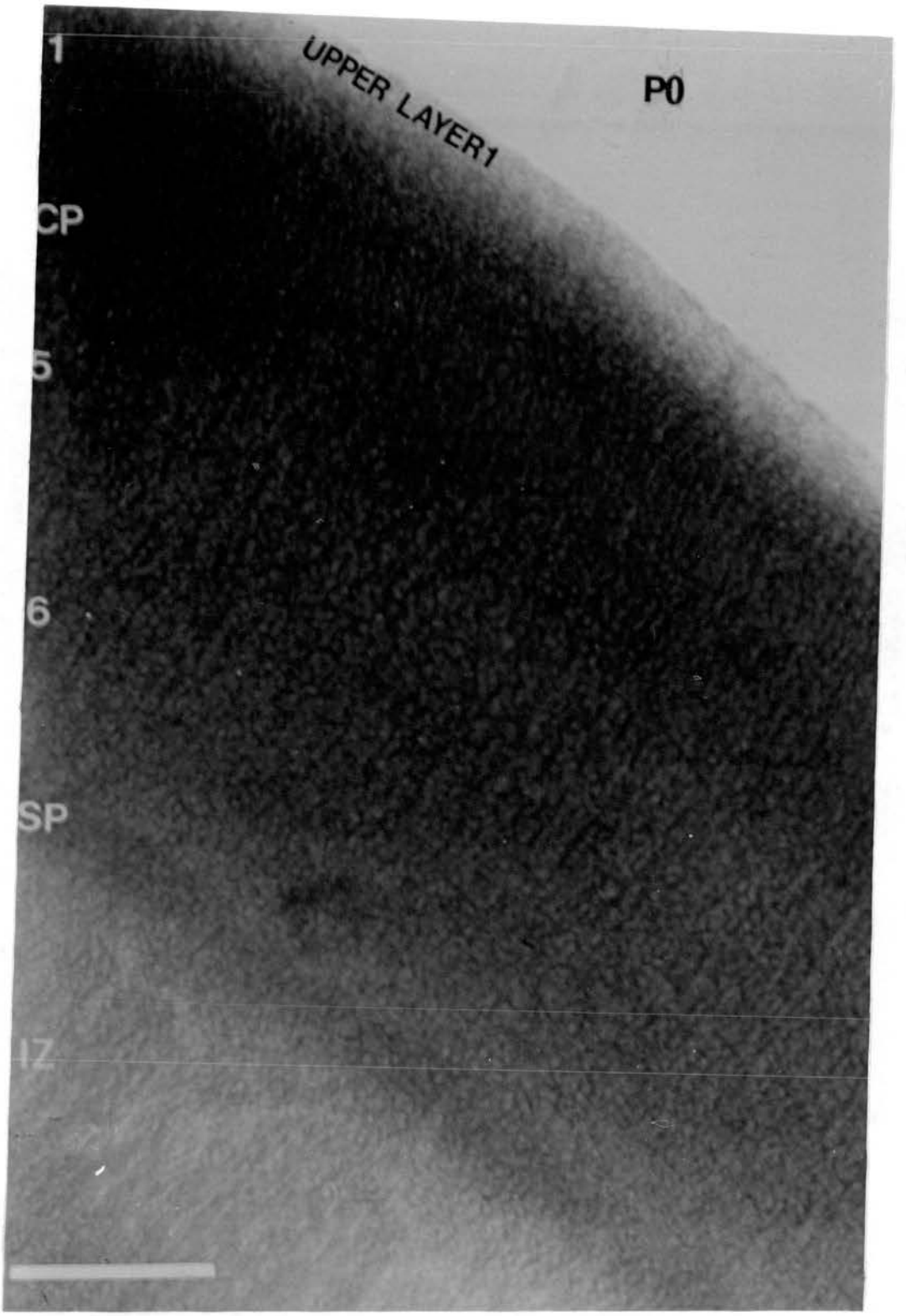
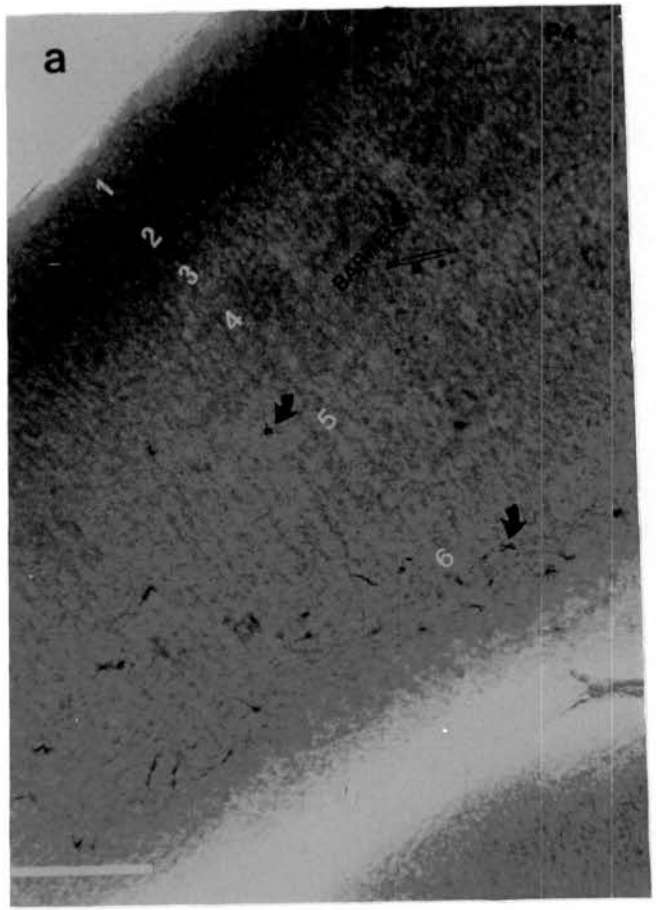

1
UPPER LAYER1
P0
CP
5
6
SP
IZ

Figure 3.5

Acute *in vivo* parasaggital slice of postnatal day 0 (P0) cortex stained with NADPH-diaphorase. No distinct Golgi-like cell body label was apparent. A diffuse label was present across the cortex which was more heavily labelled than at embryonic day 15 (E15). Layer 1, cortical plate (CP) and possibly the subplate(SP) were heavily labelled in comparison to the upper layer 1. IZ is the intermediate zone.
(scale =144 μm)


a
P4
1
2
3
4
5
6

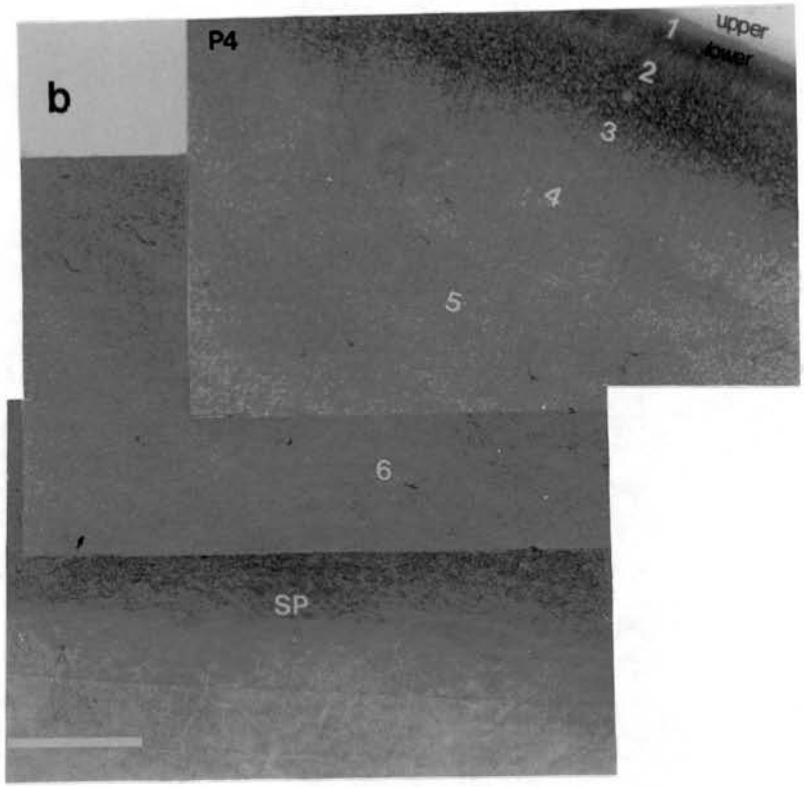

b
P4
1
upper
lower
2
3
4
5
6
SP

Figure 3.6

(a) and (b), Acute *in vivo* parasagittal slices of postnatal day 4 (P4) cortex stained with NADPH-diaphorase. A dark diffuse background label and a Golgi-like cell body label were observed. Diffuse label: layer 1 and layers 2/3 are heavily labelled. Layers 4, 5 and 6 are lightly labelled, although the barrel fields were becoming more heavily labelled (see (a)). Golgi-like cell bodies are now distinct and are present mainly in the deeper layers 5 and 6 (dark arrows). SP is the subplate. (scale (a) =360μm, (b) =547μm).

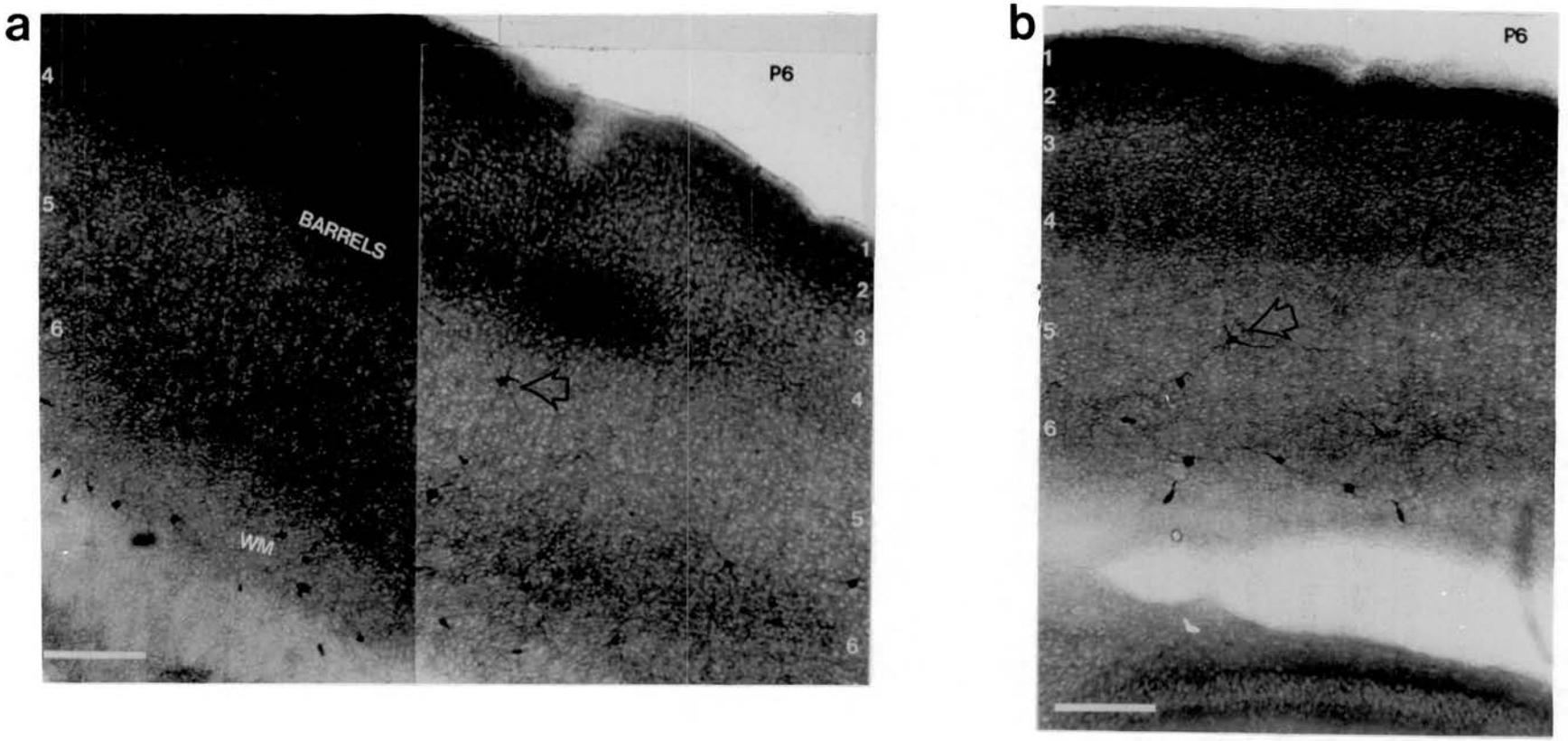
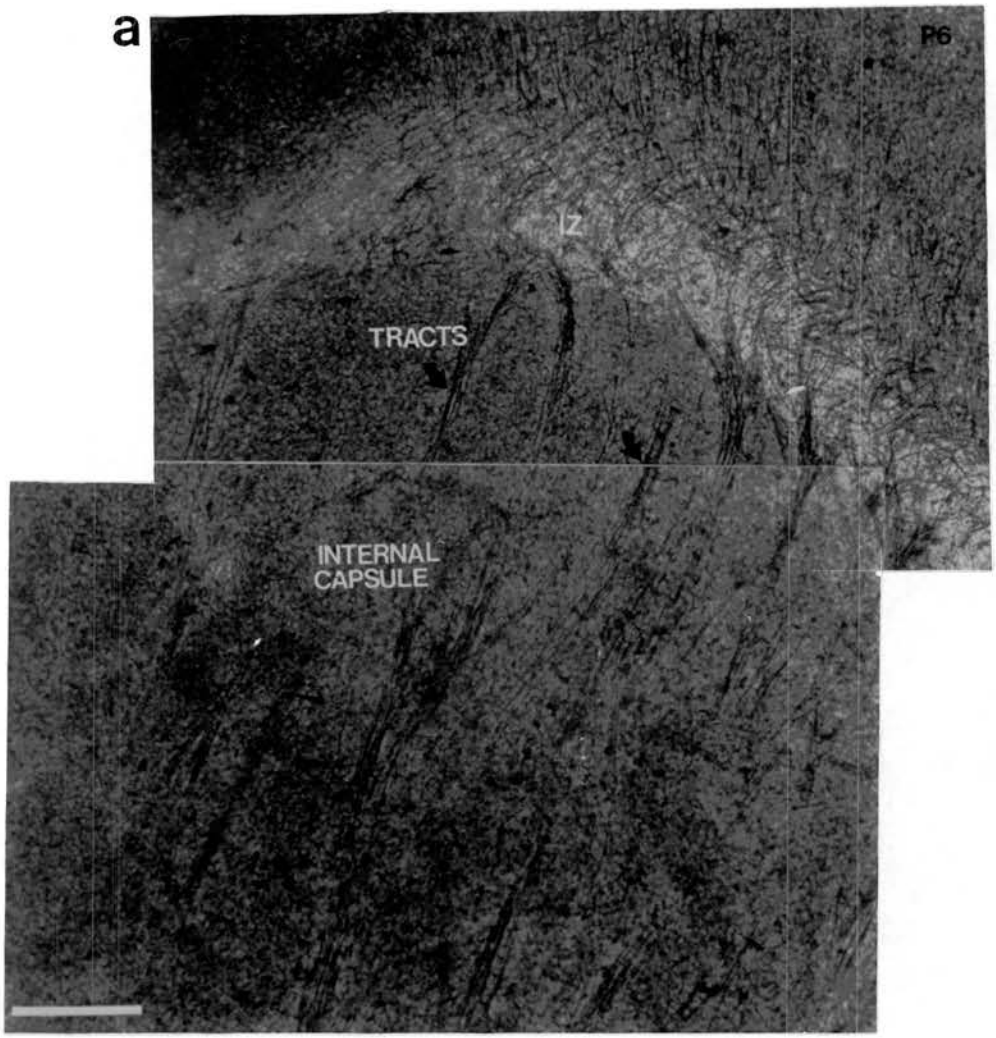

a
4
5
6
BARRELS
WM
P6
1
2
3
4
5
6
b
P6
1
2
3
4
5
6

Figure 3.7

(a) and (b), Acute *in vivo* parasagittal slices of P6 cortex stained with NADPH-diaphorase. A dark diffuse background label and a Golgi-like cell body label were present. Diffuse label: layers 6, 4 and 2 are most heavily labelled and the barrel fields are distinct in layer 4. The Golgi-like label is also upregulated and many cells are heavily labelled within the deeper layers (large arrows) although some cells within the superficial layers are also labelled. WM is the white matter. (scale (a) =144μm, (b) =350μm).


a
P6
IZ
TRACTS
INTERNAL
CAPSULE

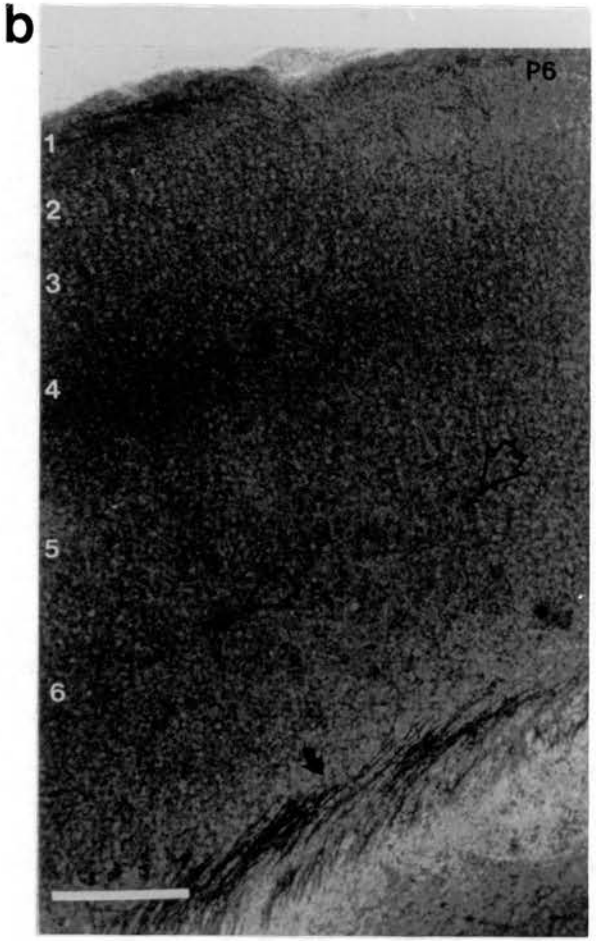

b
P6
1
2
3
4
5
6

Figure 3.8

(a) and (b) Acute *in vivo* parasagittal slices of P6 cortex stained for one hour with NADPH-diaphorase (normal incubation period is 20 minutes). Prolonged staining has labelled NO synthase within the thalamocortical tracts. These tracts are fasiculated within the internal capsule and subplate, and can be traced back to the thalamus. IZ is the intermediate zone. Large open arrows indicate Golgi-like cell bodies in the deeper cortical layers. Small solid arrows indicate thalamocortical tracts. (scale (a) =144μm, (b) = 350μm).

Distribution Of NADPH-Diaphorase *In Vitro.*

E15 thalamus and P6 cortex co-cultures were observed for NADPH-diaphorase after 6 days of culture (fig 3.9). The cortex contained no label even after an incubation period of 1 hour. Co-cultured thalamic explants were heavily labelled after 1 hour, and were also labelled after the normal incubation period of 20 minutes. Some small Golgi-like cell body label could be observed within thalamic explants using the fine focus control on the microscope. Results from chapter 2 demonstrated that in organotypic co-cultures cortical cell viability was reduced in the superficial layers. Therefore the absence of NADPH-diaphorase in the cortical explants of these co-cultures may be a direct result of cortical cell death.

These co-cultures were thick (~350μm) and therefore difficult to photograph.

No NADPH-diaphorase label was observed in isolated cortex which had been cultured for 6 days, however label was present in isolated thalamus which had also been cultured for 6 days. A control experiment, where the cortices were prepared for culture but were instead placed in paraformaldehyde, was conducted to prove that cortical explants were NADPH-diaphorase positive before they were cultured.

Effects Of Blocking NOS On Neurite Outgrowth And Target Recognition Iin Thalamocortical Co-cultures *In Vitro.*

Addition of 7-nitroindazole to co-cultures consisting of E15 thalamus and P6 cortex prevented the growth of thalamic axons from being restricted within their target layer 4 *in vitro* (fig 3.11). Control co-cultures were similar to those described previously in Chapter 2. It was difficult to distinguish the border between layer 4 and 3 in these cultures, so these layers have been grouped together. Fig 3.11 demonstrates that in control cultures luminance decreased within the cortical explant mainly within layer 5 and 4/3. Fig 3.11 also demonstrates that in 7-nitroindazole treated cultures luminance decreased evenly across the cortical plate. 40% luminance was present at the outer edge of the cortical explant suggesting that many axons grew through layer 1 onto the collagen membrane. Photomicrographs demonstrate these results in fig 3.10.

FIGURE 9

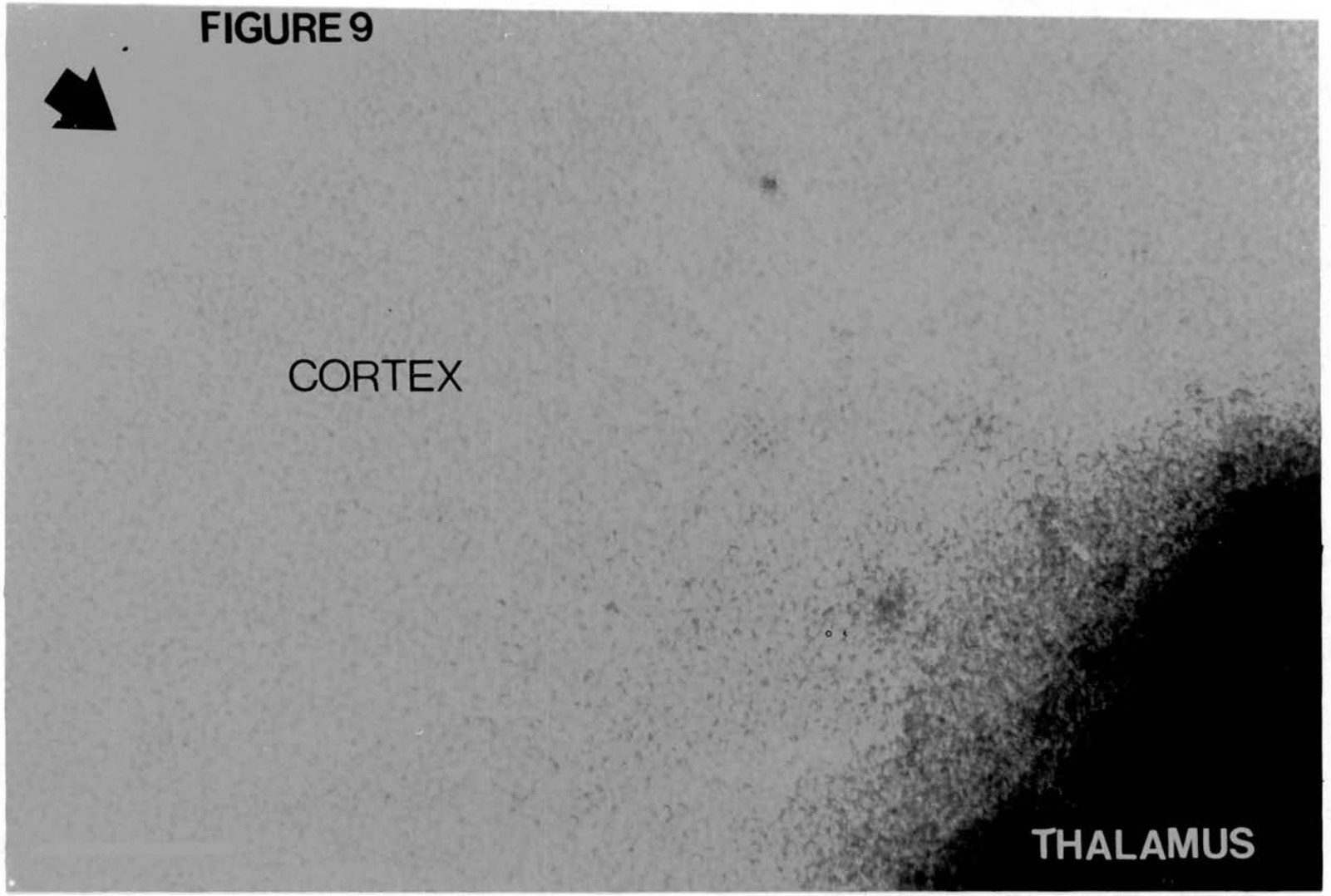


Figure 3.9

This is a co-culture consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) cortex which has been cultured for 6 days and fixed before staining for NADPH-diaphorase. The cortex did not stain for NO synthase even after an incubation period of 1 hour. However, the thalamus was heavily stained with a dark diffuse label. (scale 144μm). Results from chapter 2 demonstrated that in organotypic co-cultures cortical cell viability was reduced in the superficial layers. Therefore the absence of NADPH-diaphorase in the cortical explants of these co-cultures may be a direct result of cortical cell death.

FIGURE 10

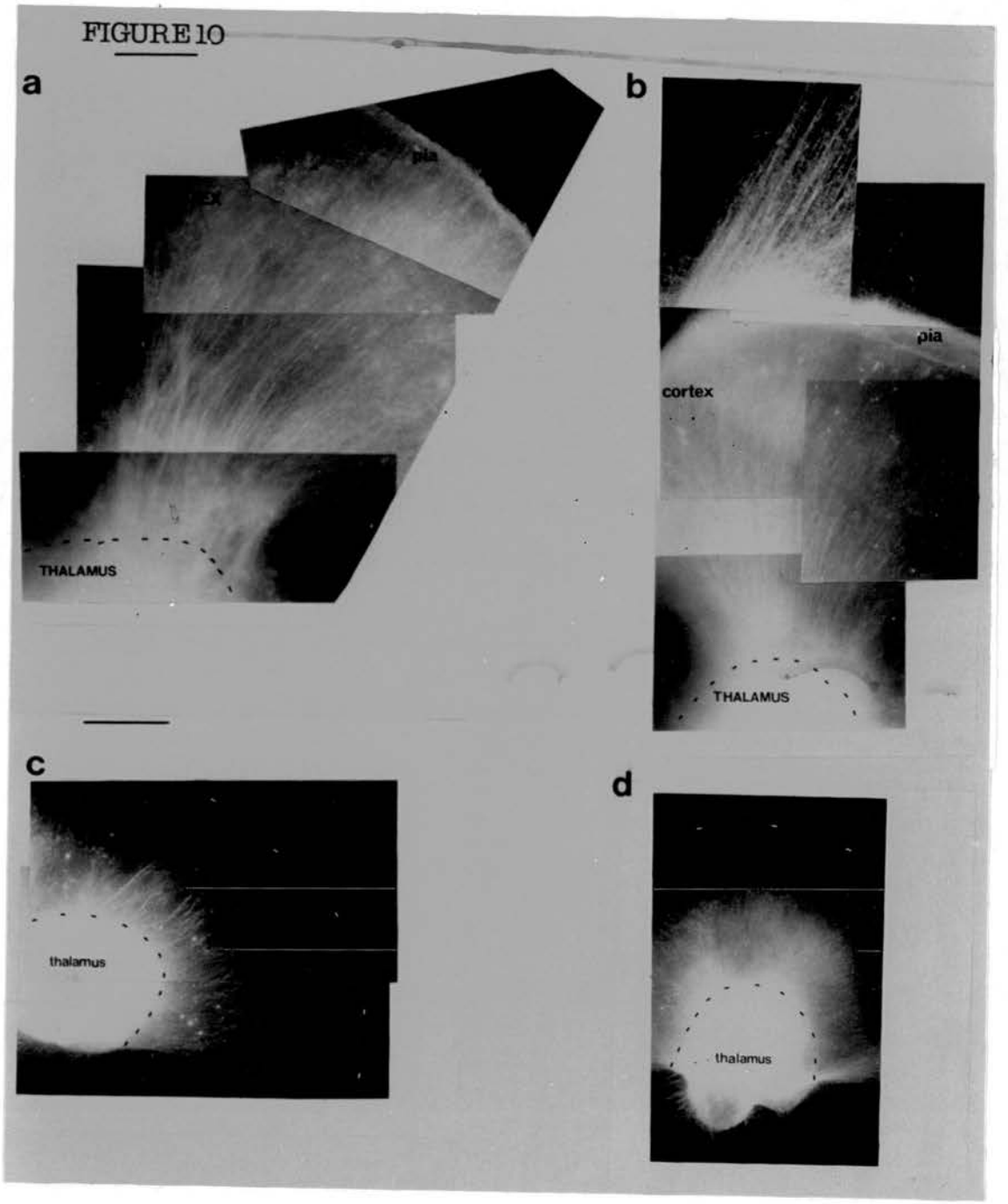


Figure 3.10

Photomicrographs of co-cultures consisting of embryonic day 15 (E15) thalamus and postnatal day 6 (P6) cortex which have been cultured for 6 days in the presence of a nitric oxide synthase (NOS) inhibitor. (a) and (b) 7-nitroindazole inhibited the growth of thalamic neurites from being restricted within target layer 4. Many axons grow past layer 1 and onto the collagen membrane (scale =220μm). (c) and (d) in control co-cultures the growth of thalamic neurites was restricted within the cortical explant within the region of target layer 4. White dotted lines indicate the outer edge of the cortical explant. Black dotted lines indicate the boundaries between thalamic explants and cortical explants (scale=580μm).

GRAPH DISPLAYING THE PERCENTAGE DECREASE IN LUMINANCE AT EACH CORTICAL LAYER BORDER

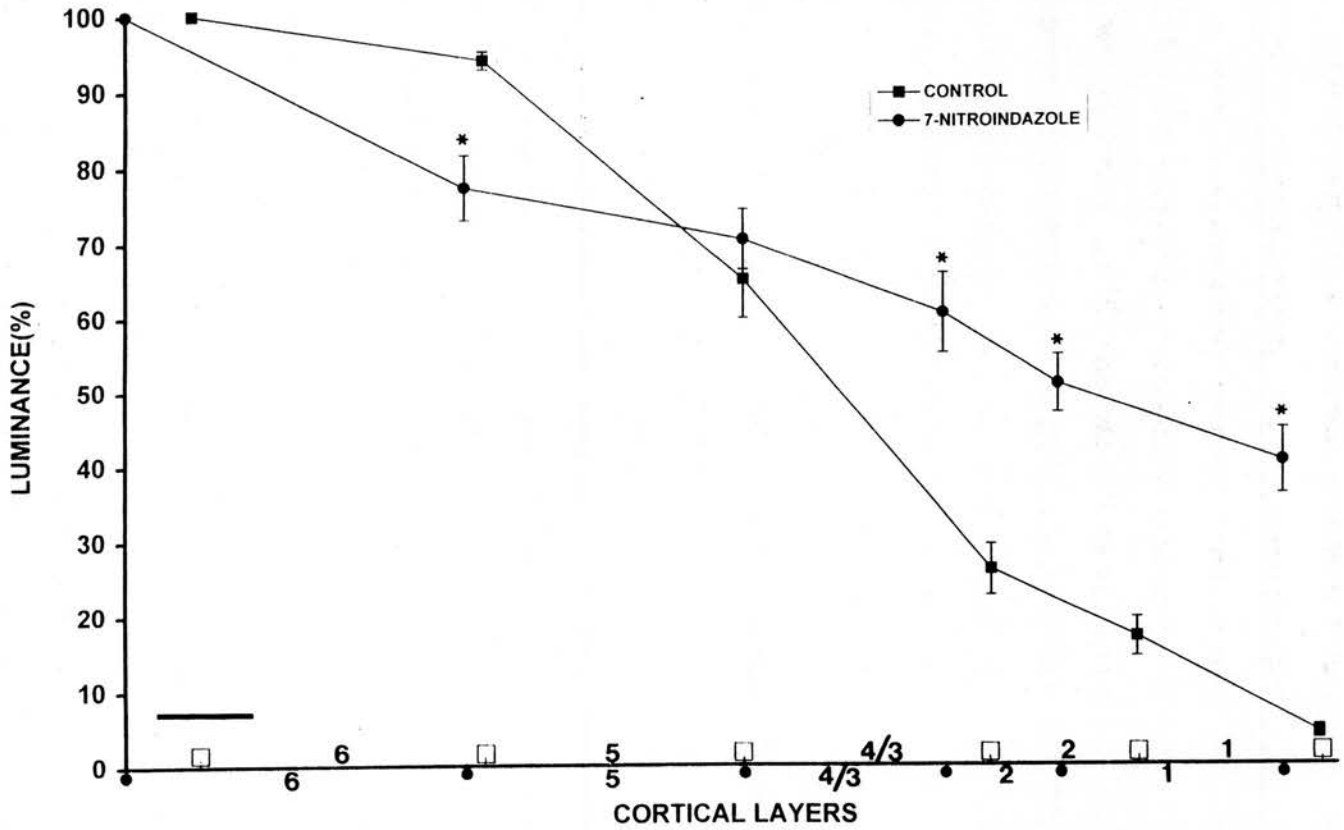


Figure 3.11

This graph compares the ingrowth of embryonic day 15 (E15) thalamic axons into postnatal day 6 (P6) visual cortex in the presence and absence of 7-nitroindazole (NO synthase blocker). The co-cultures were cultured for 6 days in serum free medium and 7-nitroindazole was added at culture day 1.

Using computer software the intensity of luminance was measured within two cross sections of each co-culture. At each layer border the decrease in luminance was calculated as a percentage of total luminance (i.e. the luminance intensity at the thalamic explant and cortical layer 6 border which was designated to be 100% luminance). The width of each cortical layer was measured within each cultured cortex and the average width of each layer was calculated. Black squares and black circles on the X-axis represent the average width of each cortical layer for control and 7-nitroindazole treated cortices respectively. A graph displaying the average decrease in luminance at each averaged cortical layer border was plotted. Luminance can be directly correlated to thalamic ingrowth. The luminance intensity drops off as the density of thalamic axons decreases in the cortex.

The graph displays mean luminance values (+/- S.E.M) and mean cortical layer width values (see scale bar = 83μm). The graphs have been aligned on the X-axis at the layer 5/4 border. n = 5 for control luminance data and n = 5 for 7-nitroindazole luminance data. The mean luminance values at each cortical layer border were compared statistically between control and 7-nitroindazole data. The statistical test used was the Student's t-test (* indicate significantly different values ($p<0.05$), see graph). The 6/5, 5/4, 3/2, 2/1 and 1/collagen borders within controls were compared to the 6/5, 5/4, 3/2, 2/1 and 1/collagen borders within the drug treated data respectively. Only the 5/4 border was not statistically significantly different from controls ($p >0.05$).

In controls the greatest decrease in luminance occurred within layer 5 and 4/3. This suggests that thalamic neurites have been restricted within the region of their target layer 4. Layers 4 and 3 could not be differentiated in these cultures. In 7-nitroindazole treated co-cultures, luminance decreased steadily across the cortical

plate. Over 60% luminance was present in layer 2 suggesting that many thalamic neurites were not restricted within the region of their target layer 4.

Effects Of 7-Nitroindazole On Cell Viability In Thalamocortical Co-Cultures.

Addition of 7-nitroindazole to co-cultures consisting of E15 thalamus and P6 cortex produced adverse effects on cell viability. Results from control co-cultures were similar to previous findings in chapter 2 (refer to fig 3.12a and fig 2.20a). In controls thalamic cells and cells found in the deeper layers of the cortex were viable whereas cells in the superficial layers of the cortex were less viable. In 7-nitroindazole treated co-cultures many of the thalamic cells were viable but most cortical cells were pyknotic (fig 3.12b). Blocking nitric oxide synthase (NOS) in these co-cultures has decreased cortical cell survival. It is possible that the inhibition of NOS has produced massive cortical cell death and loss of the recognition signal within target layer 4.

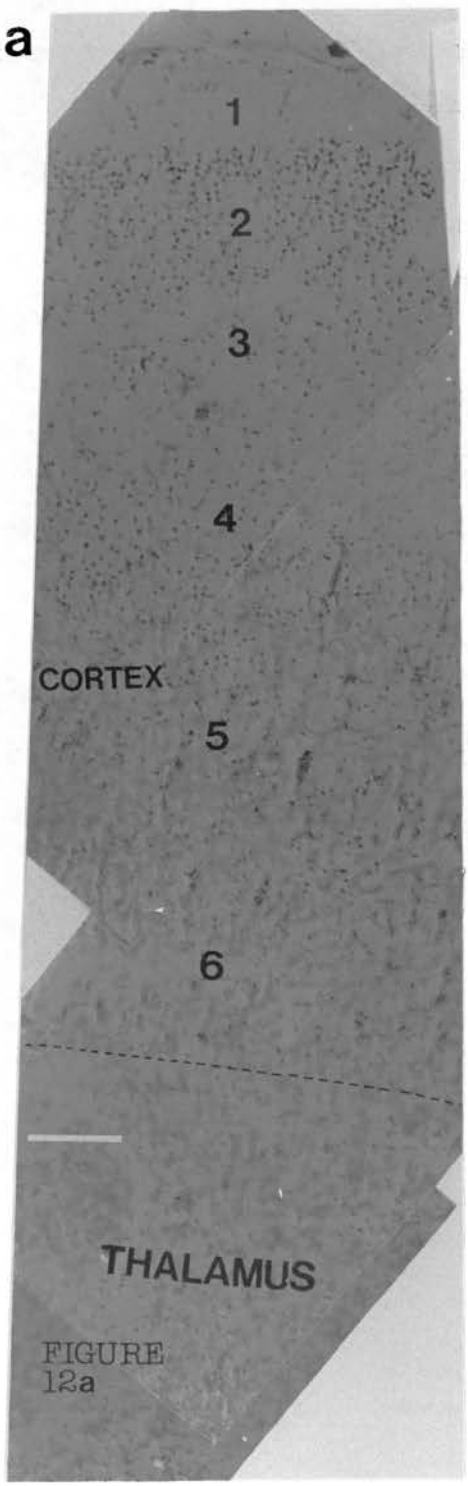


FIGURE
12a

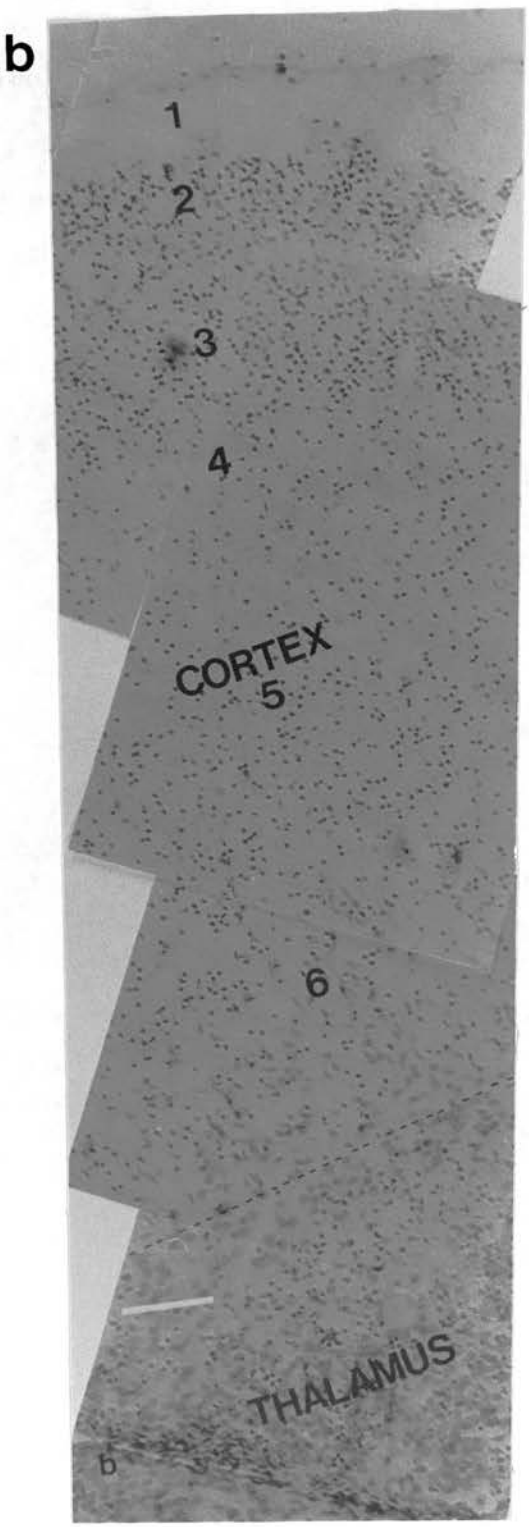


Figure 3.12

Photomicrographs of Nissl stained cross sections of an *in vitro* co-culture consisting of E15 thalamus and P6 cortex which have been cultured for 6 days in the presence and absence of 10μM 7-nitroindazole (NOS inhibitor). (a) In controls thalamic cells and cells in the deeper layers of the cortex are viable, whereas cells in the superficial layers of he cortex are pyknotic. (b) In 7-nitroindazole treated co-cultures many thalamic cells are viable but nearly all cortical cells are pyknotic (scale=70μm). It is possible that the inhibition of nitric oxide synthase (NOS) has produced massive cortical cell death and loss of the recognition signal within target layer 4.

DISCUSSION

The aim of this chapter was to investigate a possible role for NO in the development of the thalamocortical system. The enzyme NOS is expressed in both the developing cortex and thalamus *in vivo*. When NO is applied to advancing growth cones *in vitro* immediate cessation of growth occurs. The addition of NO inhibitors to thalamocortical co-cultures disrupts the growth of thalamic neurites from being restricted within target layer 4 of co-cultured cortex. Presented together these data provide strong evidence that NO plays a role in process outgrowth in thalamocortical development.

Time Lapse Experiments.

The data gathered from time lapse recordings suggest that NO could be playing a role in the development of thalamocortical projections. NO has an inhibitory effect on thalamic growth cones inducing them to collapse and even retract. Similar effects of SIN-1 on growth have been reported by Hess et al (1993), who observed rapid and reversible inhibition of growth on neurites of rat dorsal root ganglion *in vitro.* They recorded the growth of dissociated dorsal root ganglion neurites using time lapse videomicroscopy. Addition of 1mM SIN-1 to culture wells via a perfusion system induced growth cones to collapse within minutes. These effects were reversible after wash out. They suggested that NO may affect the modulation of dynamic protein fatty acylation in neuronal growth cones. I cannot determine the mode of action of NO in my experiments although the time course of NO action is similar to that reported by Hess et al (1993). SIN-1 exerted its effect on embryonic dissociated thalamic growth cones within minutes of its addition to the culture well. There is also evidence that NO modulates the expression of MAP-2, a cytoskeletal protein involved in neurite outgrowth (Johnston & Morris, 1994). Experiments were conducted in which application of NMDA, or agents releasing nitric oxide (NO), onto the dendrites of hippocampal granule cells increased the levels of the mRNA encoding MAP2.

Interestingly, Hess et al (1993) also conducted experiments on PC12 cells, these neurites had slower growth rates. Although growth cones collapsed after SIN-1 was added to these cultures the neurites did not retract. This is in agreement with my observations. SIN-1 induced very little retraction in embryonic dissociated thalamic growth cones which were advancing slowly, and the greatest retraction produced by the addition of SIN-1, was observed in a growth cone which was advancing very rapidly. My results also provide evidence that NO removes small axonal processes from the axon. NO released locally therefore would potentially allow a sustained inhibition of outgrowth to be confined to those regions of a neuron's dendritic arbor situated within the diffusion range of NO.

Expression Of NADPH-Diaphorase *In Vivo.*

Two types of staining patterns were observed. The well characterised Golgi-like cell body stain which was first described by Thomas & Pearce (1964) over thirty years ago, and a diffuse background stain probably due to low concentrations of NADPH-diaphorase in the neuropil (i.e. synapses, soma and dendrites). I cannot be sure of the identity of these stained structures. However, this background stain increases throughout the development of the thalamocortical system and follows an interesting pattern which mainly coincides with thalamocortical input. I will first describe the staining pattern of the diffuse background stain.

At E15 a diffuse dark band of label was expressed in the lower marginal zone and possibly the subplate. The subplate receives the earliest thalamic afferent input, at around E15-17 *in vivo* (Rennie & Price, 1992). Between E17 and birth, afferents are thought to form synapses in the subplate and interact with migrating cells destined for the cortex (Ghosh & Shatz, 1993). This period is termed the waiting period and its existence is debated in the rodent, although it is firmly established in the cat (Wise & Jones, 1978; Catalano et al, 1991; De Carlos & O'Leary, 1992). In the cat pioneer subplate axons which receive synaptic inputs from waiting thalamic axons, in turn make axonal projections into the marginal zone, and raise the possibility that they may function as a cellular scaffold that forms a crucial but transient link between developing thalamic axons and their ultimate target cells in

layer 4 (Friauf et al, 1990). Although, there is no evidence as yet that subplate neurons project to the marginal zone in the rodent, the expression of NOS in the marginal zone is interesting. By P0, most thalamic afferents have grown through the subplate and are advancing towards their developing target layer 4 (Lund & Mustari, 1977). At this age areas which I presume to be the subplate, cortical plate and the lower layer 1 are all labelled. At P4, layer 4 cells should be in position and maturation would be almost complete. At P4 layer 1 and migrating layers 2/3 are heavily labelled and the deeper layers are lightly labelled. The barrel fields are also labelled in layer 4. At P6, when thalamocortical afferents are established, neuropil within layers 6, 4 & 1 are heavily labelled. These layers receive thalamocortical input *in vivo*. Layer 4 receives 80% of all thalamic afferents *in vivo*, and the barrel fields within layer 4 are very heavily labelled in these cortical slices. The barrel fields mature within the first postnatal week and thalamocortical afferents have been suggested to play a primary role in barrel formation (Belford & Killackey, 1980; Jeanmonod et al, 1981). It is possible that cortical derived factors upregulate NO production in the thalamus, and the resultant NO is involved in segregating thalamocortical axons into layer 4 as well as segregating somatosensory afferents into the barrel fields. It is established that correlated afferent activity is necessary for both the segregation of eye-dominant inputs in the vertebrate visual system (Shatz & Stryker, 1988) and the formation of barrel fields in layer 4 (Van Der Loos & Woolsey, 1973; Killackey & Belford, 1979; Woolsey et al, 1979; Belford & Killackey, 1980; Jeanmond et al, 1981). It would be interesting to examine NOS expression in older brain slices to see whether the expression is transient. Cortical cells are densely packed within the barrel fields. However, I do not believe that the increased density of cells in this area can explain the dramatic increase in NOS expression. Interestingly, when parasagittal slices of P6 brains were stained for 1 hour with the NADPH-diaphorase reaction mixture, I found that NOS was distributed throughout the thalamocortical tracts. These tracts are fasiculated and are clearly stained. Axon tracts can be traced from the subplate down to the thalamus, where individual nuclei are labelled. The expression of NOS within thalamocortical tracts suggests that NO could be involved in the development of these neurites *in vivo*.

However, it is not known whether NOS is expressed within these tracts during development. I would like to study the expression of NADPH-diaphorase at intermediate ages between E15 and P0. During this period thalamic axons sprout and grow towards the cortex. I would also have to optimise and sensitise the NADPH-diaphorase reaction procedure to achieve optimal staining in these slices. It would be interesting to determine the role of NOS in the development of the thalamocortical pathway. NOS inhibitors could be perfused into the correct regions of developing embryonic brains. However, mice embryo's are considered too small for this experimental surgery and studies would probably have to be conducted on larger mammals. Homozygous neuronal NOS "knockout mice" mice (Huang et al, 1993) could also be sectioned to determine any developmental abnormalities in the thalamocortical system. However, to date, these knock out mice have demonstrated only minor developmental abnormalities despite their lack of NOS-1 catalytic activity in the brain and loss of NOS-1 immunostaining in central and peripheral neurons. It is possible that new isoforms could adopt the developmental role of NOS-1 in "knock-out mice".

In contrast to the diffuse background stain, the Golgi-like stained neurons do not become readily visible until P4. At this age they are concentrated in the deeper layers, but as development proceeds some also appear in the superficial layers. The NADPH-positive stained cells may be present earlier in the developing cortex as small undifferentiated neurons, but these are hard to distinguish in the relatively thick sections. NOS-positive nerve cells are reported to be present in P1 rat visual cortex, as small undifferentiated neurons (Luth et al, 1995). Later on in development (as reported also in this study) they are found in all layers and the majority of these cells are reported to survive until adulthood (Luth et al, 1995). It would be interesting to observe NOS distribution in older cortical explants to investigate whether the Golgi-like labelled neurons are expressed more evenly throughout the cortical plate. I cannot determine the morphological or chemical cell type of these cells. However, their expression pattern is very similar to that described in the rat, where these cells are identified as Martinotti cells which are GABAergic (Luth et al, 1995). Martinotti

cells have an ascending axon which normally projects up to layer 1 and are found in all layers of the cortex. Therefore these cells could potentially co-ordinate cortical activity.

Expression Of NADPH-Diaphorase *In Vitro*

P6 cortex did not stain positively for NADPH-diaphorase after a 6 day culture period with co-cultured thalamus. Acute slices of P6 cortex prepared in an identical manner, but not cultured, stained positively for NADPH-diaphorase suggesting that NOS downregulates during culture. This might be due to the removal of extrinsic connections *in vitro* or the inability of these cells to survive in culture. These co-cultures were set up as described in chapter 2 and preliminary evidence from chapter 1 demonstrates that in organotypic co-cultures the superficial layers of the cortex do not survive. Furthermore, in this chapter a few control co-cultures were sectioned and the viability of cells within these sections was compared to sections of co-cultures treated with 7-nitroindazole. The viability of cells in the superficial layers of the cortex in these control co-cultures was also reduced. It is possible that NADPH-diaphorase staining may be absent in the cortex of these co-cultures as a result of reduced cortical cell survival. However, many cells in the deeper layers survived in control co-cultures and this hypothesis does not explain the absence of NADPH-diaphorase in these layers.

The co-cultured thalamic explant contained NADPH-diaphorase positive label. These cells were small and due to the thickness of sections, it was difficult to determine their morphology although some small golgi-like cells were observed. Results from these experiments demonstrate that NOS expression is downregulated in the cortex and suggests that extrinsic connections may be important for maintaining NOS production in the cortex.

Isolated thalamic explants were cultured for 6 days and were labelled for NADPH-diaphorase after the culture period. The label was diffuse but was spread consistently across the explant. Again at high microscopic magnification the resolution of thalamic cells was poor due to the thickness of these slices. It was difficult to assess the exact location of the NADPH-diaphorase label. These results demonstrate that

these cells can upregulate or maintain NOS without extrinsic connections. NO is not present in cultured cortical explants and it is therefore unlikely that NO is released by cortical cells *in vitro*, however NO released by thalamic cells *in vitro* may play a role in thalamocortical development..

Control For The NADPH-Diaphorase Colour Reaction

The ideal control for this reaction would be to compare previous results with background levels produced by an explant of tissue which is known to contain no NOS or NADPH. However, this control is not possible since I was unaware of a suitable piece of tissue. However, experiments of co-cultured thalamus and cortex provide evidence that the reaction produces very little background when no NOS is present. In these co-cultures the thalamus stains positively for NOS following a 6 day culture period (proving that the reaction was working), whereas the cortex fails to react and there is no background stain. Acute P6 cortical slices stain positively for NOS before culture proving that NOS has downregulated in culture and that the background reaction is minimal.

Effects Of NO Blockers On Thalamocortical Development *In Vitro.*

Results demonstrate that in control cultures axons are restricted mainly within the region of their target layer 4. Exposure to 7-nitroindazole prevents thalamic axons from being restricted within their target layer 4 *in vitro*. In control cultures luminance decreased mainly within layer 5 and 4/3. It is possible that many axons were still growing towards their target layer 4 when these cultures were fixed therefore accounting for the large decrease in luminance within layer 5. However, few axons grow past their target layer 4 since only 25% luminance was present at the layer 3/2 border. Previous control experiments described in Chapter 2 rule out the possibility that these axons would grow past layer 4 if cultured for a longer period, since even after 10 days of culture axons were restricted within cortical layer 4. However, thalamic explants can grow for very long distances when cultured alone for 6 days, providing evidence that thalamic neurites are restricted within cortical layer 4 *in vitro*.

I also wax embedded, sectioned and Nissl stained these thalamocortical co-cultures to observe any adverse effects of 7-nitroindazole on cell viability. Control cultures were similar to previous findings in chapter 2. In controls thalamic cells and cells found in the deeper layers of the cortex were viable whereas cells in the superficial layers of the cortex were less viable. In 7-nitroindazole treated co-cultures many of the thalamic cells were viable but most cortical cells were pyknotic. It is therefore possible that the reason thalamic cells did not terminate in their target layer 4 was because these target cells were dead and could no longer produce a stop signal. These results are also interesting because results from NADPH-diaphorase staining experiments reveal that NOS expression downregulates in the cortex after culture. Again this could be due to reduced cortical cell survival in control co-cultures and suggests that the cortex does not produce any NO *in vitro*. However, blocking NOS with 7-Nitroindazole reduced the survival of cortical cells in these co-cultures. I therefore suggest that the thalamus which expresses NOS throughout the culture period could produce a factor via a mechanism involving NO production which acts to maintain cortical cell viability. If NO had an inhibitory effect on neurite outgrowth then blocking NOS expression in co-cultures could also lead to increased outgrowth from thalamic explants. Increased outgrowth may mask the restriction of some thalamic axons within target layer 4. The ability of NO to inhibit outgrowth could be tested by comparing the outgrowth of neurites from isolated thalamic explants in culture which have been treated with 7-nitroindazole or with NO releasing compounds. The experiments with 7-nitroindazole cannot eliminate the possibility that NO is involved in the termination mechanism in layer 4, however it does not provide positive evidence to suggest NO is involved.

Conclusion

It is clear from the results that NO is present in both the developing thalamus and cortex *in vivo* and is also expressed within thalamocortical tracts which connect these two structures. Advancing growth cones of dissociated thalamic cells are responsive to NO *in vitro*. This chapter provides evidence that NO could be involved in regulating thalamocortical outgrowth both *in vivo* and *in vitro* and helps support

previous observations (Bohme et al, 1991; O'Dell et al, 1991; Schuman & Madison, 1991; Shibuki & Okada. 1991) that NO may participate in signalling mechanisms that serve to regulate axonal growth.

The endogenous production of NO can be regulated by the activation of neuronal glutamate receptors (Garthwaite, 1991; Garthwaite et al,1988; Bredt & Snyder, 1990), many of which are present in the visual cortex of the neonatal rat (Coyle & Yamamura, 1976; Monaghan & Cotman, 1985). For instance, NMDA receptors contribute to both mEPSCs and evoked EPSCs in the developing rat neocortex (Burgard & Hablitz, 1993), and these events could induce Ca^{2+} influx and subsequent activation of intracellular signalling mechanisms. Thalamic neurons in culture also form predominantly glutamatergic synapses (Gottmann et al, 1994) and my results reveal that the embryonic thalamus expresses NOS *in vitro*. The above stimuli could induce inhibition of process extension (Pearce et al, 1987), synapse stabilisation (Constantine-Paton et al, 1990), and perhaps elimination (Rabacchi et al, 1992) via NO production. NMDA receptors in the visual cortex of kittens are highest during the developmental stage which is considered the critical time for experience-dependent modifications of the cortex (Bode-Greuel & Singer, 1989). Altering the electrophysiological characteristics of NMDA EPSCs or the expression of NMDA receptors during development could regulate axonal growth or neural plasticity.

The fact that thalamocortical tracts express NOS, expression of NOS in the cortex coincides with thalamocortical innervation *in vivo*, NOS decreases in the cortex in culture and addition of 7-nitroindazole (NOS inhibitor) to thalamocortical co-cultures reduces cortical cell survival suggests NO is important in regulating thalamocortical development. A hypothesis which involves NO in thalamocortical development and incorporates the evidence provided by this study is that cortically derived factors produced by the cortex upregulates NO production in thalamic cells. NO acts as a secondary messenger to regulate thalamic outgrowth and cortical cell survival. It would be interesting to examine in more detail the role of NOS inhibitors on thalamocortical development.

CHAPTER 4:

TROPHIC AND OUTGROWTH -PROMOTING EFFECTS OF K^{+}-INDUCED DEPOLARIZATION ON DEVELOPING THALAMIC CELLS IN ORGANOTYPIC CULTURE

CHAPTER 4: TROPHIC AND OUTGROWTH-PROMOTING EFFECTS OF K^+-INDUCED DEPOLARIZATION ON DEVELOPING THALAMIC CELLS IN ORGANOTYPIC CULTURE

ABSTRACT

The aim of this study was to investigate how different levels of K^+-induced depolarization affect the survival and growth of isolated, cultured thalamic explants from mice aged embryonic day 13 to postnatal day 2. K^+ was added to explants in serum-free culture medium. After culture for 3 days, explants were sectioned and Nissl-stained or photographed under phase-contrast for quantification of neurite outgrowth. Viable and pyknotic cells were counted in sectioned material. The results revealed that, with no added K^+, both viability and neurite outgrowth decreased as the age of the thalamic explant increased: most cells survived in embryonic day 13 explants, most died in postnatal day 2 explants. Adding K^+ had an age and dose-dependent effect on viability and neurite outgrowth. The greatest viability-promoting effect of adding K^+ was at embryonic day 19: adding 5 mM K^+ rescued the majority of these cells, although there was no effect on neurite outgrowth at this age (i.e. enhanced viability did not necessarily produce increased outgrowth). This same dose of K^+ had its greatest effect on neurite outgrowth at embryonic day 17. No dose of added K^+ had a stimulatory effect on viability and neurite outgrowth after embryonic day 19. The highest dose of K^+ used here (50 mM) inhibited thalamic cell survival.

We suggest that the survival and growth of the prenatal thalamus can occur without external afferent input. This innate mechanism becomes increasingly reliant on neural activity for its maintenance as it ages. After birth, when thalamic cells may switch their dependence to cortex derived growth factors, this mechanism may become ineffective.

INTRODUCTION

In the mouse, the birth of posterior thalamic neurons is completed early in the second half of gestation, between embryonic day 13 (E13) and E14 (Rennie S., 1992). Axonal outgrowth from the thalamus begins at around E14-E15 and thalamocortical axons form the internal capsule and reach the subplate (a transient layer below the developing cortex) by approximately E17 (Blakemore & Molnar, 1990; Catalano et al, 1991; De Carlos & O'Leary, 1992; Ferrer et al, 1992; Lund & Mustari, 1977; Molnar & Blakemore, 1990). After E17, thalamocortical fibres begin their radial migration into the cortex and shortly after birth most of them have terminated in layer 4 (Lund & Mustari, 1977).

Many recent studies have investigated interactions between the developing cortex and thalamus in organotypic and dissociated co-cultures (Bolz et al, 1990,1992,1993; Hisanaga & Sharp, 1990; Lotto & Price, 1994,1995; Molnar & Blakemore, 1991,1995; Novak & Bolz, 1993; Rennie et al, 1994; Tuttle & O'Leary, 1993; Yamamoto et al, 1989,1992) . It has been shown that early postnatal neocortical explants promote the growth of neurites from thalamic explants cultured in serum-free medium (Lotto & Price, 1994,1995; Rennie et al, 1994). Conditioned medium and conditioned substrate experiments have shown that this effect is mediated by diffusible cortex-derived growth factors (Rennie et al, 1994). Similar conclusions have come from *in vivo* experiments demonstrating that thalamocortical neurons can be rescued from axotomy-induced death by the addition of a macromolecular fraction of medium preconditioned with cultured postnatal cortical slices to the sites of the lesions (Cunningham et al, 1987). These results suggest that postnatal cortex releases factors that promote thalamic survival and outgrowth, in agreement with the recognised postnatal upregulation of known growth factors in the developing brain (Castren et al, 1992; Large et al, 1986; Maisonpierre et al, 1990; Thomas et al, 1991).

However, evidence from co-cultures of embryonic thalamus with prenatal cortex suggests that, before birth, the cortex has little or no influence on the growth of the thalamus (Price et al, 1995; Rennie et al, 1994). Furthermore prenatal thalamus can survive when cultured alone for three days (Rennie et al, 1994). These findings

suggest that the factors that support the viability and growth of the embryonic thalamus are produced within the thalamus itself.

During the prenatal period, neural activity in the thalamus will be increasing, largely due to the onset of spontaneous activity in the peripheral sensory receptors (Shatz & Stryker, 1988). Neural activity is known to induce the production of growth factors in the brain (Ghosh et al, 1994; Riva et al, 1992; Zafra et al, 1992), and it is possible that enhanced levels of neural activity stimulate the embryonic thalamus to grow. The aim of this study was to investigate the ability of cultured thalamic explants of different ages from E13 to postnatal day 2 (P2) to survive and grow in isolation with and without increased activity. Activity was enhanced by adding K^+ to the culture medium (Lipscombe et al, 1988), a method that is widely used to non-specifically depolarize neuronal membranes, and the effects on cell survival/death and neurite outgrowth were examined.

Ionic Basis Of The Resting Potential

The electrical potential difference between the inside and outside of a nerve cell membrane depends on the ionic concentration gradients across the cell membrane and the relative permeability of the membrane to the ions present. For a steady state membrane potential to be maintained, the total distribution of ions on either side of a cell membrane must satisfy three major constraints: 1. the bulk of the solutions inside and outside the cell must be electrically neutral; 2. the osmotic concentration of intracellular ions and molecules in the solution must be equal to that in the extracellular fluid; and 3. there must be no net flux of any permeant ions across the membrane.

Each permeant ion has different intracellular and extracellular concentrations and is subject to 2 gradients which drives it into or out of the cell: a concentration and an electrical gradient. Potassium is more concentrated inside the cell than out, so outward movement of potassium ions along the concentration gradient would be anticipated. However, the inner surface of the membrane is negative with respect to

the outside, which restrains the outward movement of positively charged ions. In resting cells, these concentration and electrical gradients are in balance, tending to restrain the outward movement of potassium ions. The membrane potential at which their is no net flux of potassium ions is called the potassium equilibrium potential (E_K). The equilibrium potential for any ion, in terms of extracellular and intracellular ionic concentrations, is given by the Nernst equation.

Sodium is much more concentrated in the extracellular fluid than in the cytoplasm. Therefore, in a normal cell with a negative resting potential, the concentration gradient and membrane potential both favour inward movement of sodium. However, the resting membrane is only sparingly soluble to sodium. The inward sodium leak depolarizes the membrane slightly from the potassium equilibrium potential, so that there is an accompanying outward leak of potassium. To maintain a steady state in the face of these continual leaks, sodium is transported outward, and potassium inward, across the cell membrane by ionic pumps.

The Dependence Of The Resting Potential On Extracellular Potassium.

In neurons the membrane potential is sensitive to changes in extracellular potassium concentration. The extracellular potassium concentration can be increased from 3mM to 6mM, by replacing 3mM NaCl with 3mM KCl, thereby keeping the osmolarity unchanged. Assuming an intracellular K^+ concentration of 140mM the result of doubling the extracellular potassium concentration, would depolarize the cell membrane from -96.8 to -79.3 mV. The intracellular potassium concentration is increased slightly, and the intracellular chloride concentration almost doubled. This is because potassium and chloride both enter the cell. When the external potassium concentration is increased, potassium is no longer in equilibrium and enters the cell. As positive charges accumulate on the inner surface of the membrane, chloride ions, being no longer in equilibrium, move in as well. This entry of potassium and chloride continues until a new equilibrium is established and is accompanied by the

entry of water to maintain osmotic balance. This results in a slight increase in cell volume.

Importance Of Apoptosis

A conspicuous feature of the development of the nervous system is that many cells are born to die. Examples of developmentally programmed cell death are the regression of the Mullerian ducts in male embryos, the removal of interdigital webs, and amphibian tail regression during metamorphosis (Walker et al, 1988). Apoptosis is also particularly important in the development and effective functioning of the immune system (reviewed by Golstein et al, 1991; Williams, 1994). Failure or suppression of apoptosis is likely to contribute to the initial development of cancer and to the appearance of tumour cells resistant to cytotoxic therapy (reviewed by Williams, 1991).

Morphological And Cellular Features Of Necrosis And Apoptosis

Two common forms of cell death have been described in vertebrate tissues (Kerr et al, 1972). Necrosis refers to the morphology most often seen when cells die from severe and sudden injury, such as ischaemia, sustained hyperthermia, or physical or chemical trauma. this is sometimes referred to as accidental death. In necrosis their are early changes in mitochondrial shape and function, and the cell rapidly becomes unable to maintain homeostasis. The cell loses its ability to regulate osmotic pressure and as a consequence the cell swells and the plasma membrane is ruptured. The cells contents are spilled into the surrounding tissue space and trigger an inflammatory response. This inflammatory response enables the debris to be cleared away and activates the repair process.

Apoptosis is often equated with programmed cell death and it refers to a series of morphological changes which are observed during cell death that are different from those seen in necrosis. Apoptosis occurs in the death of cells with short half-lives (such as the neutrophil); the elimination of self-reactive T cells; involution of cells deprived of necessary growth factors; morphogenetic death of cells during embryonic

and early postnatal development; and killing of cells which serve as targets for T cells. The reason and the triggering mechanism for cell death is different between cell types. Often the stimulus that induces cell death is provided by the environment. In other cases (e.g. neutrophil turnover), cell death appears to be activated by an intrinsic autonomous clock. Truly cell autonomous cell death has been identified in the nematode, and the genes controlling it have been identified (Yuan & Horvitz, 1990). However, although the pathways triggering apoptosis may be different in different cells, the mechanism of death itself may always be the same.

The cellular changes in apoptosis are numerous. The plasma membrane becomes ruffled and blebbed, in a way more pronounced than is seen in necrosis; the phenomenon has been called 'zeiosis' (Sanderson, 1982; Goodman et al, 1975). The nucleus then shrinks and chromatin becomes very dense, collapsing into patches, then it forms crescents in tight apposition to the nuclear envelope, and finally the nucleus frequently breaks up into dense spheres. At the molecular level, these changes are thought to be accompanied by activation of a Ca^{2+} , Mg^{2+} -dependent endonuclease that results in the formation of multiple DNA fragments consisting of approximately 180 base pairs. These fragments can readily be seen after agarose gel electrophoresis, wherein a characteristic "ladder" develops (Wylie et al, 1980). The cell may then break up into apoptotic bodies, but these are sealed and maintain there osmotic gradients. There is no spilling of intracellular contents, and no inflammatory response.

The cell undergoing apoptosis also shrinks. The most likely explanation is that the cell loses water and ions isoosmotically. Biochemically, most cells decrease their synthesis of RNA and proteins (Cidlowski J.A., 1982; MacDonaald & Cidlowski, 1981).

Different Activation Mechanisms For Apoptosis.

New gene expression can be induced after exposure to the apoptotic stimuli. Apoptosis could therefore be inhibited if mRNA or protein synthesis is blocked. These processes are collectively referred to as induction mechanisms (see review, Cohen, 1991). In other systems apoptosis is triggered by the inhibition of mRNA or

protein synthesis e.g. cell line HL-60 (Martin et al, 1990). Because these cells behave as though the suicide program is constitutively expressed but inhibited by factors with short half-lives, the mechanism is referred to as release. These two mechanisms are related biochemically. In other systems inhibitors of macromolecular synthesis have no effect, positive or negative (Duke et al, 1993). These processes are called transduction mechanisms, and their existence means that cells may have all the necessary molecules for apoptosis within them at all times.

Caenorhabditis elegans- A Model System For Active Cell Death

The nematode *C.elegans* is a very powerful model system for analysis of the cell and molecular biology of multicellular animals. The embryonic development of C.elegans is reproducible and has been precisely mapped. 1090 somatic cells are eventually formed in the adult hermaphrodite and of these 131 undergo cell death (Ellis et al, 1991). The death of these cells is just as predictable as the division and differentiation occurring during development. This developmentally programmed cell death displays several areas of similarity with apoptosis in mammalian cells and recently the genes controlling cell death have been shown to be phylogenetically conserved between these animal kingdoms.

Mutant nematodes have been identified with defects in different parts of the cell death process and this has allowed a genetic pathway of cell death to be produced. The genes identified can be divided into several groups: genes involved in triggering cell death (Egl-1, Ces-1 and Ces-2); those involved in the cell death process itself (Ced-3, Ced-4, Ced-8 and Ced-11); those that suppress cell death (Ced-9); those required for engulfment (Ced-1, Ced-2, Ced-5, Ced-6, Ced-7 and Ced-10) and those implicated in the disposal of the corpse (Nuc-1). In mammals the process is inevitably more complicated and involves more genes. Several of the nematode cell death genes have been cloned and sequenced (reviewed by Yuan, 1995). Ced-9, a suppresser of apoptosis in the nematode, is homologous to the Bcl-2 family of proteins and Ced-3 is homologous to the interleukin-1β converting enzyme (ICE) family of cysteine proteins (Yuan & Horvitz, 1992) which exist in mammalian cells.

Proteases In Apoptosis

Recent attention has focused on the possibility that intracellular proteases might play a critical role in the initiation of apoptosis. Evidence implicating proteases in apoptosis comes from a variety of physiological, biochemical, and genetic studies: specific and reproducible proteolytic cleavage of cellular proteins has been identified early in apoptosis (Martin et al, 1995); certain protease inhibitors have been shown to inhibit apoptosis(Kwo et al, 1995); viral proteins capable of inhibiting apoptosis appear to act as protease inhibitors (Komiyama et al, 1994); and gene knockout experiments have also demonstrated an essential role for specific proteases in some physiological models of apoptosis (Kuida et al, 1995).

The sequence similarities between Ced-3 and the human interleukin-1β converting enzyme (ICE) suggested that ICE or an ICE-like protease might play a role in the control of programmed cell death in mammals (Yuan et al, 1993). The most highly conserved regions share 43% sequence identity. ICE is a protease that cleaves the inactive precursor of interleukin-1β to its active form (Kostura et al, 1989). Interleukin-1β (IL−1β) is a cytokine involved in mediating inflammation, septic shock, wound healing, haematopoiesis and the growth of certain leukemias (Dinarello, 1991). There is now an abundance of evidence which suggests that ICE plays a role in apoptosis (see review, Patel et al, 1996). However, the specific substrates for ICE activity during apoptosis remain unknown. ICE and possibly ICE homologues are believed to mediate their actions on substrates other than pro-IL-1β. The existence of other substrates is suggested by the observation that expression of ICE in rodent fibroblasts, which do not express pro-IL-1β, induces cell death by apoptosis (Miura et al, 1993).

The identification of specific substrates for apoptotic proteases is being actively pursued. To date, only a few apoptotic substrates have been identified (see review, Patel et al, 1996): the lamins (intranuclear intermediate filament proteins that form a fibrous layer interposed between the inner nuclear membrane and the chromatin);

poly ADP-ribose polymerase (an abundant nuclear enzyme that catalyses the conversion of the dinucleotide NAD^+ to nicotinamide and protein-linked chains of ADP-ribose); and the 70kDa polypeptide of U1 RNP (a particle required for splicing of precursor mRNA). Other substrates which include: endonucleases, histones, nucleolin, and nuclear matrix proteins have also been implicated as potential targets for proteases.

MATERIALS AND METHODS

Animals And Surgery

BALB/c mice from an isolated laboratory colony were mated overnight. Plugged females were separated from the male the following day, deemed E1. Thalamic explants were obtained from E13, 15, 17, 19 and postnatal day 2 (P2) mice. Pregnant mice were deeply anaesthetised with urethane (0.3ml of a 25% solution in normal saline, i.p.) and foetuses were removed by Caesarean section and decapitated. P2 pups were instantly decapitated using a guillotine. The brains were removed, and the posterior thalamus was carefully dissected out as described before (Lotto & Price, 1995; Rennie et al, 1994) and stored in ice-cold oxygenated Earl's balanced salts (Sigma). $1.5mm^3$ (about 1.5 x 1 x 1mm) thalamic explants from the posterior thalamus were sliced at 350μm with a McIlwain tissue chopper. These explants were centred on the lateral geniculate nucleus (LGN) and they included neighbouring nuclei (Lotto & Price, 1995; Rennie et al, 1994).

Culture Methods

Thalamic slices were positioned on a collagen-coated filter (Costar UK, Transwell-COL chambers with 3μm pores) suspended in a chemically defined serum-free medium (Bottenstein & Sato, 1979; Lotto & Price, 1995; Molnar & Blakemore, 1991; Rennie et al, 1994; Romijn et al, 1988; Yamamoto et al, 1989). Serum-free medium contains: 100mls of Dulbecco's Modified Eagles (DME) formulae D5671, 100 mls of Ham formulae F12 (N4888), 1mg Insulin, 2mg apo-Transferrin, 0.24gm $NaHCO_3$, 3mls Hepes buffer, 2mls L-Glutamine, 2mls of Putrescine, 20μl of Na_2SeO_3, 20 μl of Progesterone (all from Sigma chemicals). The filters and medium had been pre-incubated at 37°C with 5% CO_2 for at least 2 hrs. Two millilitres of medium were placed in the lower chamber of each culture-well and 200-250μl of the same medium were placed in the upper chamber, ensuring the explants were just covered. Thalamic explants were cultured for three days at 37°C with 5% CO_2 in the presence of various concentrations of K^+ (Table 4.1). Over the culture period,

thalamic explants spread slightly but maintained their original shape. Once the culture period was complete, the medium was replaced by a similar volume of 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) for a minimum of 1 hour to fix the tissue.

TABLE 4.1. This table displays the number and age of thalamic explants cultured under each experimental paradigm. The first column gives the concentration of added K^+ (concentration after addition); the second column gives the final concentration of K^+ in each culture well (the serum-free medium contains 4 x 10^{-3}M K^+).

		Age of Explant				
		E13	**E15**	**E17**	**E19**	**P2**
Added [K^+]	**Final [K^+]**					
viability expt						
0	**4mM**	5	4	4	4	3
50m M	**54mM**	2	2	2	2	2
5m M	**9mM**	2	2	2	2	2
0.5m M	**4.5mM**	2	2	2	2	2
0.05m M	**4.05mM**	2	2	2	2	2
outgrowth expt						
0	**4mM**	7	6	7	8	4
5mM	**9mM**	13	16	12	9	4

Viability Experiments

Following fixation, the paraformaldehyde was replaced with a similar volume of 0.1M PBS for at least 1 hr. Explants were then wax-embedded and sectioned (10μm). Sections were mounted on poly-$_L$-lysine coated slides and Nissl-stained to reveal live and pyknotic cells.

Outgrowth Experiments

Following a 1 hour fixation period, the paraformaldehyde was replaced by a similar volume of 0.1M PBS. In some cultures (n=20), neurites growing from the thalamic explants were labelled by placing a crystal of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) onto the fixed tissue and allowing it to diffuse over the following 3-4 weeks; this was not always done as neurites on the collagen filters were readily visible with phase-contrast. All thalamic explants were photographed under phase contrast for analysis.

Control Experiments

(i) Time-Course Of Neurite Growth

In addition to the experiments in Table 4.1, E17 thalamic explants (n=8) were cultured using essentially the methods described above, except that they were removed to the stage of an inverted microscope surrounded by an incubator (37°C with 5% CO_2) for photographing after 24, 48 and 72 hrs of culture. The photographs were then analysed to determine the growth rates of thalamic neurites in control medium and medium with added K^+ (5 mM). These experiments were designed to test whether added K^+ increased the growth rates of thalamic neurites or induced earlier neurite outgrowth.

(ii) Possible Osmotic Effects

E17 thalamic explants (n=3) were cultured with a concentration of 10^{-2}M sucrose to eliminate the possibility that results obtained with K^+ were due to an osmotic effect. Outgrowth and viability data from these cultures were compared to control data (no added sucrose or K^+).

(iii) Neurofilament Staining Of Neurite Outgrowth

To ensure that outgrowth was axonal rather than glial (these can appear similar) (Torren-Allerand, 1990), E13, 15, 17 and 19 thalamic explants were cultured as above and labelled with an antimicrotubule antibody (namely anti-β-tubulin). Thalamic explants were fixed for 8 minutes in 4% paraformaldehyde in 0.1M PBS (room temperature), incubated in 90% methanol at -20°C for 10 minutes, washed (x3) in 0.1M PBS with 2% Triton-X-100 for 5 minutes, incubated in 0.1 M PBS with 10% horse serum (10 minutes) and placed in anti-β-tubulin (1:300; Sigma), diluted in 2% Triton X-100 in 0.1M PBS, for 24 hrs at 4°C. They were then washed (x3) in 0.1M PBS with 2% Triton X-100 for 5 minutes and incubated in biotinylated horse anti-mouse IgG (1:200) in 2% Triton X-100 in 0.1M PBS for 24 hrs at 4°C. After incubation they were washed (x3) with 0.1M PBS for 5 minutes, followed by 1 hr 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.

ANALYSIS

(i) Cell Viability

Nissl staining revealed both live and pyknotic cells. Pyknotic cells were identified by their small, dark nucleus, which was often fragmented into smaller pieces; the cytoplasm of these cells was often absent. In contrast, healthy cells were pale coloured with clear nuclei and nucleoli. All cell counts were carried out blind. For each explant, the numbers of live and pyknotic cells were counted in twelve

randomly selected areas (0.0186mm^2). In each of these areas, the number of viable cells was expressed as a percentage of the total number of cells counted. The data generated from these twelve areas were then averaged to give an overall percentage of viable cells in each explant.

To ensure that K^+ treatment did not induce massive mitosis, the total number of cells (live plus pyknotic) in E19 thalamic explants cultured with 5 mM additional K^+ was estimated and compared to E19 controls (E19 explants were studied since the greatest increase in cell viability was observed at this age). To make these estimates, the cell count for each explant was converted to cell density (cells/mm^3) and this value was multiplied by the volume of the explant (deduced from the numbers of 10μm sections from each explant and their surface areas).

(ii) Neurite Outgrowth

Under phase contrast, the Transwell collagen filters appear to be grooved, with the narrow furrows (about 35μm wide) all running parallel (fig 4.1a). The grooves act as guides for fibres growing from the thalamic explants thus keeping the majority of outgrowth well ordered, and individual fibres and fascicles straight (Rennie et al, 1994). In E13, 15 and 17 thalamic explants, neurite outgrowth was prolific (especially in K^+ treated explants), making it impossible to measure the density of neurites by counting individual profiles, as was done previously (Lotto & Price, 1995; Rennie et al, 1994). All quantifications were carried out blind. The area of collagen-coated membrane covered by thalamic fibres and the lengths of the five longest neurites were measured in each explant. Photographs of thalamic explants (under phase contrast) were scanned into a Joyce Loebl computerised image analysis system. The outlines of the explant and the area covered by neurite outgrowth were carefully traced with a Magiscan pen (fig. 4.1b); values for the area of the explant (area 1 in fig. 4.1b), the circumference of the explant and the area covered by neurite outgrowth (area 2 in fig. 4.1b) were obtained. The longest neurites were marked using the Magiscan pen, by placing a dot at the point where they exited the explant and another dot at their growth cones. The distance between the dots was then calculated. To check the reproducibility of this method of analysis, repeated

measurements of an irregular area of thalamic outgrowth were measured (maximum error = 5%).

FIGURE 1
a

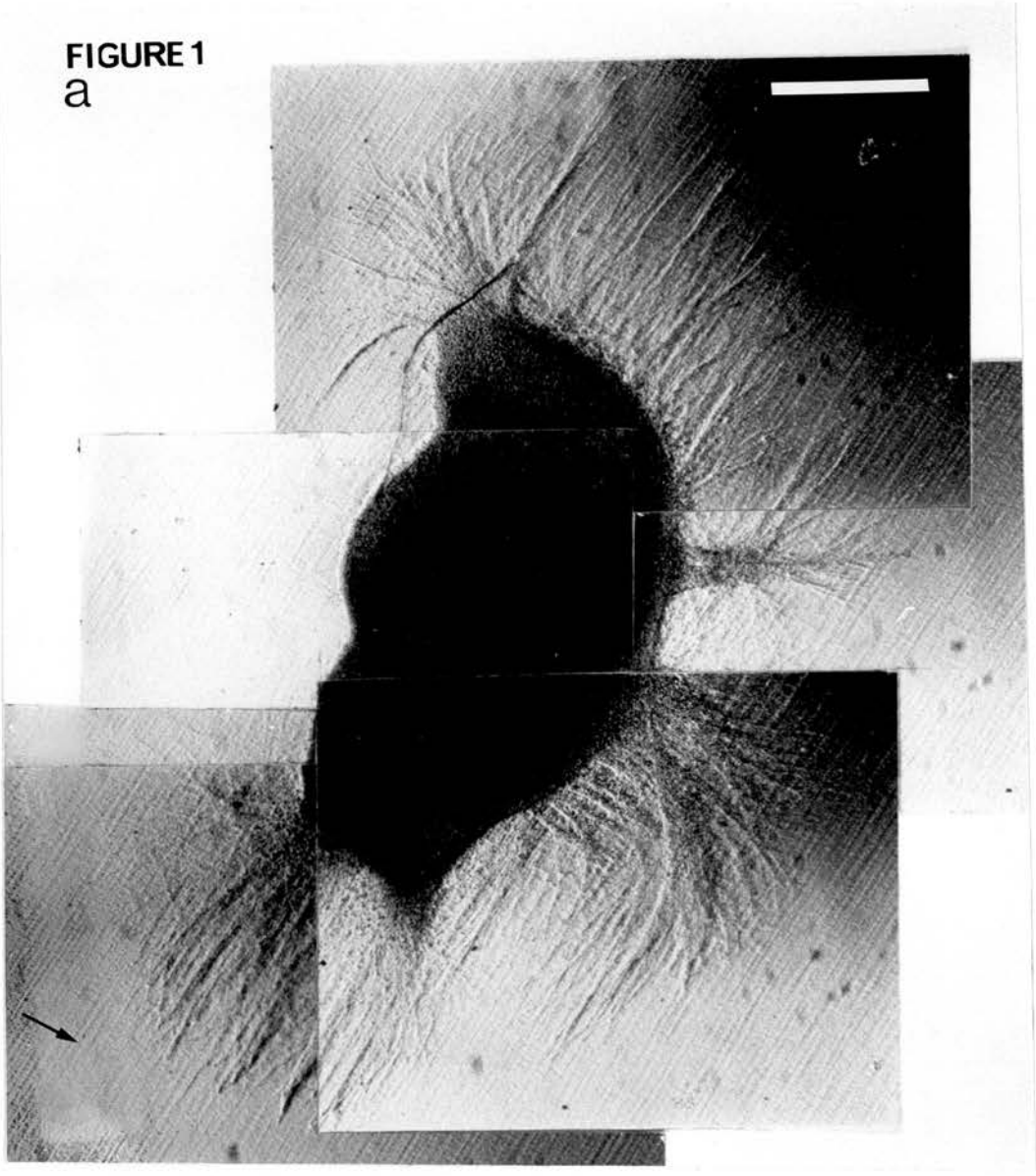


Figure 4.1

(a) Phase contrast photograph of a K^+ (5mM) treated E17 thalamic explant. Arrow indicates grooves on the collagen membrane. Scale bar, 576μm

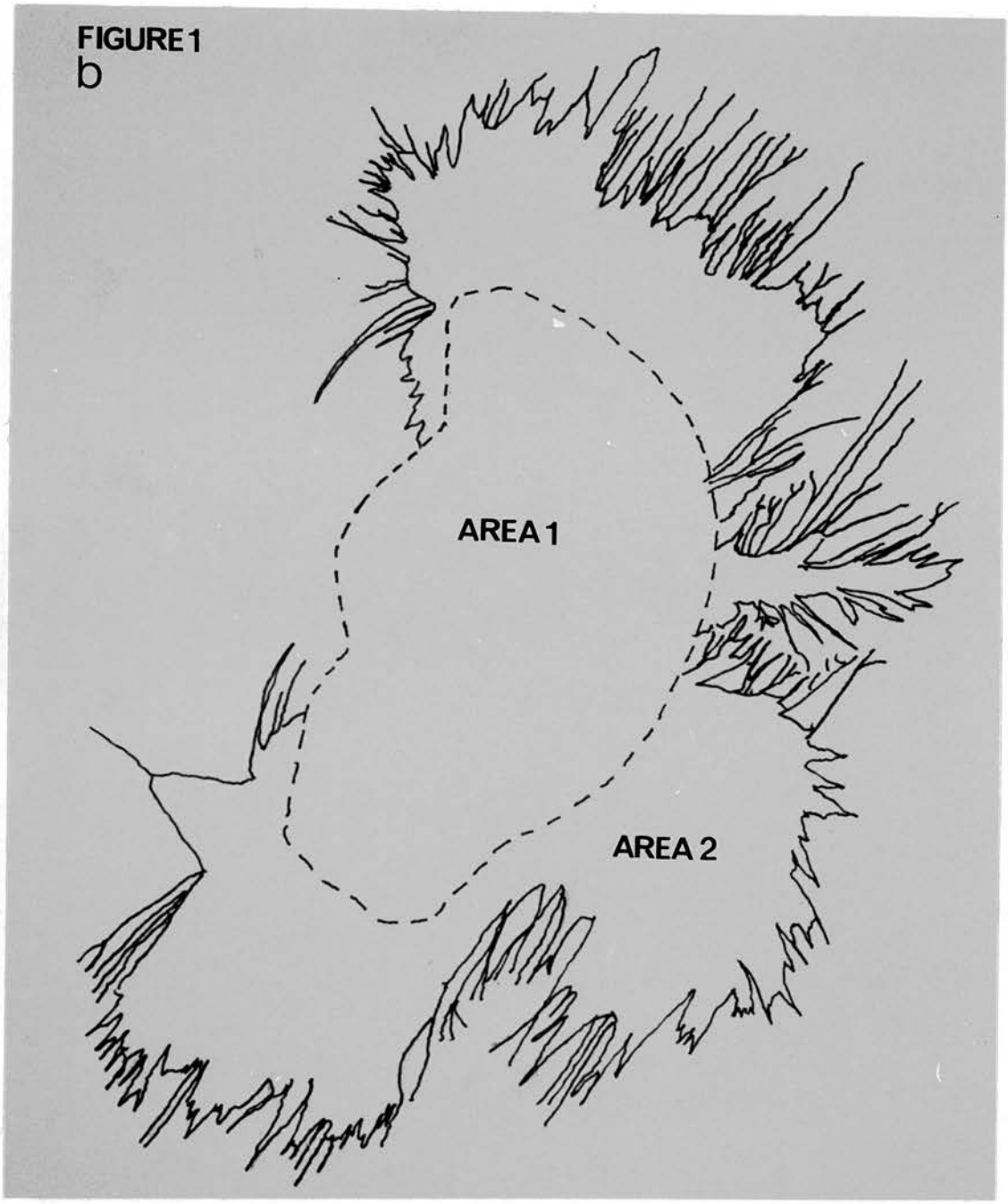


FIGURE 1
b
AREA 1
AREA 2

Figure 4.1

(b) Diagram displaying the areas which were traced with a Magiscan pen for analysis. Area 1: area of explant. Area2: area covered by neurite outgrowth.

RESULTS

Viability Of Cultured Explants

The cellular content of thalamic explants after three days in culture was examined in Nissl-stained sections. Other nuclear dyes could have been used to identify cell nuclei in these explants e.g. Hoescht dyes. I am unable to identify neurons or glia in this study. I can only identify pyknotic and viable cells. To identify glial cells, I could have double labelled Nissl-stained cells with glial fibrillary acidic protein (GFAP), this protein is found on the surface of glial cells.

Examples are shown in fig 4.2 and quantitative data are given in fig. 4.3. In control explants, the percentage of viable cells decreased as the age of the explant was increased between E13 and P2. 91% of cells in E13 thalamic explants were viable (fig. 4.2a and 4.3a), whereas only 28% of cells in E19 thalamic explants (fig 4.2b and 4.3a) and 12% of cells in P2 thalamic explants were viable. Fig. 4.3a also displays the dose-dependent effects of adding K^+ on viability in thalamic explants of different ages. At all ages, addition of 50mM K^+ to the culture medium reduced cell survival ($p<0.05$ at all ages; Student's t-test); this is illustrated for E19 explants in fig. 4.2c. However addition of 5mM K^+ increased the percentages of viable cells in E15, E17 and E19 thalamic explants ($p<0.007$ for all; Student's t-test), but not in E13 explants where survival was already very high in controls. 5mM K^+ may have had a toxic effect at P2 ($p=0.05$; Student's t-test). Fig. 4.2d illustrates the survival-promoting effect of 5mM K^+ at E19. 0.5mM K^+ produced a weaker stimulatory effect on viability in E17 and E19 explants ($p<0.02$; Student's t-test); there was no effect at the other ages. 0.05mM K^+ had no effect on cell survival (this dose represents only a very small increase in K^+ levels over those in the controls: Table 4.1).

I concluded that the effects of K^+ were dependent on the concentration used and the age of the thalamic explant. Fig. 4.3b displays these effects more clearly. In this histogram, mean control values of cell survival are subtracted from the mean values

FIGURE 2

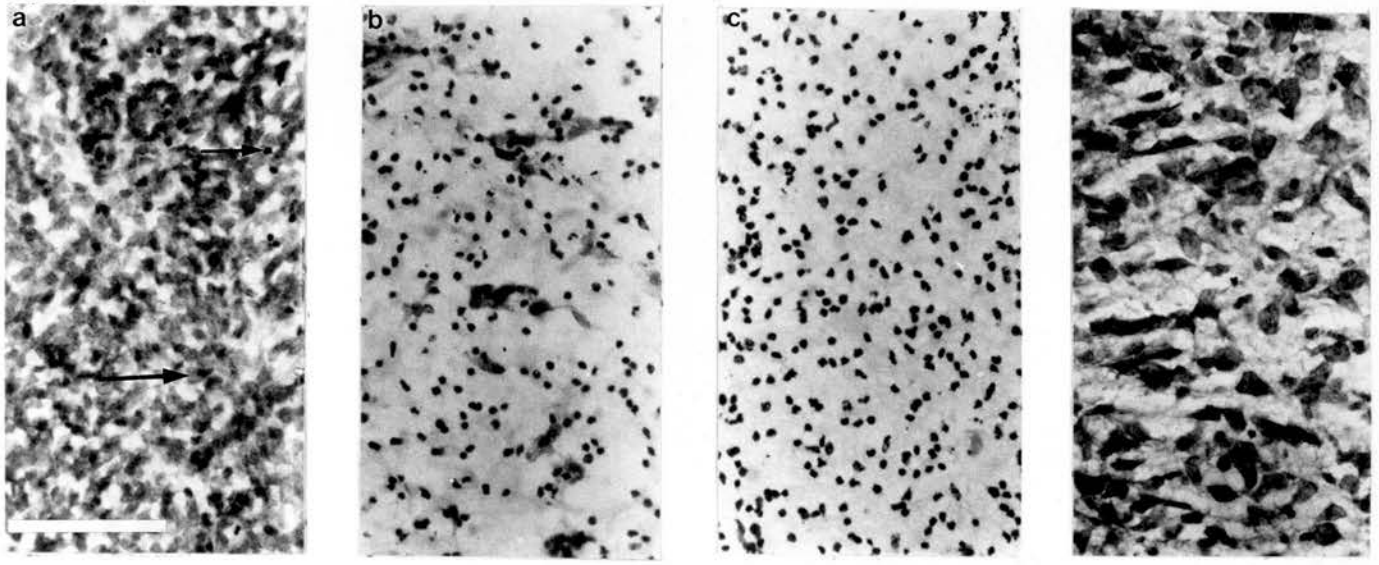
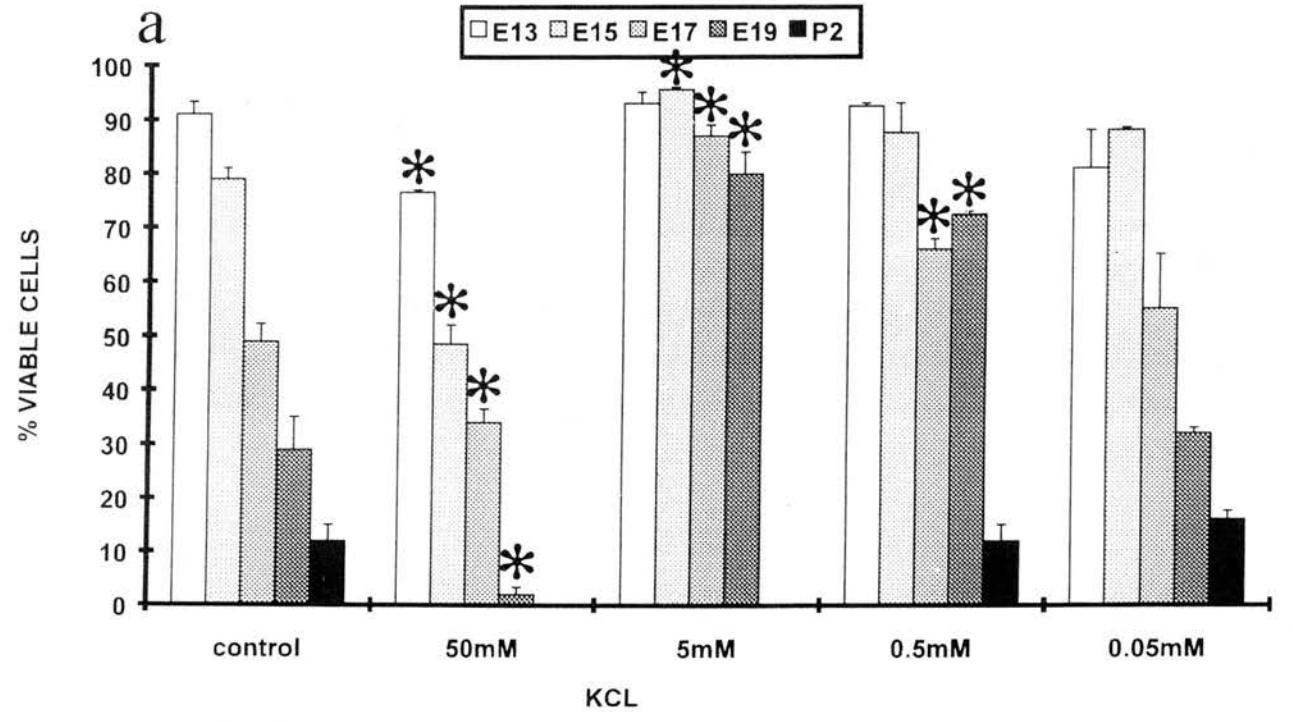


Figure 4.2 Photomicrographs of Nissl-stained sections of cultures of thalamus. (a) E13 explant in control medium (no added K^+): most cells were viable. (b) E19 explant in control medium: few cells were viable. (c) E19 explant with 50mM K^+: all cells were pyknotic (d) E19 explant with 5mM K^+: many cells that died in controls were rescued. Large arrows indicate viable cells, small arrows indicate pyknotic cells. Scale bar, 72μm


a
E13 E15 E17 E19 P2
% VIABLE CELLS
100
90
80
70
60
50
40
30
20
10
0
control
50mM
5mM
0.5mM
0.05mM
KCL

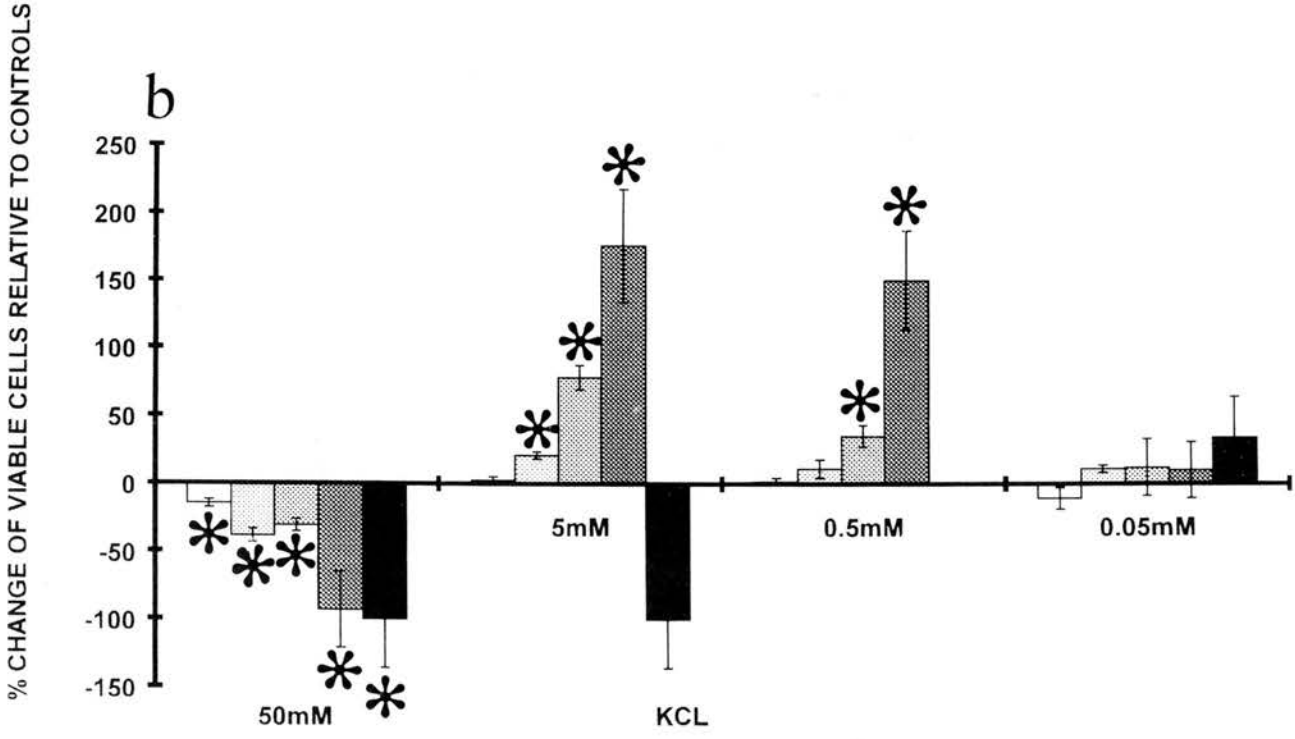

b
% CHANGE OF VIABLE CELLS RELATIVE TO CONTROLS
250
200
150
100
50
0
-50
-100
-150
50mM
5mM
KCL
0.5mM
0.05mM

Figure 4.3

(a) Histogram comparing the mean percentages (± S.E.M) of viable cells in control and K^+ treated thalamic explants of different ages (E13-P2). K^+ had a dose-dependent effect on cell survival. For control values at each age n=3-5. At each age, for all concentrations of K^+ n=3. At each age, the effect of K^+ treatment was compared to controls using a Student's t-test. * on the histogram indicates that the data are significantly different from controls ($p < 0.05$).

(b) In this histogram the change in cell viability after K^+ treatment is expressed as a percentage of the control value. The mean value (± S.E.M) after K^+ treatment is subtracted from the mean control value (± S.E.M) and expressed as a percentage of the mean control value (± S.E.M). For control values at each age n=3-5. At each age, for all concentrations of K^+ n=3. At each age, the effect of K^+ treatment was compared to controls using a Student's t-test. * on the histogram indicates that the data are significantly different from controls ($p < 0.05$).

after addition of K^+, and the product is expressed as a percentage of the mean control value. This histogram emphasises that K^+ had its greatest survival-promoting effect on E19 thalamic explants (the percentage of viable cells in these explants almost doubled with addition of 5mM K^+), although younger explants also responded. Cells in P2 thalamic explants did not follow the dose-response pattern displayed by cells in younger K^+ treated thalamic explants: both 50mM and 5mM K^+ were toxic, and lower doses had no effect on these cells.

Although the most likely explanation for the effects of added K^+ on E19 explants was that the treatment had rescued cells from death, it was conceivable that death-rates had remained high in these explants but were offset by increased mitosis. To assess whether this was possible, the total numbers of cells (live plus dead) in each E19 K^+ treated thalamic explant were estimated and compared to E19 controls (as described in Materials and Methods). Neither 5mM nor 0.5mM K^+ (stimulatory doses) increased the total numbers of cells in E19 thalamic explants. The total numbers of cells in E19 thalamic explants after K^+ treatment (n =4) were compared to controls (n =4) using a Student's t-test. The counts were not significantly different ($p > 0.05$). Futhermore, previous work in our lab reveals that the clearance rate of dead cells from explant cultures is minimal (Rennie, 1992), therefore an increase in cell mitosis would be revealed by my cell counts. 50mM K^+ (toxic dose) treated thalamic explants contained decreased numbers of cells ($p<0.05$; Student's t-test).

Outgrowth of Cultured Explants

The effects of varying thalamic age and adding K^+ on neurite outgrowth are illustrated in fig. 4.4 and quantifications are shown in fig. 4.5. 5mM K^+ was chosen since higher doses had toxic effects and lower doses had less survival-promoting effect. In control explants, the area and length of outgrowth decreased as the age of the explant increased (fig. 4.4a-c and fig. 4.5a,b); note that the amount of outgrowth from P2 explants was so low that insufficient data were obtained for the length of neurites at this age. 5mM K^+ increased neurite outgrowth for E13-E19 thalamic explants ($p<0.05$ for area and length of outgrowth; Student's t-test), but not for P2 explants.

FIGURE 4

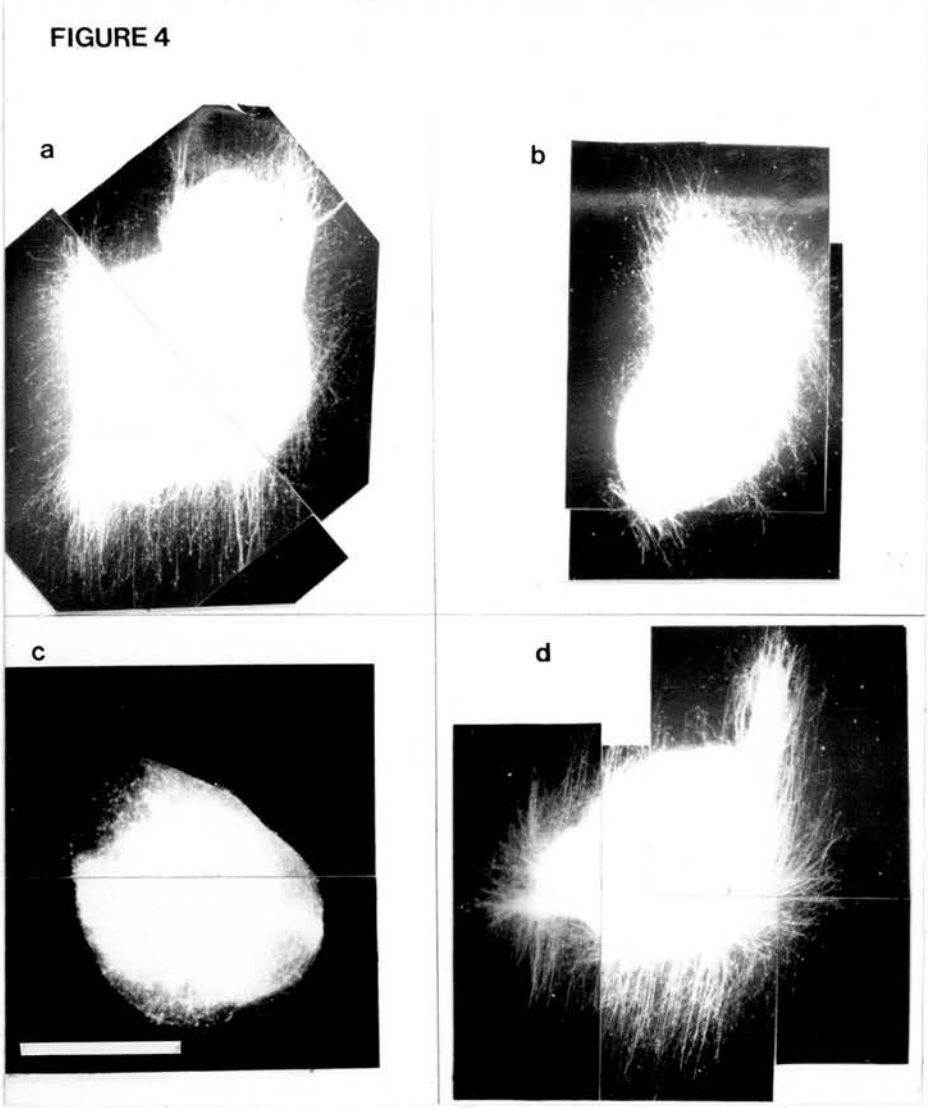
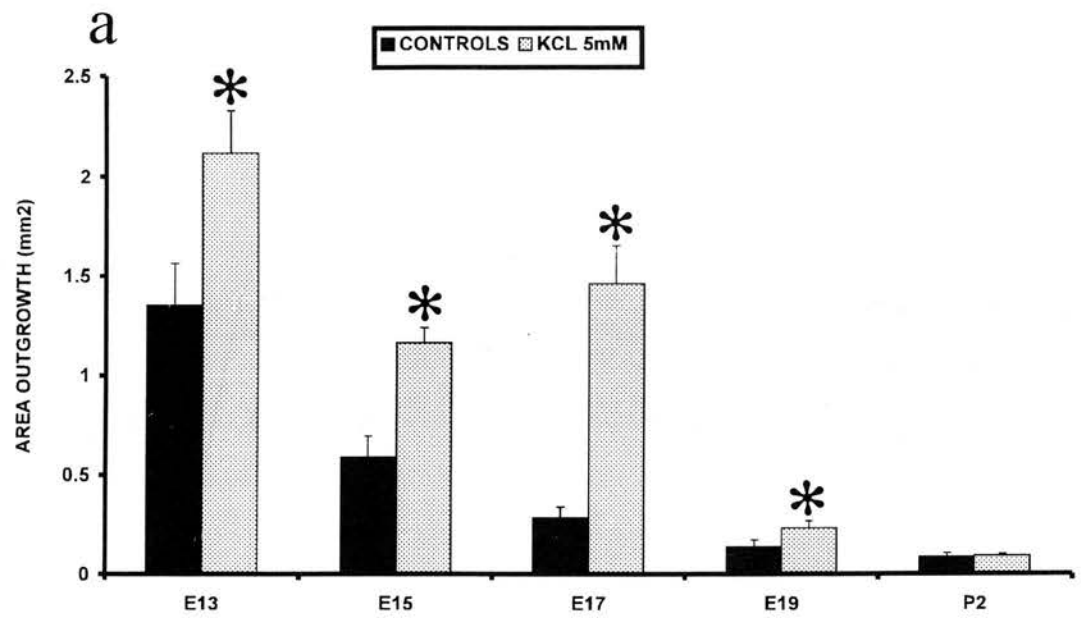


Figure 4.4 Photographs of DiI labelled neurite outgrowth from thalamic explants of different ages. (a) E13 explant, (b) E17 explant and (c) E19 explant in control medium. (d) E17 explant with 5mM K^+ added. The amount of outgrowth decreased as the age of the explant increased. 5mM K^+ stimulated prolific outgrowth. Scale bar, 814μm.


a
CONTROLS
KCL 5mM
AREA OUTGROWTH (mm2)
2.5
2
1.5
1
0.5
0
E13
E15
E17
E19
P2

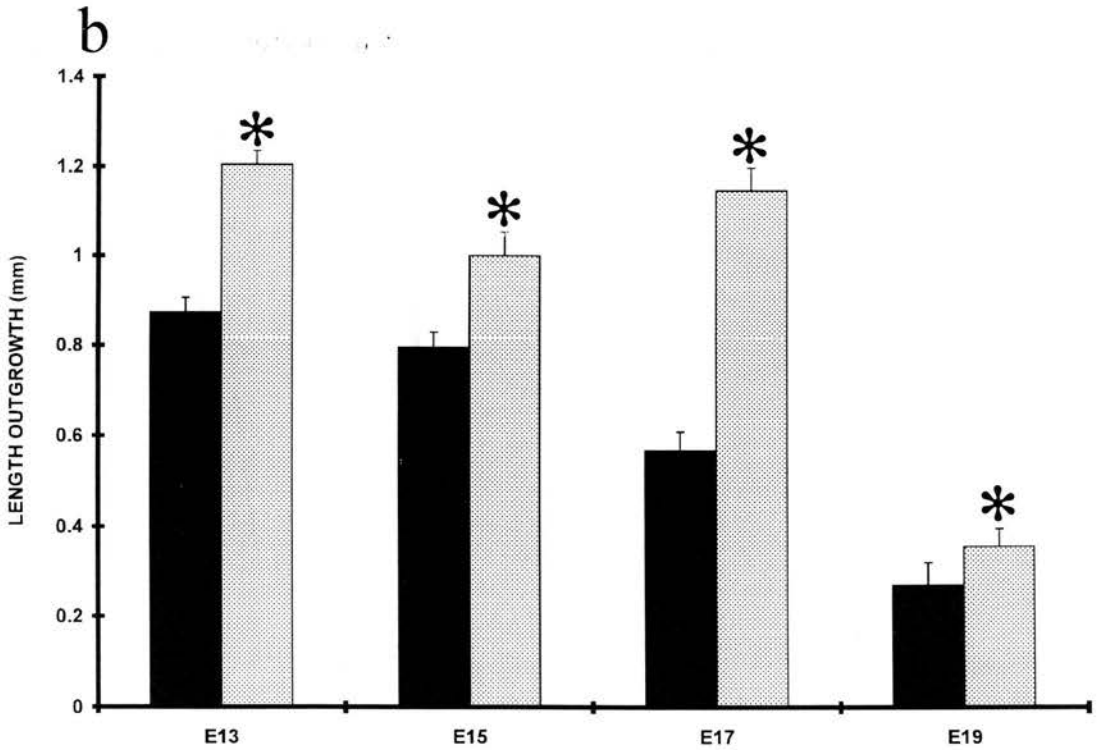

b
LENGTH OUTGROWTH (mm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E13
E15
E17
E19

Figure 4.5

(a) Histogram comparing the average area (mm^2; $\pm$ S.E.M) of neurite outgrowth between control and 5mM K^+ treated thalamic explants of different ages (E13-P2). For control data n= 4-8. For 5mM K^+ treated data n= 4-16. For each age, 5mM K^+ treated thalamic explants were compared to controls using a Student's t-test. * on the histogram indicates that the data are significantly different from controls.

(b) Histogram comparing the average length of neurite outgrowth (mm; $\pm$ S.E.M) between control and K^+ treated thalamic explants of different ages (E13-P2). For control data n= 4-8. For 5mM K^+ treated data n= 4-16. For each age, 5mM K^+ treated thalamic explants were compared to controls using a Student's t-test. * on the histogram indicates that the data are significantly different from controls.

TABLE 4.2. In this table the mean increase in both the area and the length of neurite outgrowth after K^+ treatment (n = 4-16) is expressed as a percentage of the mean control value (n = 4-8). Area of neurite outgrowth from E17 thalamic explants is increased 5-fold. Length of neurite outgrowth from E17 thalamic explants is increased 2-fold.

K^+ treated values expressed as a percentage increase over the control value.

NEURITE OUTGROWTH	**E13**	**E15**	**E17**	**E19**	**P2**
area (mm^2)	**57**	**99**	**408**	**68**	**3**
S.E.M	23	27	101	36	23
length (mm)	**38**	**26**	**102**	**32**	**-**
S.E.M	5	7	13	24	-

Table 4.2 displays the increased outgrowth produced by K^+ as a percentage of the relevant control value. These values emphasise that E17 thalamic explants were particularly sensitive to 5mM K^+ : the area of outgrowth increased 5-fold and the length of outgrowth increased 2-fold. This effect is illustrated in Fig. 4.4d. K^+ treatment increased outgrowth (both area and length) from E13, 15 and 19 thalamic explants by similar proportions to each other but these values were always less than those observed with K^+ on E17 thalamic explants.

Although every attempt was made to keep the sizes of the thalamic explants the same, some slight variation was inevitable. Within the narrow range of sizes used, no control or experimental group contained an over-representation of explants of a particular size. Furthermore, to check that the minor variations that did exist did not bias the results, data on outgrowth from control and K^+ treated E17 thalamic explants were examined for correlations with the area or circumference of the cultured explant. No correlations were observed (fig 4.6 a,b,c,d,e,f.).

Staining with an anti-β-tubulin antibody confirmed that the outgrowth studied here was neuronal (fig. 4.7).

Effect Of K^+ On Thalamic Neurite Growth Rates

Fig. 4.8 displays the amounts of neurite outgrowth from E17 explants after 24, 48 and 72 hours of culture in both control and K^+ treated medium. E17 thalamic explants were examined because this was the age at which added K^+ produced the greatest neurite outgrowth. Prior to 48 hours, growth rates were similar with and without added K^+. There was no evidence that additional K^+ induced an earlier initiation of outgrowth.

By 72 hours, the amount of neurite outgrowth in K^+ treated explants was higher than in controls ($p<0.007$ for area and $p<0.02$ for length; Student's t-test). The difference was greater for neurite area (nearly 3-fold) than for length (1.5-fold). In these explants, the total amounts of outgrowth produced after three days in culture were less than those obtained in previous experiments, most probably because of the need

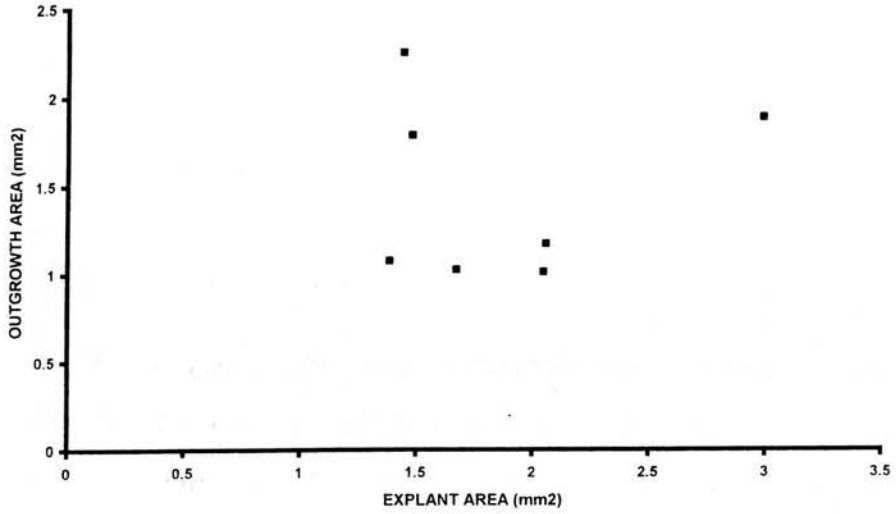
to transfer the cultures to a different incubator for photography (see Materials and Methods). The data presented in Fig. 4.8 suggest that, without added K^+, the growth of neurites is constrained after 2 days, whereas the growth of neurites with added K^+ continues.

Possible Osmotic Effects

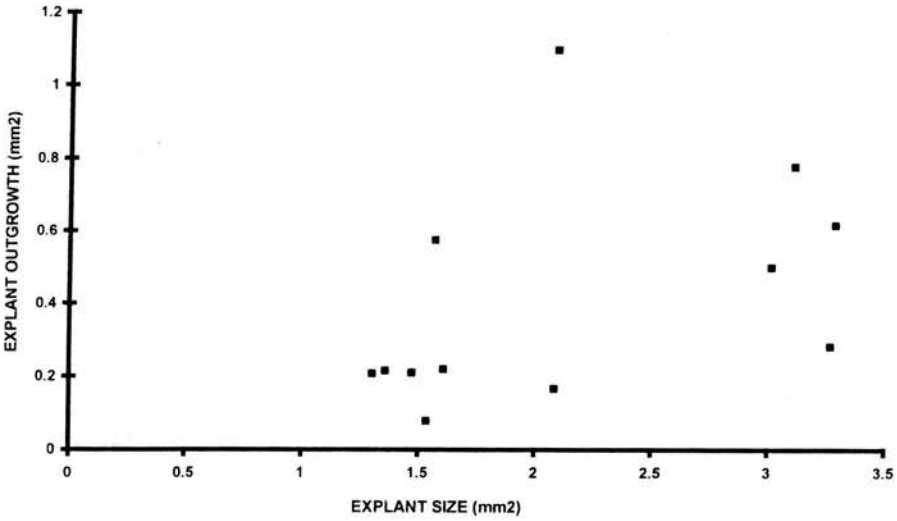
I conducted experiments to determine the effects of altering the cells osmolarity. The extracellular potassium concentration can be increased, by replacing NaCl with KCl, thereby keeping the osmolarity unchanged. However, in these experiments the culture medium was ordered from Sigma Biochemicals and I was unable to simply replace NaCl for KCl. The osmotic effects of 5mM KCl would be expected to be similar to 10^{-2} M sucrose.

Culturing E17 thalamic explants (n=3) with 10^{-2}M sucrose for 3 days did not enhance viability nor outgrowth from thalamic explants, excluding the possibility that osmotic effects mediated the results described above.

a GRAPH OF EXPLANT OUTGROWTH (E17 CONTROL) AGAINST EXPLANT SIZE



b GRAPH OF EXPLANT OUTGROWTH (E17 KCL TREATED) AGAINST EXPLANT SIZE.



c GRAPH COMPARING NEURITE OUTGROWTH AGAINST PERIMETER LENGTH OF EACH EXPLANT (E17 CONTROL).

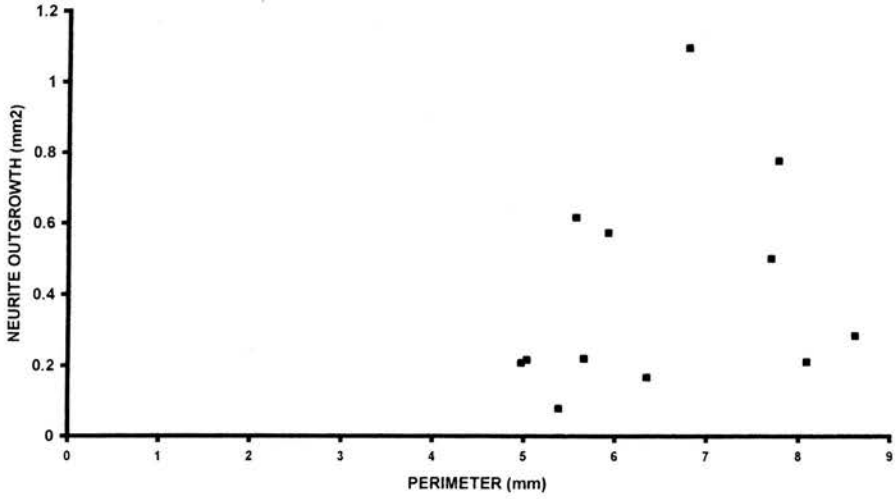
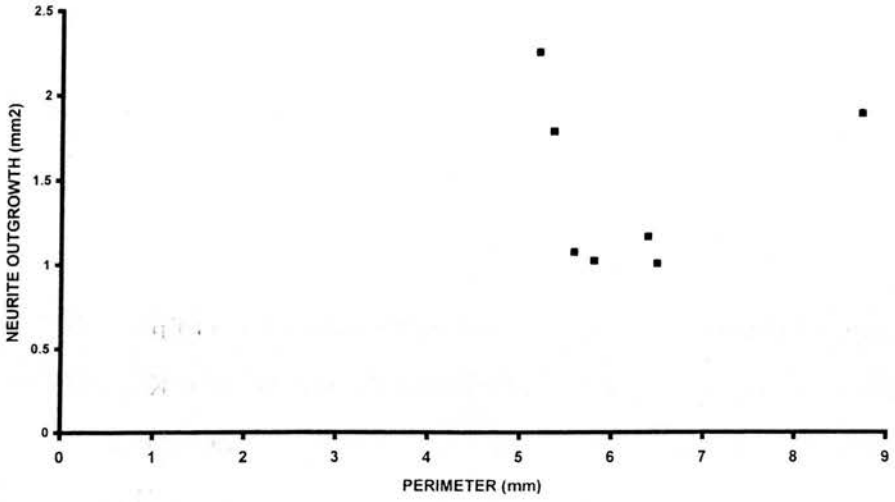


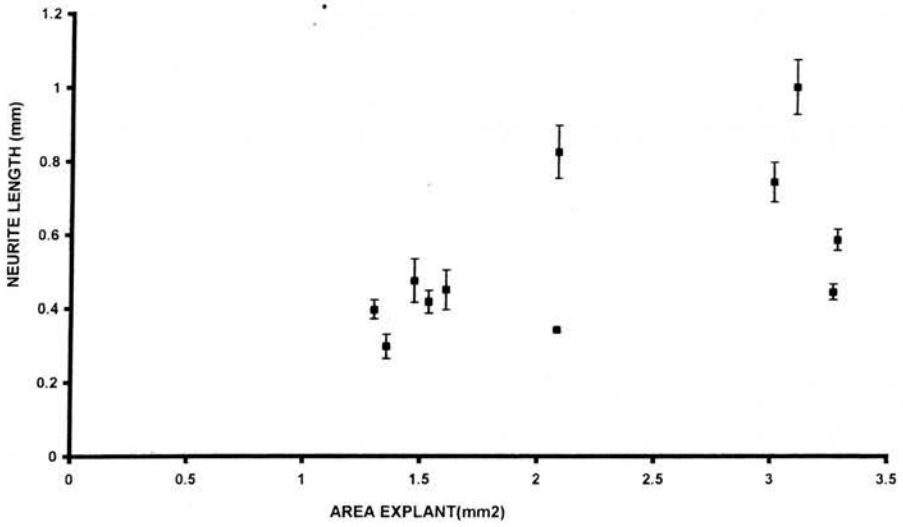
Figure 4.6

Scatter graphs displaying that there is no correlation between the area of neurite outgrowth and the size of the E17 thalamic explant in (a) control explants, r = 0.088 and is not significant (b) K^+ treated explants, r = 0.435 and is not significant. Scatter graph displaying that there is no correlation between the area of neurite outgrowth and the circumference size of the E17 thalamic explant in (c) control explants, r =0.062 and is not significant (see next page for d, e and f).

d GRAPH COMPARING NEURITE OUTGROWTH AGAINST PERIMETER LENGTH OF EACH EXPLANT (E17 KCl TREATED).



e GRAPH OF LONGEST NEURITE OUTGROWTH (N=4) AGAINST SIZE OF EXPLANT (E17 CONTROL).



f GRAPH OF LONGEST NEURITE OUTGROWTH (N=4) AGAINST SIZE OF EXPLANT(E17 KCL).

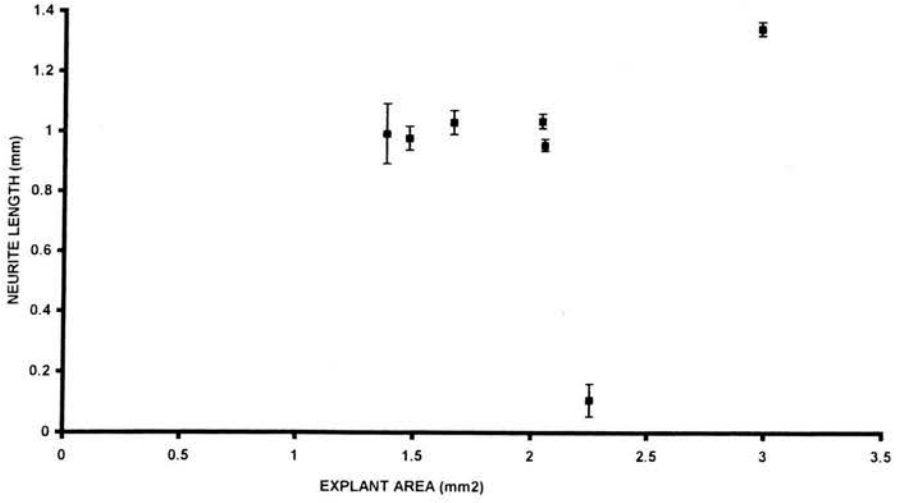


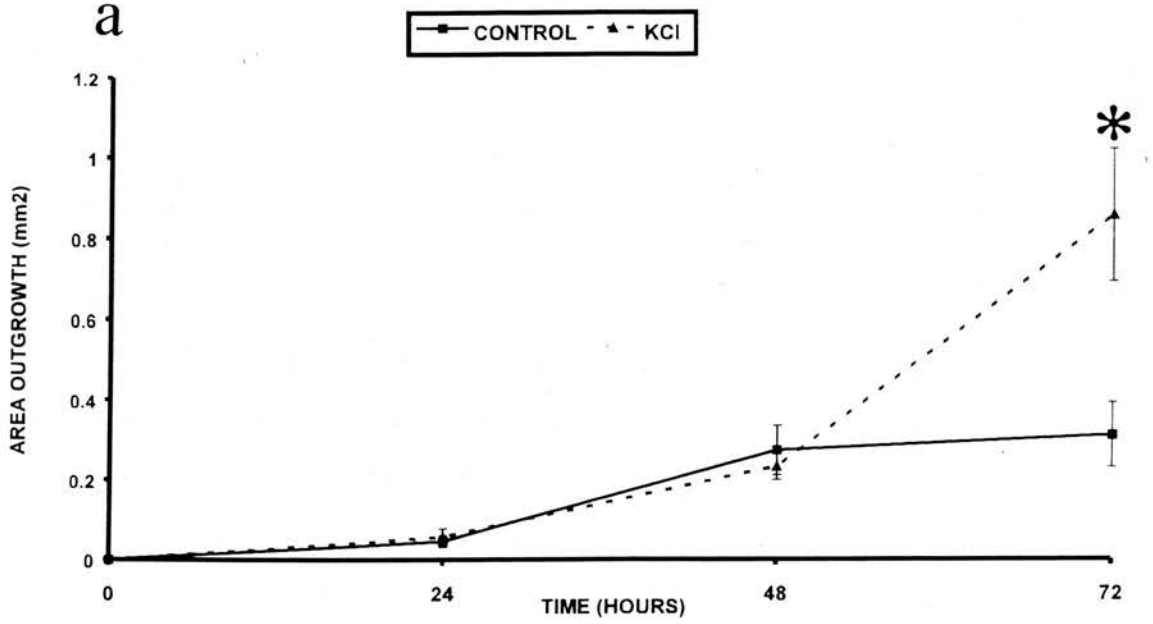
Figure 4.6

Scatter graphs displaying that there is no correlation between the area of neurite outgrowth and the circumference size of the E17 thalamic explant in (d) K^+ treated explants, r = 0.081 and is not significant. Scatter graphs displaying that there is no correlation between the average neurite length (n =4) and the size of the E17 thalamic explant in (e) control explants, r = 0.571 and is not significant (f) K^+ treated explants, r = -0.099 and is not significant. In (e) and (f) the average neurite length (n =4) is plotted against explant area and error bars relate to the S.E.M.


thalamic
explant
anti-β-tubulin
labelled axon

Figure 4.7 Photomicrograph displaying outgrowth from an E17 K^+-treated (5mM) thalamic explant stained with anti-β-tubulin, to confirm that outgrowth was neuronal. Scale bar, 683μm.

a


CONTROL
KCl
AREA OUTGROWTH (mm2)
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
TIME (HOURS)
*

b

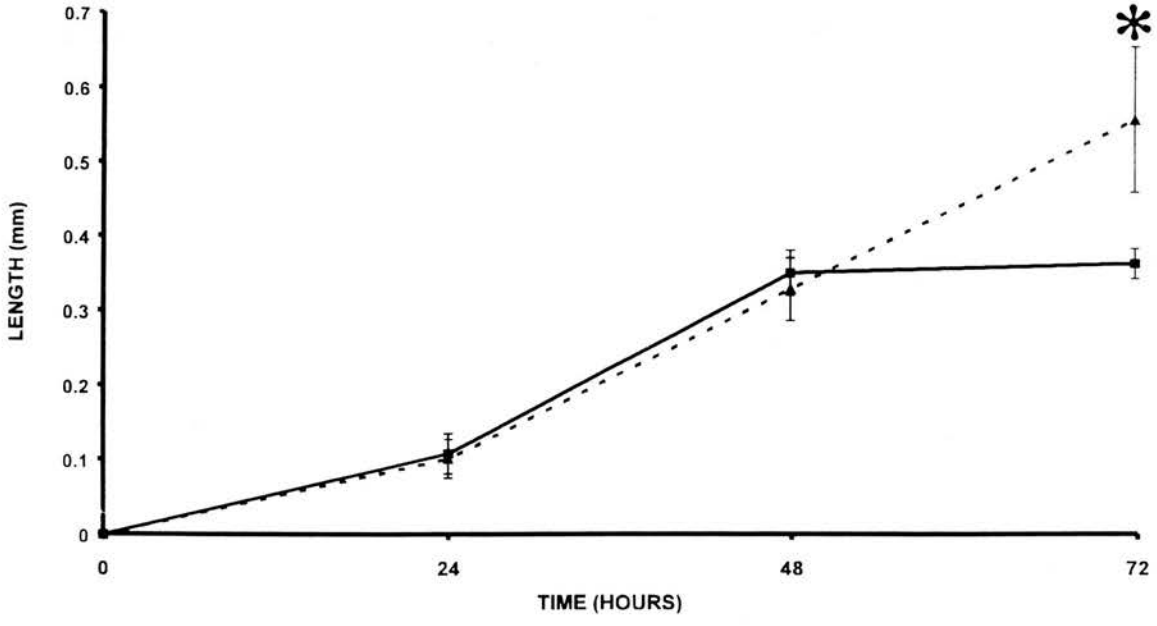

LENGTH (mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
72
TIME (HOURS)
*

Figure 4.8

Graphs comparing the neurite outgrowth from E17 K^+-treated (5mM) thalamic explant at 24, 48 and 72 hours of culture: (a) the average area (mm^2; $\pm$ S.E.M.) of neurite outgrowth in control (n =4 or more) and K^+ treated explants (n =5 or more). At 24, 48 and 72 hours K^+ treated data were compared to controls using a Student's t-test. * on the line graph indicates values that are statistically different from controls ($p < 0.05$).

(b) the length (mm; $\pm$ S.E.M) of neurite outgrowth in control (n =5 or more) and K^+ treated explants (n = 8 or more). At 24, 48 and 72 hours K^+ treated data were compared to controls using a Student's t-test. * on the line graph indicates values that are statistically different from controls ($p < 0.05$).

Squares and continuous lines: control cultures; triangles and broken lines: K^+ treated cultures.

DISCUSSION

The aim of this study was to assess whether embryonic thalamic viability and neurite outgrowth were influenced by increased depolarization *in vitro*. My results revealed that depolarization with added K^+ could increase or decrease cell survival, depending on the age of the explant and the concentration of K^+ used. Added K^+ could also increase neurite outgrowth from prenatal but not postnatal thalamic explants. Furthermore, the effects of added K^+ on neurite growth were not manifest until 48 hours of culture had elapsed. These findings suggest that, *in vivo*, neural activity in the thalamus could have an important role in regulating cell survival and promoting neurite outgrowth. K^+-induced depolarization also has trophic effects in other systems, and these are dependent on such variables as the cell-type and age, the dose of K^+ and the length of time of exposure to it (Berdan et al, 1993; Collins & Liile, 1989; Garyantes & Regehr, 1992; Koike et al, 1989; Lipscombe et al, 1988; Mattson & Kater, 1994; Zafra et al, 1990).

Mechanism Of Action Of Added K^+

K^+ is widely used to non-specifically depolarize neuronal membranes *in vitro* (Lipscombe et al, 1988). With regard to the developing thalamus, Gottman et al. (1994) showed that cultures of E17 thalamus from the rat have spontaneous activity in the form of miniature excitatory postsynaptic currents (EPSCs), and that their frequency is greatly increased after K^+ depolarization. Their work indicated that this effect of added K^+ is dependent on Ca^{2+} influx. In cultures of developing neurons from other systems, K^+-induced depolarization can maintain survival and stimulate growth (Acheson et al, 1987; Bennett & White, 1979; Chalazonitis & Fischbach, 1980; Cohen-Cory et al, 1991; Scott, 1977; Wakade et al, 1983) and these effects are mediated by Ca^{2+} influx (Benari et al, 1994; Collins & Lille, 1989; Collins et al, 1991; Koike et al, 1989; Koike & Tanaka, 1991).

It is most likely that Ca^{2+} influx also mediates the trophic effects of K^+ treatment described here. Many Ca^{2+}-activated intracellular signalling systems have recently been discovered in developing neurons. Most likely, an increase in intracellular Ca^{2+}

would have survival and/or growth-promoting effects by activating Ca^{2+}/calmodulin dependent enzymes, such as Ca^{2+}/calmodulin-dependent protein kinases (CaM-kinases) (Williams et al, 1995). This activation might occur as a result of Ca^{2+}-induced Ca^{2+} release, which can activate CaM-kinase II (Kocsis et al, 1994). How these intracellular changes have trophic effects is not clear. The possibilities include: (i) an increased production of endogenously active growth factors such as the neurotrophins (whose levels of production are known to be increased by increased neural activity in other systems (Castren et al, 1992; Catalano et al, 1991; Garyantes & Regehr, 1992; Kocsis et al, 1994; Lund & Mustari, 1977; Patterson et al, 1992; Riva et al, 1992; Zafra et al, 1990) (ii) increased activity of neurotransmitter systems (these are known to play a role in neuronal growth as well as in the mature nervous system) (Bessho et al, 1994; Mattson & Kater, 1994); (iii) regulation of growth cone motility and the stability of actin filaments in the cytoskeleton of growth cones (Lankford & Letourneau, 1989).

In this study, added K^+ had a dose-dependent effect on thalamic cell survival and high concentrations were toxic. According to Mattson & Kater (1994), the harmful effects of chronic depolarization with high doses of K^+ might be caused by a depletion of essential intracellular Ca^{2+} stores, or the accumulation of toxic levels of intracellular Ca^{2+} in the cytoplasm. The youngest thalamic explants (E13) were less sensitive to high concentrations of K^+, perhaps because of a relative immaturity of their voltage-gated channels.

Added K^+ Had An Age-Dependent Effect On Cell Survival

The ability of added K^+ to increase thalamic cell survival was greatest at E19; added K^+ had no effect on older explants. Previous work has indicated that postnatal thalamic neurons (that have innervated their cortical targets) become dependent on cortex-derived growth factors for their survival (Cunningham et al, 1987; Haun & Cunningham, 1993; Price et al, 1995). My results suggest that postnatal thalamic cells are unable to survive without these target-derived factors even if their electrical activity is increased. One hypothesis to explain the age-dependent effects of

culturing, with and without added K^+, is that the survival of the prenatal thalamus is under the control of an intrinsic mechanism that has an increasing requirement for neural activity as it ages. This requirement may be maximal at E19. After birth, the switch to a need for paracrine cortex-derived factors may be completed and the mechanism in the thalamic explant may cease to function.

Added K^+ Had An Age-Dependent Effect On Neurite Outgrowth

Without added K^+, the youngest thalamic explants produced more outgrowth than the older thalamic explants. As for viability, K^+ depolarization increased neurite outgrowth from prenatal but not from postnatal thalamic explants. Adding K^+ to thalamic explants aged E13-17 produced similar amounts of neurite outgrowth (hence the largest increase over controls was at E17), but by E19 additional K^+ no longer enhanced neurite outgrowth even though it did produce a large increase in viability. These data indicate that, while less viable explants produce less outgrowth, the converse is not necessarily true: enhanced viability does not necessarily produce enhanced outgrowth. The relationship between thalamic viability and growth may not be simple. There is evidence for this in other systems: for example, K^+-induced survival and outgrowth are mediated by different proteins in cultured cerebellar granule cells (Graham & Burgoyne, 1993).

My observation that the growth-promoting effect of adding K^+ to E17 explants needed 2 days to appear, and that it appeared to comprise a release from a rate-limiting process that constrained the growth of the control explants, is compatible with suggestions made above. The E17 thalamus may start to lose its innate ability to survive and grow after 2 days in culture (as it would *in vivo*), and the presence of increased K^+ may prevent this loss by keeping the explants intrinsic or extrinsic mechanisms in a more active state. K^+ depolarization may have a direct effect on cell viability and/or neurite outgrowth by activating voltage gated ion channels (e.g. calcium channels), which in turn could trigger various intracellular signalling mechanisms which mediate neurite outgrowth or cell survival in all thalamic cells. Alternatively, this direct effect of K^+ depolarization on cell survival may be limited

to different subsets of neurons within the thalamic explant at specific developmental ages. After the viability of this subset of neurons has been enhanced, these neurons may then release a factor or factors (e.g. neurotrophins), which subsequently enhances the viability or neurite outgrowth of neighbouring cells. It is therefore possible that K^+ could also have indirect effects on thalamic cell viability and neurite outgrowth by increasing the release of extrinsic factors or the cell's sensitivity to these factors.

A Possible Activity-Dependent Regulation Of Cell Survival And Growth *In Vivo*

There is substantial evidence in other systems that intrinsic mechanisms regulate neuronal viability and growth before their axons innervate their target, and that after innervation neurons become dependent upon a supply of target-derived neurotrophins for survival (see review by Davies, 1994). My laboratory and others have previously described how cortex-derived growth factors can regulate the survival and growth of thalamic cells once they have innervated their targets (Cunningham et al, 1987; Price et al, 1995). This present study suggests that, towards the end of gestation (between E17-E19), cultured thalamic cells begin to lose their ability to survive and grow. Furthermore, they show that depolarization of the ageing cells, which may occur *in vivo* as thalamic afferents develop and spontaneous activity increases (Shatz & Stryker, 1988), can maintain this mechanism until birth but not thereafter. The stimulatory effects of K^+-depolarization on cell survival and neurite outgrowth is most likely to be produced by a subset of neuronal cells, since very few non-neuronal cells (<5%) are present in cultured E15 thalamic explants (Lotto et al, unpublished observations) . However, in older cultures it is possible that glial cells are involved in producing the stimulatory effects on neuronal outgrowth and survival.

What growth factors mediate this effect of depolarization? The most obvious candidates are the neurotrophins. First, high affinity receptors for the neurotrophins are present in the developing thalamus of other species (Allendoerfer et al, 1994), and of the embryonic mouse (Price et al, 1995; J.A.Clausen and D.J.Price, unpublished observations). Second, it is known that central neurons increase their

production of neurotrophins in response to increased neural activity (Castren et al, 1992; Gall, 1992; Isackson et al, 1991; Lu et al, 1991; Patterson et al, 1992; Riva et al, 1992; Zafra et al, 1990). Neurotransmitters might also mediate the effects of depolarization: in other parts of the nervous system, they are thought to have direct and interactive effects on both neurite outgrowth and cell survival (Benari et al, 1994; Bessho et al, 1994; Lipton & Kater, 1989). Cultured rat thalamic neurons express glutamate and gamma-aminobutyric acid (GABA) receptors (Lebmann et al, 1992), and K^+-depolarization has been shown to induce an increased frequency of glutamatergic miniature EPSCs and a decreased frequency of GABAergic miniature inhibitory postsynaptic currents in cultured E17 thalamic neurons from rats (Gottmann et al, 1994). Both neurotransmitters and neurotrophins may be involved in a thalamic mechanism: for example, it is known from other studies that glutamate can increase brain derived neurotrophic factor mRNA levels in cultured neurons (Lindholm et al, 1993).

In conclusion, I suggest that an intrinsic and/or extrinsic mechanism, that becomes increasingly reliant on neural activity for its operation, can promote the survival and growth of embryonic thalamic cells before they switch their dependence to cortex derived factors at around the time of birth in the mouse.

CHAPTER 5:

EFFECTS OF GLUTAMATE ON THALAMIC CELL SURVIVAL IN ORGANOTYPIC CULTURES.

CHAPTER 5: EFFECTS OF GLUTAMATE ON THALAMIC CELL SURVIVAL IN ORGANOTYPIC CULTURES.

ABSTRACT

The aim of this study was to investigate how different levels of glutamate affect thalamic cell survival in isolated, cultured thalamic explants from mice aged embryonic day 13 to embryonic day 19. Effects of glutamate were dependent on the concentration used and the age of the thalamic explant. In control explants cell survival decreased as the age of the explant increased (E13-E19). Very high concentrations of glutamate generally decreased cell survival and very low concentrations of glutamate had no effect on cell survival. 0.05mM Glutamate had its greatest survival-promoting effect on E17 thalamic explants (the percentage of viable cells in these explants almost doubled), although E15 explants also responded. Cells in E13 explants did not follow the dose dependent pattern displayed by cells in older glutamate treated explants. Only 5mM glutamate produced a statistically significant effect on cell survival in these explants, reducing survival by 50%. Cells in E19 explants also did not follow the dose-response pattern displayed by cells in younger glutamate treated explants and no significant effect on cell survival was obtained at this age. APV (NMDA antagonist) and CNQX (AMPA/Kainate antagonist) were added to cultures of E17 thalamic explants treated with 0.05mM glutamate, in an attempt to identify which glutamate receptor was mediating the effects on cell survival. Results revealed that both subtypes could be involved in mediating the increased cell survival produced by added glutamate. These results suggest that two different cell populations may express different glutamate receptor subtypes. Therefore, *in vivo*, glutamate acting directly on both NMDA and kainate/AMPA receptors within the thalamus could play a role in regulating survival of different neurons during the embryonic phase of development.

INTRODUCTION

Glutamate is a major neurotransmitter in the brain and can act as either a trophic neurotransmitter or neurotoxin depending on its concentration (Lipton & Kater, 1989). Glutamate stimulates several types of receptors which have been classified according to their molecular structure and pharmacology (Nakanishi, 1992). Glutamate interacts with both N-methyl-D-aspartate (NMDA) and non-NMDA receptors. NMDA receptors are linked to Ca^{2+} channels and are preferentially activated by NMDA, whereas non-NMDA receptors are stimulated by quisqualate, α-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid (AMPA) or kainate. Quisqualate/AMPA receptors are divided into ionotropic receptors (selectively activated by AMPA, linked to sodium channels and mediating fast synaptic responses) and metabotropic receptors (selectively activated by quisqualate and linked to secondary messsenger pathways such as phospholipase C). The NMDA receptor subtype is therefore distinguishable from the other subtypes by its Ca^{2+} permeability.

Glutamate Induced Neurotoxicity

Calcium is implicated as a secondary messenger mediating glutamate neurotoxicity (Berdichevsky et al, 1983; Choi, 1985; Donaldson et al, 1983; Mattson et al, 1988; Mayer & Westbrook, 1987). Prolonged Ca^{2+} entry through the NMDA receptor/channel complex can induce neuronal death (Meldrum & Garthwaite,1990). *In vivo* studies demonstrated increases in calcium influx into cortical and hippocampal neurons under conditions believed to enhance glutamate excitation (Kudo et al, 1987) as well as in response to direct application of excitatory amino acids (Retz & Coyle, 1984). Glutamate neurotoxicity is greatly reduced by incubation in medium containing low levels of calcium (Choi, 1985) or by pretreatment with calcium channel blockers (Mattson et al, 1988). The ability of glutamate to kill neurons (excitotoxicity) seems to be mediated, in most cases, by an interaction with NMDA receptors, leading to an uncontrollable rise in intracellular

calcium concentrations and hence cell lysis and death. However, recent studies have revealed that glutamate -mediated excitotoxic death of cultured striatal neurons is mediated by non-NMDA receptors (Chen et al, 1995). Furthermore, non-NMDA receptor activation can cause excitotoxicity in cerebellar Purkinje neurons by mechanisms not involving Na^+ influx, but rather depending on direct Ca^{2+} permeation and activation of Ca^{2+} dependent enzymatic processes (Brorson et al, 1994).

There appear to be two forms of glutamate-induced neurotoxicity, acute and delayed. Acute neurotoxicity is probably caused by depolarization leading to excessive anionic and cationic fluxes and osmotic lysis. Delayed neurotoxicity (i.e. 24 hours later) is critically dependent on calcium influx. The mechanism by which calcium influx leads to neuronal degeneration is not completely understood, but may involve calcium dependent proteases which degrade cytoskeletal components (Nixon, 1986) as well as rearrangement of metabolic systems and membrane integrity. Recent studies on cultured cortical neurons have suggested that the radical form of nitric oxide (NO) mediates the NMDA receptor mediated neurotoxicity of glutamate (Dawson et al, 1991; Tamura et al, 1992; Lipton et al, 1993). NO is synthesised by NO synthase (NOS) from L-arginine via a Ca^{2+}/calmodulin-dependent mechanism (Bredt & Snyder, 1990). Thus, increases in intracellular Ca^{2+} resulting from activation of voltage or ligand-gated Ca^{2+} channels could activate the enzyme. Alternatively, increases in intracellular calcium could induce activation of immediate early genes (IEG) (Morley et al, 1994). Since IEGs function as transcriptional regulating factors, the differential expression of specific target genes may be of importance for determining death or survival of cells.

Under normal circumstances, protective mechanisms such as inhibitory neurotransmitters or neuromodulators presumably limit neuronal glutamate exposure and prevent toxicity. GABAergic afferents may provide trophic support for cells that would otherwise be vulnerable to excitoxicity since GABA prevents the growth suppressive -effects of glutamate in cultured hippocampal neurons by reducing Ca^{2+} (Mattson & Kater, 1989). Nerve growth factor (NGF) also ameliorates glutamate cytotoxicity in cortical cultures (Shimohama et al, 1993). Cholecystokinin (CCK)

and dopamine also appear to induce strong neuroprotection against glutamate cytotoxicity in cultured cortical and retinal neurons (Akaike et al, 1991; Kashii et al, 1994). Suppression of controlling substances which limit glutamate induced excitation can also cause severe dysfunction of CNS activity. For example, convulsive drugs which block the inhibitory actions of GABA or glycine, are known to induce clonic and tonic convulsions, respectively.

Glutamate: A Trophic Neurotransmitter

Recent studies have shown that there is significant plasticity and opportunity for change in neuronal architecture based upon neurotransmitter effects. Although glutamate can be inhibitory to outgrowth when applied over a large area (e.g. an entire dendrite or the full dendritic field), localised and brief application of glutamate can stimulate the rapid formation of new protrusions of small processes along the dendrites of older hippocampal dendrites *in vitro* (Patterson, 1988). Similarly, endogenous glutamate appears to stimulate neurite outgrowth from cerebellar granule cell neurons; this effect is mediated at the NMDA receptor since specific receptor antagonists block this effect (Pearce et al, 1987; Balazs et al, 1988). The discrepancy in the growth promoting versus inhibitory effects of glutamate is dependent on concentration and temporal effects (Mattson et al, 1988). The pharmacology of the actions of glutamate on neurite outgrowth may be different in disparate systems or at different stages of development. For instance, a population of rat retinal ganglion cells are resistant to the neurotoxic effects of millimolar concentrations of glutamate under otherwise normal culture conditions. Patch -clamp experiments show that this resistance is associated with a very small ionic current response to N-methyl-D-aspartate (Hahn et al, 1988).

In vivo models also suggest that glutamate can alter neural development. For instance, the amphibian optic tectum is characterised by a highly ordered arrangement of inputs from the eyes. In experiments, an additional eye primordium was implanted resulting in adults with one tectum innervated by two eyes. This produced segregated inputs from the two eyes resulting in a striped organization of eye terrains on the surface of the tectum. The role of activity in the formation of these

stripes was demonstrated with tetrodotoxin (TTX- a sodium channel blocker). Application of TTX to the optic nerve results in a dissolution of the striped pattern. The involvement of glutamate in the maintenance of these striped patterns is suggested by application of APV (NMDA antagonist) to the tectum after the striped pattern has formed. This also results in a loss of the striped pattern suggesting that glutamate acts as a signal to maintain the orderly structure (Reh & Constantine-Paton, 1985). These studies suggest that NMDA receptor activation regulates the development of both neurons and synaptic connections.

Glutamate Receptor Expression In The Developing Brain

Functional NMDA receptors are present in embryonic neocortex (LoTurco et al, 1990) and contribute to evoked synaptic activity in the embryonic spinal cord (Ziskind-Conhaim, 1990). However, developmental changes in receptor properties have been described. Postnatally, NMDA receptor density peaks during the first few weeks in the hippocampus (Tremblay et al, 1988), neocortex (M^{c}Donald et al, 1990), and spinal cord (Kalb et al, 1992), and then declines. It is likely that the structure of the NMDA receptor changes within the first few postnatal weeks since the voltage dependence (Ben-Ari et al, 1988), sensitivity to Mg^{2+} (Bowe & Nadler, 1990; Kleckner & Dingledine, 1991; Morrisett et al, 1990), and affinity for various ligands (Kalb et al, 1992) have all been reported to change during this developmental period. It is also likely that the expression of molecularly distinct subtypes of NMDA receptors (Kutsuwada et al, 1992; Monyer et al,1992) will be developmentally regulated. Studies investigating the expression of kainate receptors in the developing rat brain reveal the expression of high affinity sites in the gray matter, spinal cord, primordial cerebellum, and ventral forebrain structures as early as E14 (Bahn et al, 1994). Interestingly, all genes undergo a peak in their expression in the late embryonic/early postnatal period. Metabotropic glutamate receptors are also differentially regulated during development, in particular mGluR3 and mGluR5 are highly expressed at birth and decreased during maturation to adult levels of expression (Catania et al, 1994). These data imply a functional role of mGluR3 and mGluR5 during synaptogenesis and maintenance of adult synapses. Evidence for a

functional activation of NMDA receptor-channels in neurons of the developing brain has been correlated with cell migration and synaptogenesis in the rat cerebellum (Rossi & Slater, 1993). This increase in tonic NMDA receptor-channel activity is driven by endogenous glutamate release and regulated by NMDA receptor density and local glutamate uptake.

Glutamate Is The Excitatory Neurotransmitter In The Thalamus.

Glutamate, or some other excitatory amino acid, is suspected of being the neurotransmitter released by both retinal ganglion cell axons and corticothalamic axons in the mammalian dLGN (Crunelli et al, 1987; Kemp & Sillito, 1982; Scharfman et al, 1990; Sillito et al, 1990a,b). In the adult, there is considerable evidence suggesting that glutamate is released from terminals of geniculocortical axons (Tamura et al, 1990; Tsumoto, 1990) and thalamic neurons in culture form predominantly glutamatergic synapses (Gottmann et al, 1994). Similarly, subthalamic fibres carrying sensory/ motor information to other regions of the thalamus are also likely to utilise an excitatory amino acid as their transmitter (Eaton & Salt, 1990; Salt, 1986, 1987; Salt & Eaton, 1989). Removal of the cerebral cortex results in a marked reduction in thalamic gluatamate levels (Fosse et al, 1986; Kvale & Fonnum, 1983) as well as a reduction in high affinity uptake of glutamate and/or aspartate in the thalamus (Fonnum et al, 1981; Karlsen & Fonnum, 1978; Young et al, 1981). Cortical neurons which project to the thalamus also display immunoreactivity for high levels of aspartate and/or glutamate (Giuffrida & Rustioni, 1988) and display high affinity uptake of these excitatory amino acids (Baughman & Gilbert, 1980). The synaptic terminals of both retinal and cortical origin display high concentrations of glutamate, as determined by quantitative immunogold analysis (Montero & Wenthold, 1989) and the mammalian thalamus contains binding sites for both NMDA and non-NMDA receptors (Monaghan & Cotman, 1982; Monaghan et al, 1984). Mainly non- NMDA receptors are found in LGNd relay cells in the rat (Crunelli et al, 1987) but both types of glutamate receptor mediate optic tract stimulation in the LGNd in the cat (Sillito et al, 1990a, b; Scharfman et al, 1990). Recent extracellular recordings *in vivo* have revealed that the

prevalence of NMDA and non-NMDA receptors may vary with functional subtype of LGNd relay cell (Hartveit & Hegelund, 1990; Heggelund & Hartveit, 1990).

In Chapter 3, I reported that the survival and outgrowth of cultured thalamic cells are dependent on a mechanism that becomes increasingly reliant on neural activity for its operation as embryonic development proceeds. Increased neural activity may enhance neurotrophin or neurotransmitter production/release from thalamic cells. Since glutamate is well characterised as the excitatory neurotransmitter in thalamic cells, this study was intended to evaluate the possibility that elevated glutamate could increase the viability of thalamic cells in a concentration and age dependent manner. Results of these experiments could support the hypothesis that glutamate is indeed a likely candidate for mediating such an effect or conversely provide evidence that increased glutamate release is not responsible for the activity dependent survival reported in Chapter 3.

AIM

The aim of this *in vitro* study was to investigate the effects of exposing isolated thalamic explants at different stages of development, to elevated concentrations of the excitatory neurotransmitter glutamate.

1. To examine the effects of various concentrations of glutamate on thalamic cell survival and cell death *in vitro* at different stages of development

2. To attempt to block the possible stimulatory/inhibitory effects of glutamate in these isolated thalamic explants using APV (NMDA antagonist) and CNQX (Kainate/AMPA antagonist).

HYPOTHESIS

Increased external activity (possibly from the periphery) acts on thalamic neurons to increase endogenous glutamate release which stimulates either NMDA or non-NMDA receptors to activate an intracellular secondary messenger system which mediates an increase in cell survival. After birth, cell survival can no longer be maintained, possibly because, (a) postnatally this increased activity is less effective at stimulating glutamate release from thalamic cells. (b) expression or pharmacology

of glutamate receptors changes postnatally and endogenous secondary messengers are no longer stimulated.

Experimental Protocol.

E13, 15, 17 and 19 thalamic explants were cultured for three days in the presence of various concentrations of glutamate. They were then fixed, sectioned and Nissl stained to reveal effects on cell survival.

MATERIALS AND METHOD

Animals And Surgery

Thalamic explants were obtained from E13, 15, 17 and 19 mice. For details of the protocols used, refer to Methods in Chapter 4.

Culture Methods

Thalamic explants were cultured for three days at 37^0C with 5% CO_2 in the presence of various concentrations of glutamate (Table 5.1). Details of the protocols are outlined in Culture Methods in Chapter 4. Following fixation, the paraformaldehyde was replaced with a similar volume of 0.1M PBS for at least 1 hr. Explants were then wax-embedded and sectioned (10μm). Sections were mounted on poly-$_L$-lysine coated slides and Nissl-stained to reveal live and pyknotic cells.

Analysis

Nissl staining revealed both live and pyknotic cells (fig 5.1). Pyknotic cells, as in Chapter 4, were identified by their small, dark nucleus, which was often fragmented into smaller pieces; the cytoplasm of these cells was often absent. In contrast, healthy cells were pale coloured with clear nuclei and nucleoli. All cell counts were carried out blind. For each explant, the numbers of live and pyknotic cells were counted in twelve randomly selected areas (0.0186mm^2). In each of these areas, the number of viable cells was expressed as a percentage of the total number of cells counted. The data generated from these twelve areas were then averaged to give an overall percentage of viable cells in each explant.

To ensure that glutamate treatment did not induce massive mitosis, the total number of cells (live plus pyknotic) in E17 thalamic explants cultured with 0.05mM additional glutamate was estimated and compared to E17 controls (E17 explants were studied since the greatest increase in cell viability was observed at this age). To make

these estimates the cell count for each explant was converted to cell density (cells/mm^{-3}) and this value was multiplied by the volume of the explant (deduced from the numbers of 10μm sections from each explant and their surface areas).

Glutamate Antagonists

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2-Amino-5-phoshono-Propionic acid (APV) were purchased from Research Biochemicals International (RBI). CNQX/HBC (Hydroxypropyl-β-cyclodextrin) complex was dissolved in water to produce a stock solution of 3.9mM solution. A small amount of this stock solution (25μl) was then diluted in culture medium to produce a final concentration of 39μM in culture. CNQX is not very soluble in water, so I decided to use 39μm CNQX/HBC complex because this concentration remained in solution and similar doses of CNQX have been demonstrated to block receptors in slice cultures (Zheng et al, 1995; Miller et al, 1995; Kasof et al, 1995) APV was dissolved in culture medium to produce a stock solution of 10mM. This stock solution was then further diluted in culture medium to produce a final concentration of 10μM in culture. I decided to use 10μM APV because a similar dose has also been demonstrated to block receptors in slice cultures (Miller et al, 1995). These antagonists were added to the culture medium at the start of the 3 day culture period. Refer to Table 5.2 for the number of cultures in each experimental paradigm.

Figure 1

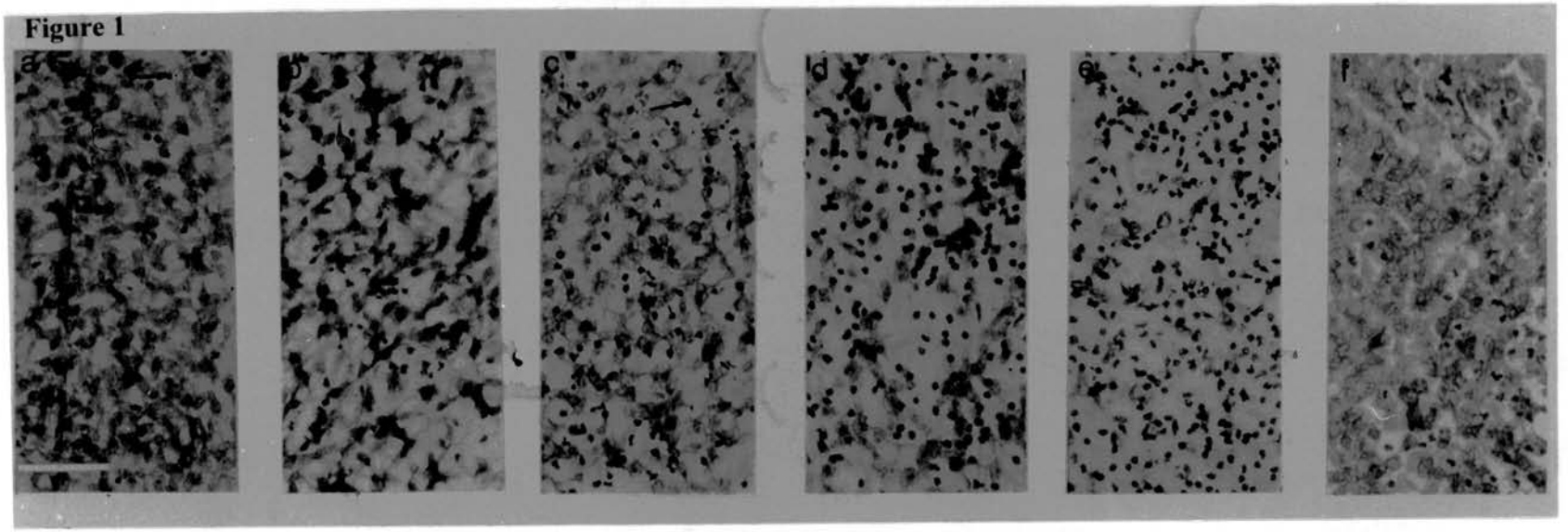


Figure 5.1

Photomicrographs of Nissl stained sections of thalamic explants which have been cultured for three days in the presence and absence of added glutamate. In control explants, the percentage of viable cells decreased as the age of the thalamic explant increased. (a) E13 control; 92.5% of cells are viable (b) E15 control; 79% of cells were viable (c) E17 control; 48% of cells were viable (d) E19 control; 28.5% of cells were viable. Large arrow indicates viable cell, small arrow indicates pyknotic cell.

Glutamate had a dose dependent effect on cell survival. High concentrations decreased cell survival and low concentrations increased cell survival. (e) E17 thalamic explant with 0.5mM added glutamate; 14% of cells were viable (f) E17 thalamic explant with 0.05mM added glutamate; 88% of cells were viable. (Scale = 44μM)

Table 5.1: This table displays the number and age of thalamic explants cultured under each experimental paradigm. The first column displays the concentration of added glutamate (concentraton after addition); the second column gives the final concentration of glutamate in each culture well (the serum-free medium contains 0.04mM glutamate).

Added [GLU]	**Final [GLU]**	**Age of the Explant**			
		E13	**E15**	**E17**	**E19**
0	0.04mM	4	4	4	4
5mM	5.04mM	4	4	5	4
0. 5mM	0.54mM	4	4	4	4
0.05mM	0.09mM	4	7	4	5
0.005mM	0.045mM	4	5	4	4
0.0005mM	0.0405mM	4	5	4	4

Table 5.2: This table displays the number and age of thalamic explants cultured under each experimental paradigm.

	control	**Glutamate (0.05mM)**	**Glutamate (0.05mM) + APV**	**Glutamate (0.05mM) + CNQX**
E17 thalamus	5	6	4	6

RESULTS

The cellular content of thalamic explants after three days in culture was examined in Nissl-stained sections. Examples are shown in fig 5.1 and quantitative data are given in fig 5.2a. In control explants, the percentage of viable cells decreased as the age of the thalamic explant was increased between E13 and E19. 92.5% of cells in E13 explants were viable (fig. 5.1a and 5.2a), whereas only 28.5% of cells in E19 explants were viable (fig 5.1d and 5.2a). Fig 5.2a also displays the dose-dependent effects of adding glutamate to thalamic explants of different ages. In all experiments basal glutamate levels were 0.04mM, therefore addition of 5mM glutamate to the culture medium actually results in there being a final concentration of 5.04mM (refer to Table 5.1). At all ages except E19, addition of 5mM glutamate to the culture medium reduced cell survival ($p<0.05$ at all ages; Student's t-test). Addition of 0.5mM (final concentration of 0.54mM) resulted in reduced cell survival in both E15 and E17 thalamic explants ($p<0.05$; Student's t-test). However, E13 and E19 thalamic explants were unaffected and levels of cell survival in these explants were comparable to controls ($p>0.05$; Student's t-test). Both E13 and E19 thalamic explants were unaffected with glutamate treatment at all other doses (0.05mM-0.0005mM) tested ($p<0.05$; Student's t-test). However, addition of 0.05mM and 0.005mM glutamate (final concentration of 0.09mM and 0.045mM respectively) increased the percentages of viable cells in both E15 and E17 thalamic explants ($p<0.02$; Student's t-test). Addition of 0.0005mM had no significant effect on either cell survival or cell death at any age of explant examined ($p>0.12$; Student's t-test). This dose represents only a very small increase in glutamate (end concentration of 0.0405mM) over those in the controls (Table 5.1).

I concluded that the effects of glutamate were dependent on the concentration used and the age of the thalamic explant. Results for control explants E13-E19 were similar to those reported in Chapter 4. Fig 5.2b displays the effects more clearly. In this histogram, control values of cell survival are subtracted from the values after addition of glutamate, and the product is expressed as a percentage of the control value. This histogram emphasises that glutamate had its greatest survival -promoting

HISTOGRAM COMPARING THE PERCENTAGE VIABLE CELLS BETWEEN THALAMIC EXPLANTS OF DIFFERENT AGE BEFORE AND AFTER EXPOSURE TO GLUTAMATE.

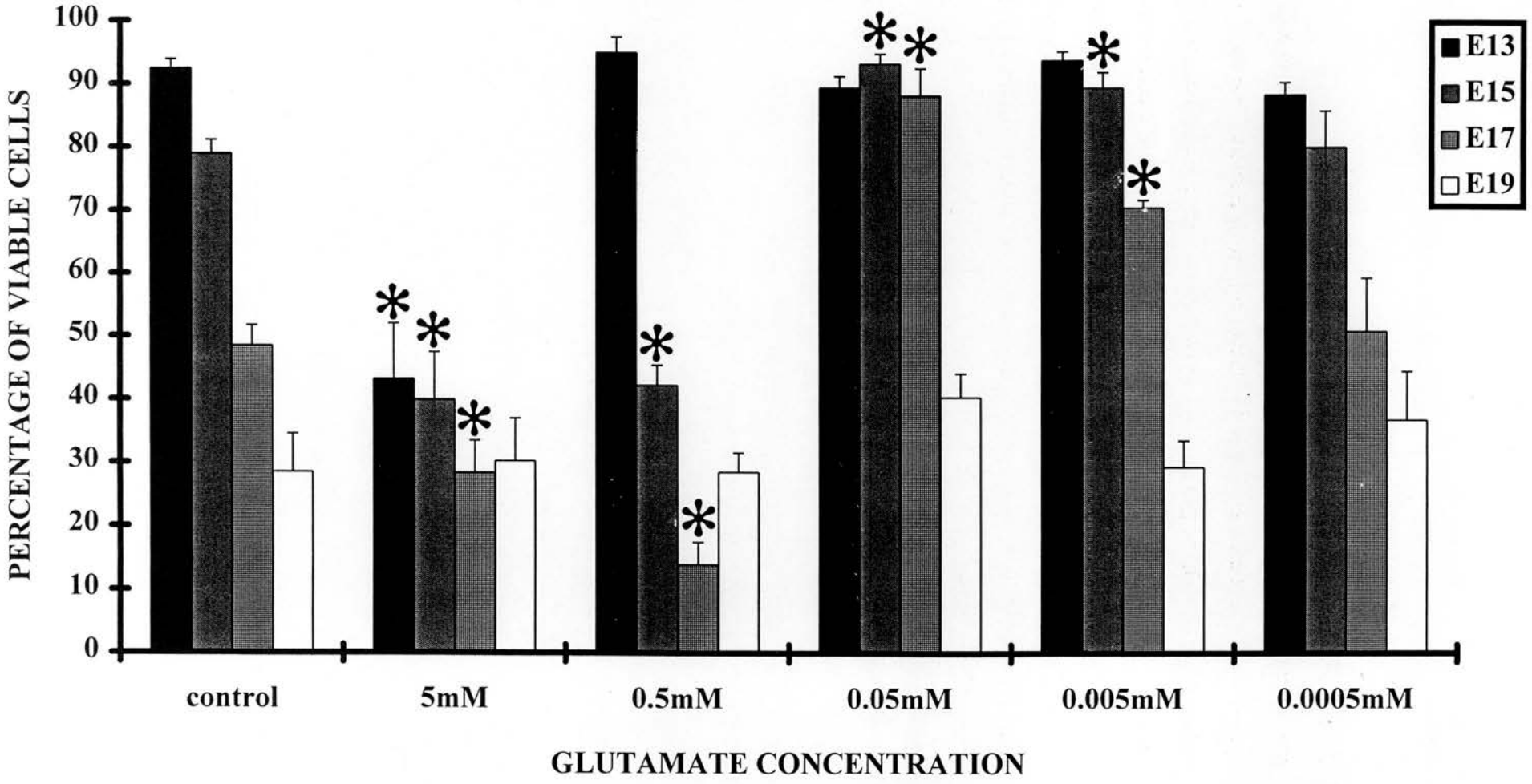


Figure 5.2a

Histogram comparing the mean percentage viable cells between thalamic explants of different ages before and after exposure to glutamate. Error bars indicate the S.E.M. In control explants (n =4), the percentage of viable cells decreased as the age of the explant increased. Glutamate had a dose dependent effect on thalamic cell survival (n =4 or more for each concentration). High doses decreased cell survival and lower doses enhanced cell survival. Glutamate treated thalamic explants were compared to control values using a Student's t-test. * on the histogram indicates values which are significantly different from controls ($p < 0.05$).

HISTOGRAM COMPARING CELL VIABILITY BETWEEN THALAMIC EXPLANTS OF DIFFERENT AGES TREATED WITH GLUTAMATE

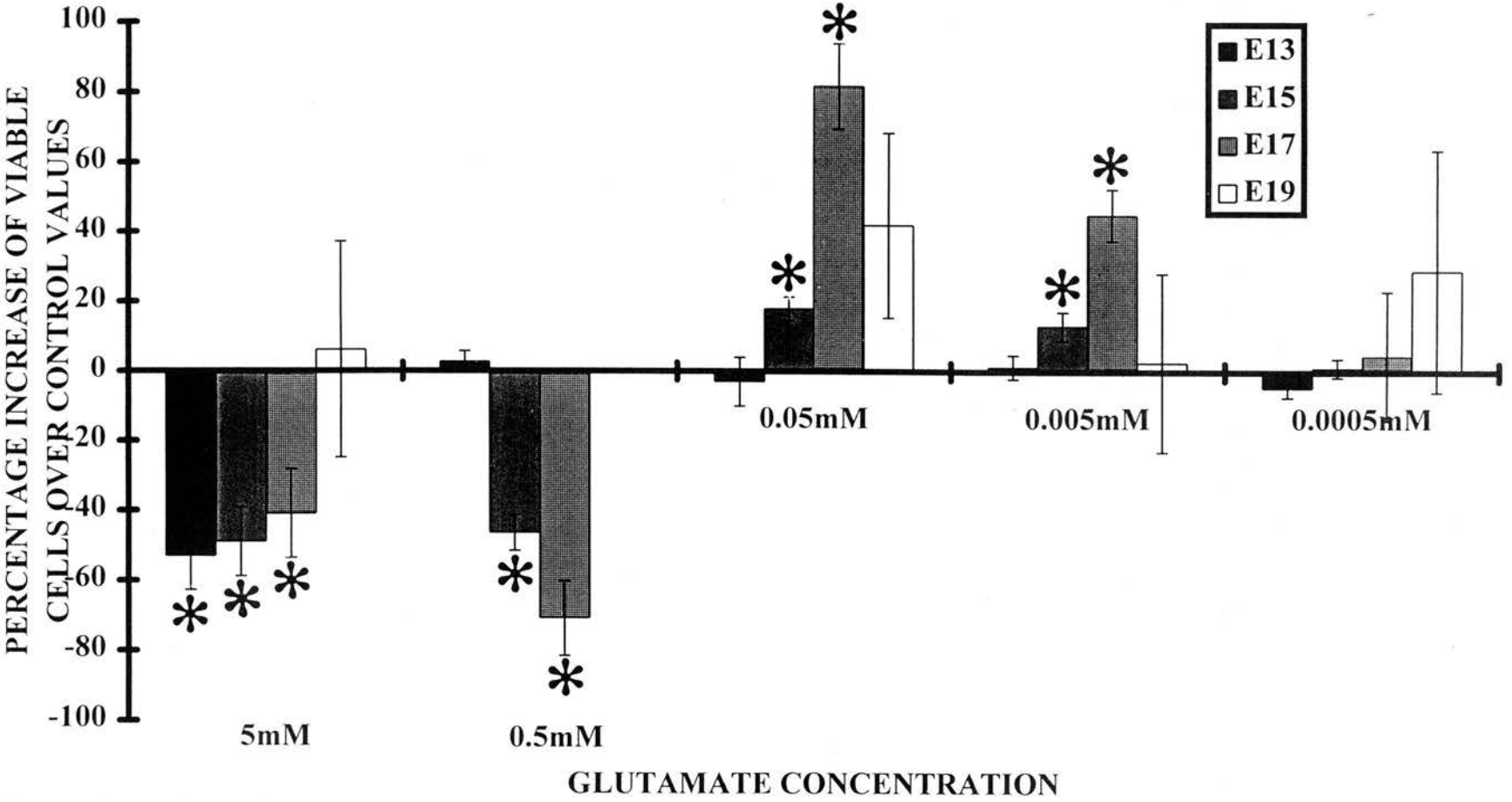


Figure 5.2b

Histogram comparing the levels of viable cells between thalamic explants of different ages treated with glutamate. This histogram demonstrates the effects on cell survival more clearly. The mean value (± S.E.M) after glutamate treatment is subtracted from the mean control value (± S.E.M) and expresses as a percentage of the mean control value(± S.E.M). For controls n = 4, and for glutamate treated values n =4 or more. Glutamate treated thalamic explants were compared to control values using a Student's t-test. * on the histogram indicates values which are significantly different from controls ($p < 0.05$).

5mM and 0.5mM of added glutamate were, at all ages examined, either inhibitory to cell survival or produced no effect. 0.05mM added glutamate had no effect on E13 thalamus but increased cell survival at all other ages. 0.005mM added glutamate had no effect on E13 and E19 thalamus but increased cell survival in E15 and E17 thalamus. 0.0005mM added glutamate had no effect on cell survival at any age examined.

effect on E17 thalamic explants (the percentage of viable cells in these explants almost doubled with addition of 0.05mM glutamate), although E15 explants also responded. Cells in E13 explants did not follow the dose-response pattern displayed by cells in older glutamate treated thalamic explants. Only 5mM glutamate produced a statistically significant effect on cell survival in these explants ($p<0.0014$; Student's t-test), reducing survival by 50%. Cells in E19 explants also did not follow the dose-response pattern displayed by cells in younger glutamate treated thalamic explants and no significant effect on cell survival was obtained at this age.

Although the most likely explanation for the effects of added glutamate on E17 explants was that treatment had rescued cells from death it was conceivable that death rates had remained high in these explants but were offset by mitosis. To assess whether this was possible, the total numbers of cells (live plus dead) in each E17 glutamate treated thalamic explant were estimated and compared to E17 controls (as described in Materials and Methods). 0.05mM glutamate did not increase the total number of cells in E17 thalamic explants ($p>0.05$; Student's t-test). Furthermore, previous work in our lab, as described in Chapter 4, reveals that the clearance rate of dead cells from explant cultures is only 4%, meaning an increase in cell mitosis would be detected in my counts (Rennie, 1992).

Effects of Adding Glutamate Antagonists

An average of 48% of cells were viable in control E17 thalamic explants and glutamate (0.05mM) treatment increased cell viability to an average of 70% in E17 thalamic explants. This effect was statistically significant (p,0.05; Student's t-test). Glutamate antagonists were also added to cultures of E17 thalamic explants in the presence of increased glutamate (0.05mM). Addition of APV (specific NMDA antagonist) to the culture medium dramatically reduced cell survival to only 17% ($p<0.05$; Student's t-test). Effects on cell survival after the addition of CNQX were not as dramatic, cell survival was only reduced to 46% ($p>0.05$; Student's t-test), (refer to fig 5.3 & 5.4).

Figure 3

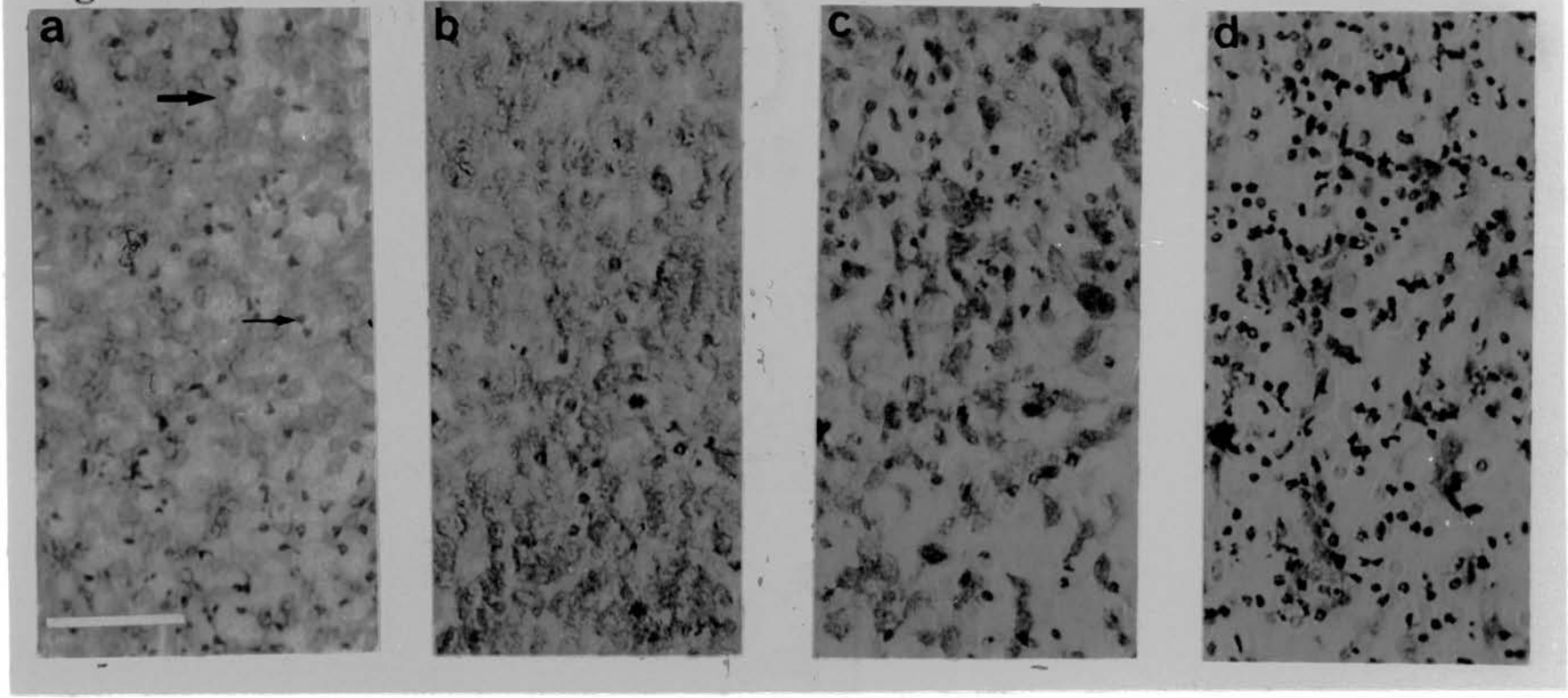


Figure 5.3

Photomicrographs of Nissl stained sections of E17 thalamic explants which have been cultured for three days in the presence and absence of added glutamate and different glutamate antagonists. (a) E17 control thalamic explant; 54% of cells were viable (b) E17 thalamic explant with 0.05mM added glutamate; 74% of cells were viable. (c) E17 thalamic explant with 0.05mM added glutamate and 39mM CNQX (Kainate/AMPA antagonist).; 48% of cells were viable. (d) E17 thalamic explant with 0.05mM added glutamate and 10μM APV (NMDA antagonist); 19% of cells were viable. Large arrow viable cell, small arrow pyknotic cell. (Scale = 44μm)

HISTOGRAM COMPARING THE EFFECTS OF GLUTAMATE AND GLUTAMATE ANTAGONISTS ON THALAMIC CELL SURVIVAL *IN VITRO*.

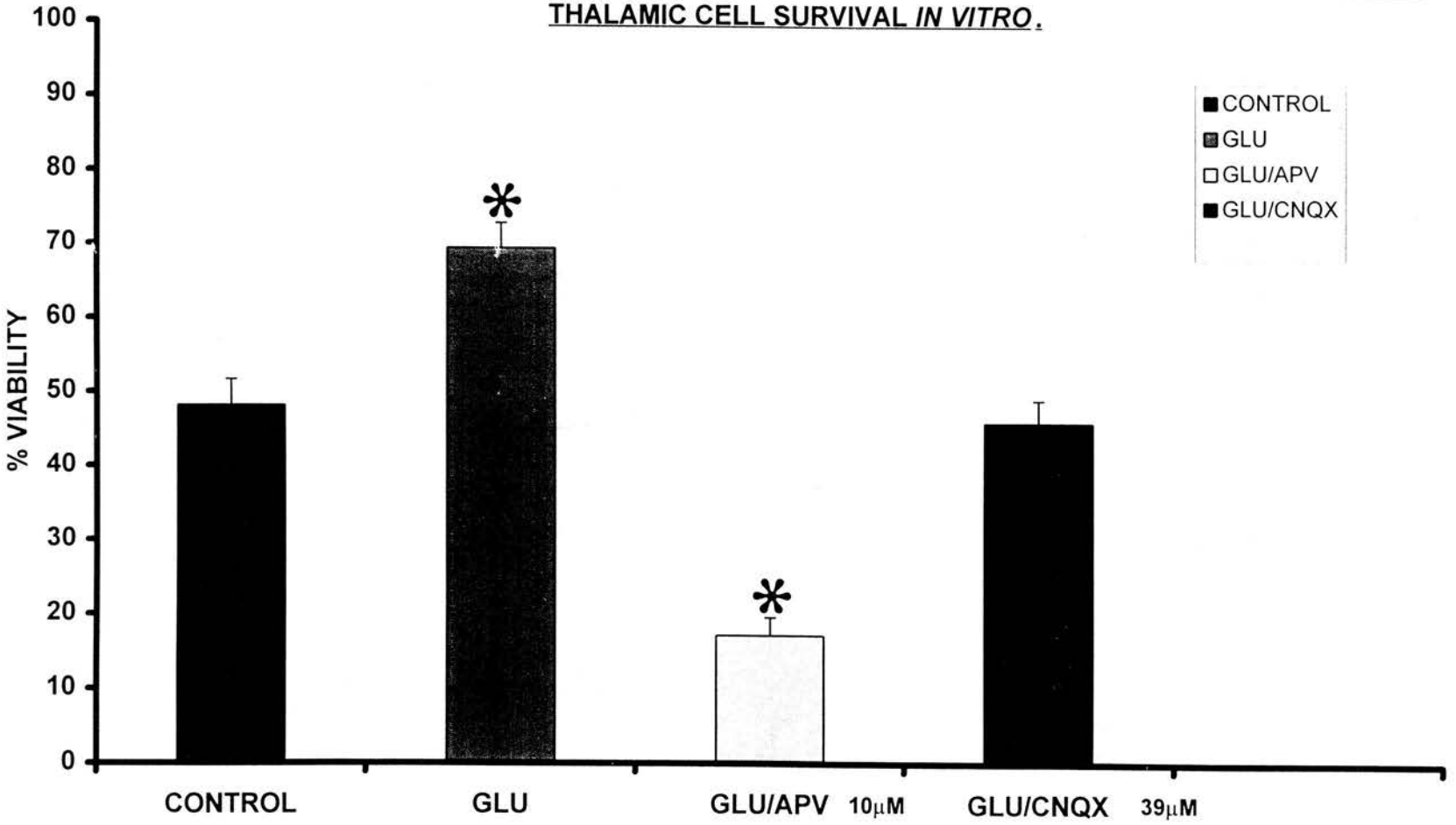


Figure 5.4

Histogram comparing the percentage viable cells in E17 thalamic explants in the presence and absence of glutamate (0.05mM) and different glutamate antagonists. For controls n =5, for 0.05mM glutamate n =6, for 0.05mM glutamate with 10μM APV n =4 and for 0.05mM glutamate with 39μM CNQX n =6. Error bars indicate the S.E.M. The effect of drugs on cell survival was compared to control values using a Student's t-test. * on the histogram indicates values which are statistically different from controls ($p < 0.05$).

Addition of 0.05mM glutamate increased cell survival from 54% to 74%. APV decreased cell survival from 74% to 19%. Addition of CNQX decreased cell survival from 74% to 48%.

DISCUSSION

The aim of this study was to assess whether the viability of embryonic cultured thalamic explants was influenced by exposure to various concentrations of glutamate *in vitro*. My results revealed that exposure to glutamate could increase or decrease cell survival, depending on the age and the concentration of glutamate used. Added glutamate did not increase or decrease cell survival in E13 or E19 thalamic explants, with one exception. E13 thalamic explants after exposure to an extremely high concentration of glutamate (5mM) had few viable cells. After E15 and E17 thalamic explants were exposed to this high concentration of glutamate, inhibitory effects on cell survival were also observed in these explants but no effect was observed in older E19 explants. At lower doses of glutamate, E13 thalamic explants were unresponsive to added glutamate, possibly reflecting the lack of functional calcium activated transduction systems present at this age. However, E19 thalamic explants were also unresponsive to added glutamate suggesting that the receptor transduction mechanism mediating the survival effect has been switched off, and possibly another mechanism supporting cell survival would now be functional *in vivo*. 0.05mM and 0.005mM glutamate had stimulatory effects on cell survival in E15 and E17 thalamic explants. The greatest increase in cell survival was observed in E17 thalamic explants (increased ~2 fold). These findings suggest that, *in vivo*, glutamate acting directly on receptors within the thalamus could play a role in regulating cell survival during the embryonic developmental phase. Glutamate has trophic and inhibitory effects in other systems, and these are dependent on such variables as the cell-type and age, the dose of glutamate, the subtype of glutamate receptor present. (Michel & Agid, 1995; Mattson et al, 1988 a & b; Patterson, 1988; Rothman et al, 1987; Hahn et al, 1988).

Mechanism Of Action Of Added Glutamate

Chapter 4 provides evidence that K^+ depolarization also induces cell survival. K^+-induced effects are known to be mediated by Ca^{2+} influx in other systems, although I was unable to begin investigating the K^+-activated pathway. It is possible that glutamate acting on NMDA receptors, which also results in increased Ca^{2+} influx,

produces the increased cell survival observed in this study. Glutamatergic receptors are present and functional in E17 rat thalamus (Gottman et al, 1994). Cultures of E17 rat thalamus have spontaneous activity in the form of glutamatergic miniature excitatory postsynaptic currents (EPSCs) and their frequency is increased after K^+ depolarization. It is possible that K^+-induced glutamate release was responsible for the increased cell survival observed in Chapter 4.

Activation of the NMDA receptor would result in calcium influx into the cell. Evidence exists in a wide range of systems that increased survival and neurite outgrowth are mediated by calcium influx (Benari et al, 1994; Collins & Lille, 1989; Collins et al, 1991; et al, 1989; Koike & Tanaka, 1991). Increased i[Ca^{2+}] could activate a wide range of calcium-dependent enzymes (Williams et al, 1995) or activate IEG (Morley et al, 1994). The mechanisms by which calcium influx leads to neurodegeneration or increased cell survival are not completely understood. Clearly, there are multiple points within the cell systems that regulate neuronal architecture at which aberrations could lead to neurodegeneration. Activation of glutamate metabotropic receptors would result in stimulating membrane bound enzymes like Phospholipase C (PLC) and adenylate cyclase. However, there is very little evidence to suggest that activation of inositol phospholipids (hydrolyzed to PKC and IP_3) and cyclic AMP can mediate effects on cell survival although there is evidence for these receptors mediating a role in neurite outgrowth (Mattson et al, 1988c; Traynor, 1984).

Added Glutamate Had An Age-Dependent Effect On Cell Survival.

The ability of added glutamate to increase thalamic cell survival was greatest at E17; added glutamate had no effect on the youngest (E13) and oldest (E19) thalamic explants examined, with exception of 5mM glutamate acting on E13 thalamic explants. This concentration represents a 125 fold increase over the basal glutamate concentration in control medium which is only 0.04mM and produced toxic effects at all ages of thalamus, except on older E19 thalamic explants which were unresponsive. It is possible that E13 thalamic explants are too immature to express a significant number of functional NMDA receptors, thus explaining why only an

extremely high concentration of glutamate produced an effect on these thalamic explants. Although E15 and E17 thalamic cells respond to glutamate, E19 cells are resistant to added glutamate suggesting that glutamate receptors which mediate these effects have changed their pharmacological sensitivity during this developmental period. This may involve a change in receptor structure, downregulation of receptors or attenuation of the secondary messenger systems mediating the response. Interestingly, the voltage dependence (Ben-Ari et al, 1988), sensitivity to Mg^+ (Bowe & Nadler, 1990; Kleckner & Dingledine, 1991; Morrisett et al, 1990), and affinity for various ligands (Kalb et al, 1992) of the NMDA receptor have all been reported to change within the first few postnatal weeks in other systems. Previous work has indicated (as described in Chapter 4) that postnatal thalamic cells are unable to survive without target-derived factors. It is clear from these results that glutamate can increase thalamic cell survival in the embryonic thalamus. However glutamate is ineffective at stimulating cell survival in the late embryonic thalamus (i.e. E19). Although corticothalamic afferents display immunoreactivity for high levels of glutamate (Giuffrida & Rustioni, 1988), these results suggest that target cells do not enhance late embryonic thalamic cell survival via release of the neurotransmitter glutamate.

Although the greatest effect on thalamic cell survival was observed at E17, no increase in cell survival was observed at E19. Interestingly, the greatest effect of K^+-induced survival was observed at E19 (refer to results Chapter 4). These results indicate that glutamate and K^+ stimulate different secondary messenger systems in these thalamic explants because the time courses of increased cell survival do not correlate. An increase in intracellular calcium could still be the initial step in both transduction systems. K^+-depolarisation could mediate a calcium influx through voltage dependent calcium channels and glutamate could activate NMDA-receptor linked calcium channels. Recent studies of calcium signal -transduction mechanisms have revealed that, depending on the route of entry into a neuron, calcium differentially affects processes that are central to the development and plasticity of the nervous system, including activity-dependent cell survival, modulation of synaptic strength, and calcium mediated cell death (Ghosh & Greenberg, 1995). For

example, Ca^{2+} influx through voltage-sensitive Ca^{2+} channels can lead to increased cell survival of embryonic neurons from the central and peripheral nervous systems (Franklin & Johnson, 1992; Spitzer ,1995). Yet, Ca^{2+} influx does not always result in cell survival, and Ca^{2+} influx via the NMDA subtype of glutamate receptors in postnatal neurons mediates excitotoxic cell death (Choi, 1988a & b; Hartley et al, 1993). Although it is not completely understood how Ca^{2+} could cause such dramatically different outcomes, the mode of Ca^{2+} entry is becoming increasingly implicated in determining cell survival (Tymianski et al, 1993). Recent studies have revealed that Ca^{2+} transients encode information in their frequency, like action potentials although they are 10^4 times longer in duration and less frequent (Gu & Spitzer, 1995). These studies have demonstrated that spike activity of calcium transients (these rise rapidly to large amplitude and have a rapid decay) and waves (these rise slowly to smaller amplitudes with a slower decay) have distinct roles in developmental regulation. Spikes appear to drive the differentiation of amphibian spinal neurons whereas waves seem to be involved in the regulation of neurite extension (Spitzer, 1995). Therefore it is possible that both K^+-induced depolarization and glutamate stimulate a rise in intracellular calcium but these calcium signals activate different secondary messenger pathways. It is also possible that K^+-induced depolarization in E17 thalamic explants not only activates voltage dependent Ca^{2+} channels but results in an increased release of glutamate from presynaptic terminals (Gottmann et al, 1994), which could act on postsynaptic NMDA receptors and contribute to the resultant increased cell survival. However, in E19 thalamic explants NMDA receptor activation does not appear to contribute to the K^+-induced increase in cell survival, since added glutamate has no effect on cell survival in E19 thalamic explants.

It should be noted however that the NMDA receptor, in contrast to most other ion channels, is ligand- and voltage gated (Collingridge et al, 1992). At resting potential the channel is blocked by extracellular Mg^+ ions, and depolarization of the membrane allows the Mg^+ block to be relieved. However, the membrane potential in embryonic mouse LGN is less negative and is probably already sufficiently depolarized to enable ion channels to open as a result of ligand binding (M ^{c}Leod N, personal

communication). It is therefore possible that older E19 thalamic explants do not have enough spontaneous activity, possibly because of the large occurrence of cell death, to enable added glutamate to open calcium channels. Interestingly, added K^+ which induces membrane depolarization and enhances presynaptic glutamate release (Gottmann et al, 1994) could open these calcium channels and enhance cell survival in E19 thalamic explants. It is also possible that these receptors become more sensitive to Mg^+ block as development proceeds, as described in other systems (Bowe & Nadler, 1990; Kleckner & Dingledine, 1991; Morrisett et al, 1990).

Effects Of Glutamate Antagonists On Glutamate Enhanced Cell Survival.

In an attempt to answer some of the possibilities outlined above two different glutamate antagonists were added to E17 thalamic explants cultured under conditions in which glutamate levels were increased to levels which provide optimal cell survival. 0.05mM glutamate increased cell survival from 48% to 70% in E17 thalamic explants. Addition of 10 μM APV (NMDA antagonist) to these E17 thalamic explants dramatically reduced cell survival to 17%. However, addition of 39μM CNQX (kainate/AMPA antagonist) only reduced cell survival to 46%; this amount of cell survival is comparable to that found in controls. These results suggest that both receptor subtypes may play a role in glutamate induced cell survival in the thalamus. Recent studies have demonstrated that glutamate acting on AMPA receptors in granule cells can increase cell survival (Hack & Balazs, 1994). AMPA mediates these effects by calcium influx via both depolarization activated voltage sensitive calcium influx and NMDA receptors stimulated as a result of AMPA induced glutamate release. My results also provide evidence that glutamate mediates trophic effects by acting at both NMDA and non-NMDA receptors. I would suggest that there are two different populations of cells in the thalamic explants each expressing a different glutamate receptor e.g. interneurons and projection neurons. It would be interesting to add both the NMDA and the AMPA antagonists together and then observe effects on cell survival. Long term, I could radiolabel either APV or CNQX and investigate the distribution of NMDA and Kainate/AMPA receptors present on viable cells. It would also be interesting to investigate the cell population

by back tracing projections from the cortex to the thalamus. This could be done by injecting a lipophilic dye into the cortex *in vivo* before culturing with APV or CNQX. These experiments would determine whether specific glutamate receptors were responsible for maintaining the viability of different cell types within the thalamus.

Conclusion

This study has provided evidence that glutamate can increase cell survival in embryonic thalamic explants in a dose dependent manner. It is clear from the results that K^+-induced depolarization (see chapter 4) could potentially have increased cell survival by stimulating presynaptic terminals to release glutamate. *In vivo*, this stimulation might arise from the increasing activity of peripheral afferents (Shatz & Stryker, 1988). Alternatively, the peripheral afferents may be glutamatergic. It is known that synaptic terminals within the thalamus of both retinal and cortical origin display high concentrations of glutamate (Montero & Wenthold, 1989). If K^+-induced depolarization is producing increased cell survival by stimulating glutamate release then its failure to stimulate P2 thalamic explants to survive could be a result of reduced presynaptic glutamate release. However, my results reveal that added glutamate cannot increase the survival of E19 thalamic explants suggesting that a change in the expression or the pharmacology of glutamate receptors may provide a better explanation for decreased K^+-induced survival. The interpretation of these results is not straight forward. There are two possible explanations for this effect. Glutamate could be activating an entirely different secondary messenger system to that activated by K^+. This is especially interesting because results from glutamate antagonist experiments suggest that glutamate mediates its effect mainly via the NMDA receptor. This would imply that the mode of calcium entry determines which secondary messenger system is activated and would strengthen existing evidence for this relatively recent theory. It would be interesting to determine whether the K^+-induced depolarization effect on cell survival is blocked after addition of glutamate antagonists to the culture medium. HPLC would be useful to determine whether glutamate levels have increased in the culture media after the three day culture

period. If K^+-induced depolarization does indeed stimulate glutamate release, the lack of increased cell survival in E19 thalamic explants by added glutamate could be explained by a decrease in spontaneous activity, and it would be interesting to investigate whether the pharmacology of glutamate receptors changes in P2 cells.

SUMMARY

My interest has been in the development of the mammalian thalamus and cortex. I began my research by exploiting a novel organotypic co-culture system to investigate the signalling mechanism(s) that allows thalamic explants to innervate specifically their target cells in cortical layer 4. I investigated the possibility that neurotransmitters and their receptors are involved. I focused my research on neurotransmitters that are present during thalamocortical development. Neurotransmitter antagonists (atropine, mecamylamine, phenoxybenzamine, propanolol, etc.) failed to block the ability of E15 thalamic axons to recognise layer 4. However, this did not exclude the possibility that that these molecules and their receptors were involved earlier or later in thalamocortical development *in vivo*. APV (NMDA antagonist) prevented thalamic axons from terminating within their target layer 4 *in vitro* but had no adverse effect on the viability of cortical cells. This result suggests that glutamate may play a role in inhibiting thalamic neurite outgrowth within layer 4. AP-5 (GABA B antagonist) and picrotoxin (GABA Cl^- channel blocker) reduced the amount of thalamic axons which would normally innervate a co-cultured cortical explant suggesting that GABA may regulate neurite outgrowth *in vitro*. I also investigated the possibility that activity may be important in target recognition (e.g. ligand gated ion channels) which change the membrane potential as a result of sodium or calcium ion influx. I attempted to induce activity in the cultures by elevating extracellular K^+ and by blocking activity by adding tetrodotoxin (Na channel blocker). K^+-induced depolarization resulted in unexpected and massive thalamic neurite outgrowth and addition of tetrodotoxin prevented thalamic axons from recognising their target layer 4. I concluded that an optimal level of activity is crucial for generating appropriate signals for thalamic outgrowth and termination within layer 4 of the cortex. I followed this up by studying the effects of K^+-induced depolarization on isolated thalamic explants from mice aged embryonic day 13 (E13) to postnatal day 2 (P2) (a period that covers the time from when thalamic outgrowth first occurs until the axons terminate in cortical layer 4) (Bolz et al, 1992). Results

revealed the survival and growth of the late embryonic thalamus can occur without external influences. This mechanism becomes increasingly reliant on neural activity for its maintenance as it ages. After birth, when thalamic cells switch their dependence to cortex derived factors, this intrinsic control may become ineffective.

Next I decided to investigate which factors were responsible for mediating the effects of K^+ induced depolarization. There are two obvious candidates, neurotrophins or neurotransmitters. I decided to investigate the effects of added glutamate to cultured embryonic thalamic cells since glutamate is the excitatory neurotransmitter in the thalamus and thalamic neurons in culture form predominantly glutamatergic synapses (Gottmann et al, 1994). Furthermore, K^+-depolarization has been shown to induce an increased frequency of glutamatergic miniature EPSCs in cultured rat E17 thalamic neurones (Gottmann et al, 1994). Results from these experiments revealed that glutamate can increase cell survival in embryonic thalamic explants in a dose dependent manner suggesting that the K^+-induced depolarization could potentially have increased cell survival by stimulating presynaptic terminals to release glutamate. However, added glutamate did not increase the survival of E19 thalamic explants even though K^+-induced depolarization had its greatest effect on survival at this age. It is therefore possible that glutamate could be activating an entirely different secondary messenger system to that activated by K^+. Addition of glutamate antagonists to the culture medium (CNQX and APV) in the presence of increased glutamate levels which increase cell viability to 70% indicated that glutamate mediates its effects via both the NMDA and kainate/AMPA receptor. Cell survival was dramatically reduced to 17% after blocking the NMDA receptor. However, blocking the kainate /AMPA receptor with CNQX also reduced cell survival although the effect was less dramatic. Thalamic cell viability was reduced to 46% with CNQX. This reduced level of survival is comparable to that found in control E17 explants. The action of added glutamate may not be simple, and increased cell survival above control levels was probably mediated through both the NMDA and kainate/AMPA receptors. I suggest that two different cell populations within the thalamus may express different glutamate receptors.

In addition to the experiments discussed above I also investigated the effects of NO (a novel neurotransmitter) on the development of the thalamocortical system. I decided to investigate the effects of NO, because previous studies had described its ability to inhibit neurite outgrowth by producing direct effects on growth cones (Douglas et al, 1993). Furthermore, NO production has often been described to be regulated by local glutamate receptor stimulation (Bredt & Snyder, 1989; Garthwaite et al, 1989; Johnston & Morris, 1994). It is possible that activity dependent glutamate release may control NO production. NO could then regulate neurite outgrowth.

NO is present in both the developing thalamus and cortex *in vivo* and is also expressed within thalamocortical tracts which connect these two structures. The distribution of NOS in the cortex coincides with thalamocortical innervation. However, the expression of NOS decreases in cultured cortex. Growth cones of dissociated embryonic thalamic neurones are responsive to added NO *in vitro*. Application of SIN-1 (which releases NO) induces growth cones to collapse and removes small axonal processes from the main axon body. Many axons also retracted towards the cell body. These results provide evidence that NO is involved in regulating thalamocortical development.

GENERAL CONCLUSION

Neurotransmitters and Axon Segregation

The results of many experiments which were performed in this thesis were not anticipated, and hence the focus of my research often changed direction. In Chapter 1 neurotransmitter antagonists Picrotoxin & AP-5 (GABA antagonists) reduced the amount of thalamic outgrowth into the cortex and APV (NMDA antagonist) blocked axon termination within target layer 4 in these cultures. These results demonstrate, that GABA may play a role in regulating thalamic outgrowth and glutamate may play a role in termination within target layer 4. However, it is likely that many mechanisms involving many different molecules (e.g. substrate bound chemicals) which were not studied in this thesis are involved in regulating the development of the thalamocortical system. For example adhesion molecules are expressed within the cortex and thalamic axons may be capable of recognising these markers. These molecules could be upregulated *in vivo* as a result of neurotransmitter mediated signals in the P6 cortex before explants are sectioned and placed in culture. Neurons and their projections may be guided either because they follow a gradient of increasing adhesivity to the substrate (e.g. Berlot & Goodman, 1984) or their growth is inhibited in a particular direction (Muller et al, 1990).

Activity Regulated Mechanisms.

Experiments in chapter 2 and 4 in which activity levels were altered suggest that optimal levels of activity are important for maintaining appropriate levels of neurite outgrowth and survival in these co-cultures. I suggest that the early outgrowth from the thalamus is activity dependent and it is not until thalamic axons have innervated the cortex that they lose this ability and become dependent on cortical derived factors. It is possible that increased glutamate release in the thalamus could mediate this activity based mechanism. However other factors such as the neurotrophins could also be involved.

In other systems activity based mechanisms can also regulate axon termination and segregation. For instance, in the cat prenatal tetrodotoxin (Na channel blocker)

infusion blocks segregation of retinogeniculate afferents into eye specific layers in the thalamus. It is thought activity blockade permits the growth of inappropriate branches that would normally be selectively eliminated. The disruption of axon termination in my co-culture system suggests that an activity based mechanism could control axon termination in target layer 4. This mechanism may involve glutamate, since addition of APV to these co-cultures also disrupted axon termination into target layer 4. Chapter 5 has also revealed that glutamate acting via the NMDA receptor can regulate cell survival in isolated thalamic explants.

Novel Neurotransmitters

NO, a novel neurotransmitter, could potentially be very important for controlling thalamocortical development. Nitric Oxide Synthase (NOS), is present in the developing thalamus and cortex *in vivo,* and is also expressed within the thalamocortical tracts that connect these two structures. My results also demonstrate that NO can inhibit the growth of dissociated thalamic axons *in vitro*. It has been demonstrated in other systems that NO can modulate neurotransmitter release and can be neurodestructive or neuroprotective depending on its oxidation reduction status. Activation of NOS is regulated in many systems through the glutamate NMDA receptor (Bredt & Snyder, 1989; Garthwaite et al, 1989). It is possible that NO acting as a retrograde molecule may regulate survival and outgrowth in the early thalamus. My results also reveal NOS is not expressed in the cortex after culture and that 7-nitroindazole (a NOS inhibitor) reduces cortical cell survival *in vitro*. It is possible that the thalamus releases a factor via a NO dependent mechanism which acts to increase cortical cell viability.

Interestingly, NOS is co-localised with neuropeptide-Y and somatostatin containing neurons in the cerebral cortex (Vincent et al, 1983; Dawson et al, 1991). Lateral geniculate axons are arrested at a time that coincides precisely with the appearance of a large number of neuropeptide-Y and somatostatin containing neurons that appear transiently at the cortical subplate (Chun et al, 1987). After these transient neurons die axon elongation resumes. It is possible that these peptide neurons in the subplate may also co-express NOS, and inhibit neurite outgrowth via the production and local

release of NO. It would be interesting to examine the distribution of these peptides throughout thalamocortical development.

It is obvious from these results that the relationship between the developing thalamus and cortex is highly complex and it is likely that several integral signalling mechanisms combine to control different aspects of thalamocortical development.

FUTURE WORK

Whilst studying the development of the murine forebrain, I noticed that K^+-induced depolarization dramatically affects the survival and growth of isolated embryonic murine thalamic explants in culture, in an age and dose dependent fashion. In control medium, the survival and growth of the youngest isolated thalamic explants were high, but both viability and neurite outgrowth decreased as the age of the explants increased. Outgrowth and survival were dramatically increased in the older thalamic explants by K^+-induced depolarization. These results suggest that thalamic cell survival and growth are controlled by a mechanism that becomes increasingly reliant on neural activity for its maintenance as it ages. Elevated K^+ neither prevented the death nor increased the neurite outgrowth of postnatal thalamic explants, suggesting that postnatal thalamic cells switch off their mechanism (other evidence suggests that they become dependent on target derived factors; Lotto & Price, 1995). This work is currently in press in *Neuroscience*. However, I have been unable to capitalize on these observations because they were made relatively late during my studies. I would like to exploit both the dissociated and explant culture systems to test specific key hypotheses concerning the mode of action of elevated extracellular K^+. My overall hypothesis is that elevated K^+ stimulates an increase in intracellular Ca^{2+} which produces an increase in endogenous growth factor(s) possibly via the activation of CaMKII. I intend to: **1**. Investigate whether neurotrophic factor(s) mRNA is upregulated using a ribonuclease protection assay (RPA). I would study any corresponding changes in protein levels using antibodies to the neurotrophins. I have already conducted some of these experiments in collaboration with Tom Pratt. We have the RPA reaction working and can detect TrKB mRNA in control E17 thalamic explants. We now need to detect TrKB in cultured control explants, before investigating the levels found in K^+-treated explants. **2**. Block the neurotrophic factors by adding various reagents to the culture medium. **3**. Block Ca^{2+} entry into the cell and attempt to knock out functional CaMKII using molecular techniques. **4**. Determine whether gene transcription is upregulated by looking at the activation of transcription factor CREB (a critical mediator of Ca^{2+} dependent gene expression). I

have applied for a two year Wellcome Prize Postdoctoral Fellowship, if this application is successful I intend to pursue this research as outlined below.

EXPERIMENTAL DESIGN.

YEAR 1 : Aim is to test the hypothesis that elevated K^+ affects the viability and growth of thalamic explants by upregulating the transcription of one or more specific neurotrophic molecules.

Experiment 1 - Neurotrophic Factor Upregulation.

I shall start by using a ribonuclease protection assay (RPA), which is a standard, extremely sensitive procedure for the detection and quantitation of small amounts of mRNA, to assess changes in the levels of mRNA for specific neurotrophic factors in cultured thalamic explants in response to elevated K^+. RPA's have been found to be more satisfactory for the detection of neurotrophins than alternatives such as quantitative PCR or northern blot analysis. Probes for nerve growth factor (NGF), brain derived growth factor (BDNF), neurotrophin 3 (NT3) and NT4 are all available in our laboratory (through agreements with Regeneron and Genentech Inc, U.S.A.). I would systematically investigate whether the mRNA for each neurotrophin (BDNF, NGF, NT-3 and NT-4), is upregulated by various doses of K^+ at the relevant developmental ages. I would follow up positive results by studying changes in corresponding protein levels using antibodies to the neurotrophins (also available in our laboratory).

Possible Outcomes. If one or more of these molecules is upregulated by K^+ treatment , I would proceed to **experiment 2**. If none are affected, I would use the methods to study other good candidates (e.g. fibroblastic growth factor, FGF). If none of these experiments gives promising results, I would proceed to **experiments 3 and 4**.

Experiment 2- Blocking the Neurotrophic Factors.

I would attempt to prevent the K^+-enhanced trophic effects in culture by adding reagents to the culture medium that would block the action and/or synthesis of neurotrophins. I would use dissociated as well as explant cultures of thalamic cells (to improve the blocking reagent's access). I already have evidence that added K^+ enhances cell viability and neurite outgrowth in dissociated culture and this will be confirmed during these experiments. I shall block with (i) K252a, a blocker of tyrosine kinases, (ii) chimeric molecules comprising IgG coupled to Trk A, B or C receptors (we have been given these by Genentech) which are more specific since they bind to neurotrophins in solution with high affinity and prevent them binding to receptors on target cells, (iii) blocking antibodies to FGF (a & b), and (iv) antisense oligonucleotides for neurotrophic factor mRNA.

Possible Outcomes. Blockers of neurotrophin action/synthesis may inhibit or abolish the effect of elevated K^+. Some neurotrophins are not completely specific for one receptor and can cross react with others. It is possible that the same neurotrophin could mediate different physiological functions by activating different receptors. Therefore these experiments could also indicate which receptors are important in the regulation of neurite outgrowth and cell survival. If **experiment 1** had indicated upregulation, but no inhibitory effect on either cell survival or neurite outgrowth is observed in **experiment 2**, this would suggest that neurotrophic factors have other K^+-dependent effects (i.e. on differentiation).

YEAR 2: Aim is either (i) to test the hypothesis that the effects of K^+ are mediated by Ca^{2+} and CaMKII (experiment 3); and/or (ii) to investigate the possibility that gene activation and upregulation of a novel trophic factor is responsible for the K^+ effect (experiment 4).

Experiment 3a- Ca^{2+} Influx.

I would test whether an elevated intracellular Ca^{2+} is required by (i) adding various Ca^{2+} channel blockers (e.g. nifedipine), to the culture medium and (ii) by reducing extracellular Ca^{2+} and replacing it with magnesium. The effects on the growth and viability of K^+ treated thalamic explants and, if appropriate, the levels of their neurotrophic factors (assessed as in expt. 1) would be studied. I could also use Ca^{2+} channel agonists (Bay K 8644) in an attempt to mimic the effect of K^+.

Possible outcomes If Ca^{2+} influx is required, I would proceed to **experiment 3b**. If Ca^{2+} influx is not required, elevated intracellular Ca^{2+} may still be important but it may come from intracellular stores. I would then attempt to mimic the K^+ effect in dissociated thalamic cell cultures by adding an IP_3 agonist (e.g. histamine) and thapsigargin, a potent cell permeable IP_3 independent intracellular Ca^{2+} releaser to determine whether intracellular Ca^{2+} release is important in the signaling pathway. I would then proceed to **experiment 3b**.

Experiment 3b-to Investigate the Molecular Event Downstream of Elevated Intracellular Ca^{2+}.

I would attempt to block CaMKinaseII (CaMKII) activation in cultured thalamic explants. This enzyme is associated particularly with neurite outgrowth (Williams et al, 1995). KN62 is a specific CaMKII blocker, although whether it retains its activity over a three day period is unknown. Therefore the culture medium, containing K^+ and KN62, or K^+ alone, would be refreshed every day. Thalamic viability, growth and neurotrophin expression would be assessed as before. If time permitted, further evidence for the involvement of CaMKII in propagating the Ca^{2+} signal to the nucleus would be obtained using two molecular techniques in parallel to knock out functional CaMKII expression in dissociated thalamic cultures. (i) I could transiently transfect with antisense cDNA construct of the CaMKII-alpha subunit (Tashima et al, 1996). (ii) I could construct an antisense oligonucleotide to CaMKII-alpha subunit and add it to the extracellular medium (Wang et al, 1995).

Possible outcomes KN62 may simply block the K^+-enhanced response. However, CaMKII is involved in the normal regulation of many cells and it will be important to run appropriate controls to investigate the effects of this blocker on basal neurite outgrowth and survival. If no effect is observed with KN62 , other Ca^{2+} regulated enzymes may be important. Future work would then investigate the involvement of either adenylate cyclase or protein phosphatase.

Experiment 4- $K^{\pm}$ Effects on CREB Expression and Novel Factors?

I would investigate the possibility that a novel compound is upregulated by K^+ in these cultures. I would test whether gene activation is required by studying a transcription factor, cyclic adenosine monophosphate-response element binding protein (CREB), that is currently believed to be a critical mediator of Ca^{2+} dependent gene expression (Yoon et al, 1994). CREB becomes rapidly phosphorylated when cells are exposed to a variety of extracellular stimuli (Ginty et al, 1993) . This event can be detected in single neurons by immunostaining with an antibody that specifically recognizes only phosphorylated CREB (PCREB) (Ginty et al, 1993). The levels of PCREB immunoreactivity would be compared in control and K^+ treated dissociated thalamic cell cultures. I will also examine the levels of CREB using a specific antibody that recognizes both CREB and PCREB (Ginty et al. 1993) to investigate whether the ratio of CREB:PCREB has shifted or whether an increase in neuronal CREB protein per se has occurred. Future work could include microinjecting W39 (a highly specific antibody for CREB protein) antiserum into dissociated thalamic cells to determine whether it diminishes their response to K^+ depolarization.

If time permits, experiments would be designed to determine the nature of any novel molecule(s) mediating the K^+ enhanced effects, starting with a test of whether they were membrane bound or freely diffusible. Outgrowth assays would be done on control and K^+ treated thalamic cell membranes prepared at different developmental stages. To determine whether the molecules were freely diffusible, pre-conditioned medium from K^+ treated thalamic explants would be dialyzed to extract the K^+ and then transferred to new culture wells into which freshly dissected thalamic explants

of different ages would be placed for a three day culture period. Any increased survival or outgrowth would be determined. Heat denaturation of the pre-conditioned medium would determine if the effect was mediated by proteins. Size exclusion chromatography of the conditioned medium would indicate the size of the active molecules. Further work might include further biochemical analysis of the active fraction.

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