IN VITRO AND IN VIVO STUDIES IN THE DEVELOPING MURINE FOREBRAIN

Natasha Warren

PhD

University of Edinburgh

1996

DECLARATION

I declare that this thesis was composed by myself. Contributions of others to the work are clearly indicated.

ACKNOWLEDGEMENTS

I would like to thank Dave most of all for his advice, encouragement and friendship during my PhD. I am extremely grateful to Katy and Grace for all their help with the histology. Many thanks to Stephen, Steve, Grace, Tom and Isabelle for help in the molecular biology lab; Suzanne and Beau for teaching me the coculture technique; John, Linda and Willie for looking after the animals; Mark for his help in generating the computer images; Brendan for help with the photography and Richard for the use of his computer. I am very grateful to Kenji for patiently teaching me the whole mount in situ hybridization technique.

Thanks also to my dear friends Jacki and Ruth.

Thanks to Norah, Anna, Ally and Tasha.

Lastly thanks to Mum, Dad, Gareth, Doms and Tams and I suppose Steve.

The work presented in Chapter 3 has been sent for publication. This and sections of other chapters have been presented at scientific meetings both in the UK and the USA.

ABSTRACT

This study focuses on the development of neuronal connections between the retina, thalamus and cortex in mice using *in vitro* and *in vivo* techniques. I examined the hypothesis that the embryonic cortex releases a diffusible factor that initiates and/or guides thalamic neurite outgrowth. I used an organotypic co-culture system to show that embryonic cortex does not stimulate increased outgrowth from embryonic day 15 (E15) thalamus (cultured at a distance) above control levels, whereas postnatal cortex causes a significant increase in thalamic neurite outgrowth. This result argues against a role for diffusible cortex-derived growth factors in the early prenatal stages of thalamocortical development.

I then examined the hypothesis that developing retinothalamic and thalamocortical tracts are guided by borders of *Dlx-2* and *Pax-6* gene expression in the forebrain. Using the neuronal tracer 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI), to label the axon tracts and whole mount in situ hybridization to study the expression of *Dlx-2* and *Pax-6*, I showed that there may be a correlation between the retinothalamic tract and the domain of *Dlx-2* expression in the region of the optic tract (ventral diencephalon), and between cell bodies of the thalamocortical neurons and *Pax-6* expression on the lateral surface of the ventral thalamus.

If the borders of *Pax-6* and *Dlx-2* expression are altered, are the routes taken by the retinothalamic and thalamocortical tracts altered? I used the *Pax-6* mutant mouse Small eye (Sey), in which a point mutation in the *Pax-6* locus results in a dysfunctional PAX-

6 protein (Hill et al.,1991), to test this hypothesis. I first characterized the diencephalon of *Sey* embryos by studying Nissl-stained parasagittal sections of E14.5 embryos, and showed disruption of morphological constrictions in the tissue and disruption of cell density values. Using whole mount in situ hybridization to study the expression of *Pax-6, Dlx-2* and *Wnt-3* in *Sey* embryos, I showed that expression of all three genes was altered in the diencephalon compared to wild-type embryos. I then showed that although the retinothalamic tract forms in Sey/Sey embryos it is clearly abnormal.

ABBREVIATIONS

a	anterior
ac	anterior cortex
aep	anterior entopeduncular area
ah	anterior hypothalamus
bdnf	brain-derived neurotrophic factor
c	centromedium
cam	cell adhesion molecules
cdgf	cortex-derived diffusible growth factors
cg	ciliary ganglion
chp	choroid plexus
cp	cortical plate
cpe	cerebral peduncle
ct	corticothalamic tract
d	diencephalon
dt	dorsal thalamus
dtmesV	descending tract of the mesencephalic nucleus of the trigeminal nerve
e	ependymal layer
ecm	extracellular matrix molecules
eg	external germinal
emt	eminentia thalami
fgf	fibroblast growth factor
h	hindbrain
hcc	hypothalamic cell cord
i	intralaminar
ig	internal germinal
iml	internal medullary lamina
is	isthmus of mesencephalon
iz	intermediate zone
ld	lateral dorsal
lg	lateral geniculate
lge	lateral ganglionic eminence
lgn	lateral geniculate nucleus
lp	lateral posterior
lv	lateral ventricle
m	mantle
md	medial dorsal
mes	mesencephalon
met	metencephalon
mg	medial geniculate
mge	medial ganglionic eminence
mlf	medial longitudinal fasciculus
mye	myelencephalon
mz	marginal zone
ncam	neural cell adhesion molecule
ncx	neocortex
ngf	nerve growth factor
ob	olfactory bulb

oc	optic chiasm
omn	oculomotor nerve
on	optic nerve
ot	optic tract
p	pulvinar
p1-p6	prosomeres 1-6
pa	parencephalon anterior
par	parencephalon
pc	posterior cortex
pep	posterior entopeduncular area
pn	perireticular nucleus
poa	posterior preoptic area
pop	preoptic recess
pp	parencephalon posterior
ppl	preplate
prp	primary prosencephalon
pt	pretectum
r	rhombomere
rgc	radial glial cells
rn	reticular nucleus
s	synencephalon
sc	spinal cord
sc	superior colliculus
sch	suprachiasmatic area
se	septum
sey	small eye
sp	subplate
spr	secondary prosencephalon
spv	supraoptic/paraventricular area
svz	subventricular zone
tc	thalamocortical tract
tpc	tract of the posterior commissure
tpoc	tract of the postoptic commissure
tu	tuberal hypothalamus
va	ventral anterior
vl	ventral lateral
vpl	ventral posterior lateral
vpm	ventral posterior medial
vt	ventral thalamus
vz	ventricular zone
wm	white matter
zli	zona limitans intrathalamica

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

During embryogenesis the brain develops from a single sheet of neuroepithelial cells that folds to form the neural tube. From embryonic day 8.5 (E8.5) discrete bulges and furrows divide the neural tube along its anterior-posterior axis into four regions-the forebrain, midbrain, hindbrain and spinal cord (Fig.1 -adapted from Puelles et al., 1987). The forebrain forms two main structures: the anterior telencephalon, which gives rise to the cerebral hemispheres, and the diencephalon, which gives rise to the thalamus and hypothalamus. The diencephalon is connected to the mesencephalon, which gives rise to the midbrain. The hindbrain is further divided into metencephalon, which gives rise to the pons and cerebellum, and myelencephalon. The metencephalon and myelencephalon are further subdivided into eight transient neuromeres called rhombomeres (Rugh, 1991).

1.1 NEUROGENESIS AND MIGRATION

Neuroepithelial cells divide within each of the above regions producing cells that migrate and then differentiate into neural structures with distinct histologies.

The proliferation and migration of specific cells can be followed using tritiated thymidine (^{3}H-T) autoradiography which labels dividing cells. Animals are injected with ^{3}H-T at various stages of development, and CNS sections are processed for autoradiography after defined periods of further development. ^{3}H-T has a short half-life of several hours in the blood and any cell that is undergoing its last round of DNA synthesis (S-phase) at the time of exposure to ^{3}H-T is heavily labelled in all subsequent autoradiographs, whilst neurons that are postmitotic at the time of injection remain free of label. The time and place of 'birth' of each type of neuron and its subsequent movement can be reconstructed.

Fig. 1. Schematic diagram showing subdivision of the neural tube (adapted from Puelles et al., 1987; Rubenstein et al., 1994). According to Puelles et al. (1987), division occurs in three stages by the sucessive establishment of interneuromeric furrows. In the proneuromeric phase at E8.5 (A and B), the primary prosencephalon is divided into secondary prosencephalon and diencephalon. In the early neuromeric phase, (C), the diencephalon is divided into synencephalon and parencephalon, and the hindbrain is divided into metencephalon and myelencephalon. In the late neuromeric phase the parencephalon is divided into anterior and posterior parts and the metencephalon and myelencephalon are divided into eight rhombomeres. prp, primary prosencephalon; mes, mesencephalon; h, hindbrain; sc, spinal cord; spr, secondary prosencephalon; d, diencephalon; par, parencephalon; s, synencephalon; met, metencephalon; mye, myelencephalon; pa, parencephalon anterior; pp, parencephalon posterior; is, isthmus of mesencephalon; r, rhombomere.

Figure 1

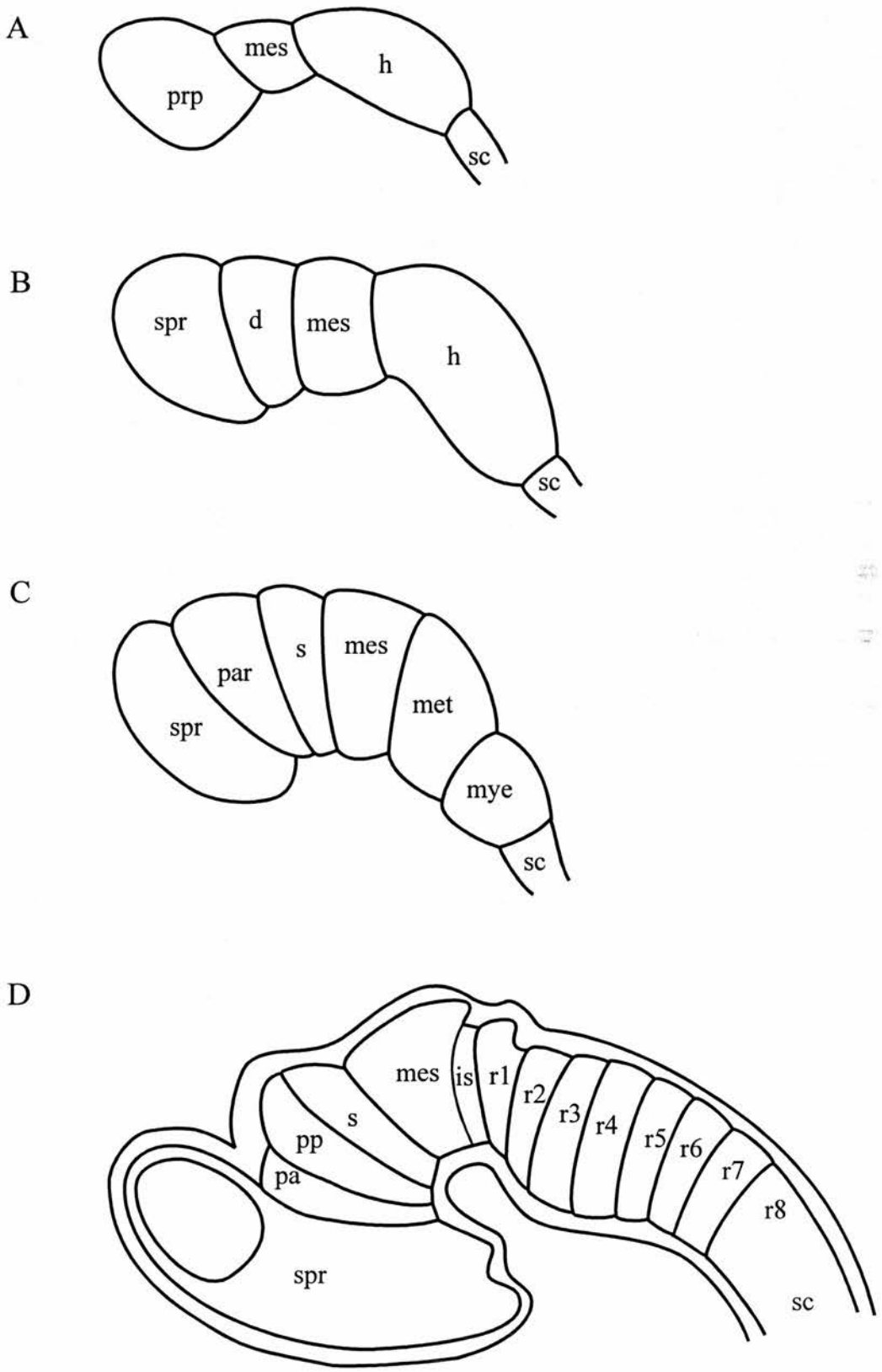
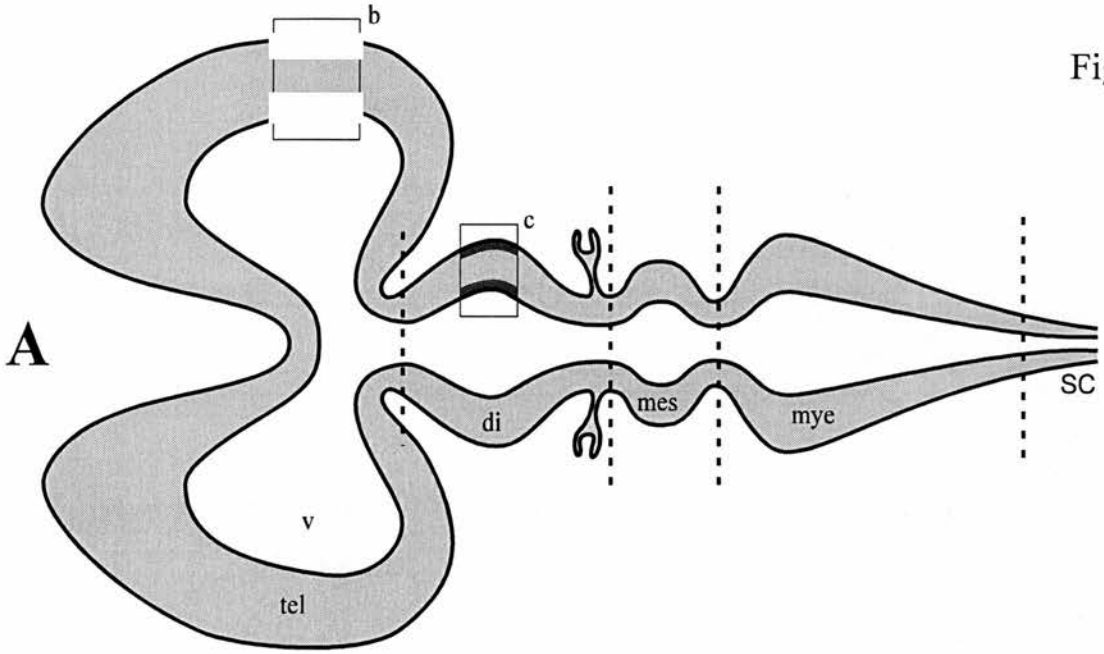
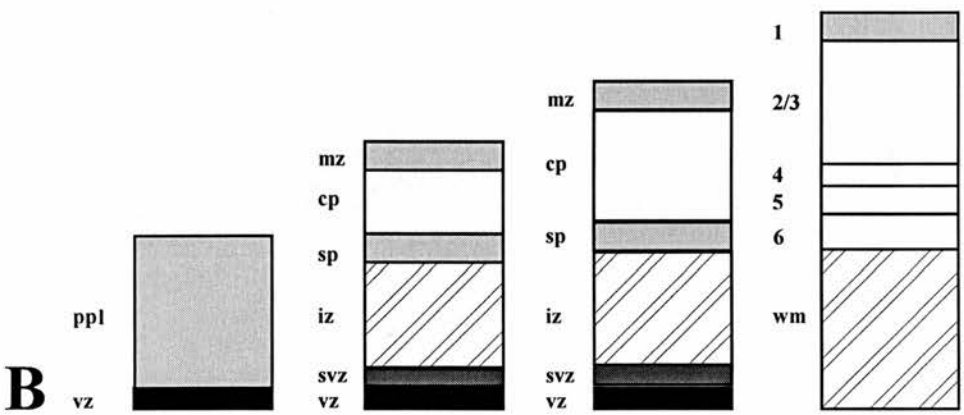
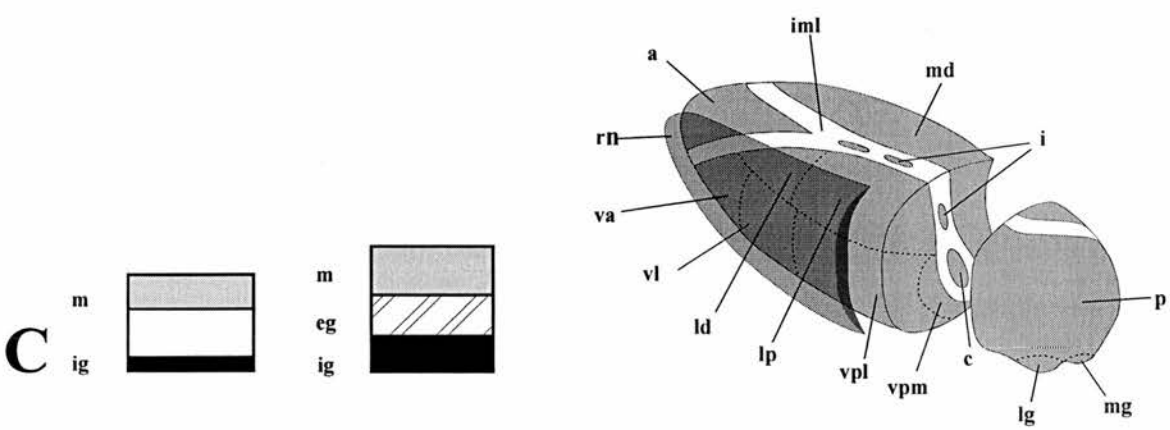

A
mes
h
prp
sc
B
spr
d
mes
h
sc
C
s
mes
par
met
spr
mye
sc
D
mes
is
r1
r2
r3
r4
r5
r6
r7
r8
s
pp
pa
spr
sc

Fig. 2. Neurogenesis and migration in the telencephalon and diencephalon. (A) Schematic drawing that shows the location of the developing neocortex in the telencephalon (box marked b) and of the developing thalamus in the diencephalon (box marked c) (horizontal plane of section). (B) Development of neocortical layers. Cells that populate the neocortex arise from the ventricular zone of the dorsal telencephalon. The first postmitotic neurons migrate along the processes of radial glial cells and accumulate immediately beneath the pial surface forming the preplate. Cells destined for deep layers of the cortex are generated next. They migrate along radial glial cells and split the preplate into the marginal zone and the subplate. Cells born later in development migrate through and settle above the earlier generated cells, forming progressively more superficial layers. This process of cell division and migration results in the inside-out formation of the six cortical layers and takes almost two weeks to complete. vz, ventricular zone; rgc, radial glial cells; ppl, preplate; svz, subventricular zone; iz, intermediate zone; sp, subplate; cp, cortical plate; mz, marginal zone; wm, white matter; e, ependymal layer. (C) Development of the thalamus. Cells born in the ventricular zone of the diencephalon migrate along radial glial cells and settle immediately beneath the pial surface. Cells generated later in development migrate and settle beneath earlier generated cells in an outside-in manner forming three distinct layers: the mantle, external germinal and internal germinal. Later in development the three layers are lost and differentiation of functional neuronal nuclei occurs. m, mantle; eg, external germinal; ig, internal germinal; a, anterior; rn, reticular; va, ventral anterior; vl, ventral lateral; ld, lateral dorsal; lp, lateral posterior; vpl, ventral posterior lateral; vpm, ventral posterior medial; c, centromedium; lg, lateral geniculate; mg, medial geniculate; p, pulvinar; i, intralaminar; md, medial dorsal; iml, internal medullary lamina.

Figure 2


A
b
c
di
mes
mye
SC
v
tel


B
ppl
vz
mz
cp
sp
iz
svz
vz
mz
cp
sp
iz
svz
vz
1
2/3
4
5
6
wm


C
m
ig
m
eg
ig
iml
a
md
rn
i
va
vl
ld
lp
vpl
vpm
c
p
lg
mg

1.1.1. Neurogenesis of the cerebral cortex

The cells of the mature cerebral cortex are organized into 6 well-defined layers. These cells are generated in the ventricular zone near the ventricles of the brain. (Figure 2A). The cells migrate through the intermediate zone to their destinations in the developing neocortex. In the mouse, neurogenesis of the neocortex begins on embryonic day 12 (E12) (Wood et al., 1992). The earliest generated neurons migrate through the intermediate zone to form a structure known as the preplate which is subsequently split into the marginal zone and transient subplate by the invasion of cells that form the cortical plate (Luskin & Shatz, 1985a; Bayer & Altman, 1990) (Figure 2B). Neurons born at early stages of cortical development form the deepest cortical layers (layers 5 and 6), while those born at later times form progressively more superficial layers (layers 2 and 3). Thus the layers of the cortex are formed in an 'inside-first, outside-last' manner (Luskin & Shatz, 1985b; Angevine & Sidman, 1961). Neurons born at later times must therefore migrate past neurons that have already reached their final position in the cortex. It appears that the final laminar position of a neuron is determined prior to migration, in the ventricular zone during the S-phase of the cell cycle (McConnell & Kaznowski, 1991).

1.1.2 Neurogenesis of the thalamus

Cells of the thalamus and hypothalamus accumulate in an 'outside-first, inside-last' sequence (Rakic, 1977) (Figure 2C). In mouse the diencephalon develops in three stages. Neurogenesis begins around E10 when cells in the ventricular zone adjacent to the third ventricle divide to form a dense single cell layer. Migration of cells to the lateral edge of the tissue occurs around E12 and between days E13-15 the cells differentiate into the internal germinal, external germinal and mantle layers. During

the third stage, from E16 to postnatal life, the organization of the three layers is lost and the differentiation of functional neuronal nuclei becomes more evident (Niimi et al., 1962).

1.1.3 The role of radial glial cells in migration

There is considerable evidence that the migration of postmitotic neurons from the ventricular zone to their final destinations in laminar structures (such as the cerebral cortex), and nuclear structures (such as the thalamus), is guided by elongated radial glial cells which extend from the ventricular to the pial surface (Rakic, 1971, 1972, 1977, 1990; Mission et al., 1991; Gasser & Hatten, 1990; Golden & Cepko, 1996). The postmitotic neurons attach themselves to the radial glia fibres by a mechanism which seems to involve N-type calcium channels (Komuro & Rakic, 1992). Once the leading neurite of postmitotic neurons reaches the final destination, several morphological transformations take place in the neuron and it detaches from the radial glia fibre. While the phenomenon of neuronal cell migration has been known for more than a century (Ramon y Cajal, 1891), identification of the molecular components underlying selective neuronal cell displacement to specific laminae and nuclear aggregates is only beginning to emerge. For example, two membrane polypeptides of radial glial cells have been identified that may contribute to the formation of the junction between migrating neurons and radial glial cell processes (Cameron & Rakic, 1994).

1.2 DEVELOPMENT OF AXONAL PROJECTIONS

As neurogenesis advances, postmitotic neurons in each cortical layer begin to receive and extend axons. Neurons in layers 5 and 6 extend axons to subcortical targets: layer 5 projects to the superior colliculus and spinal cord and layer 6 projects to the thalamus. Neurons in layers 2 and 3 extend axons to other cortical areas and neurons in layer 4 are innervated by axons extending from cells in the thalamus. Layer 1 cells project to other layer 1 cells, and receive apical dendrites and axons from other layers.

This study focuses on the development of axonal connections between three structures in the mammalian forebrain, the retina, thalamus and cerebral cortex with particular interest in the development of the retinothalamic and thalamocortical tracts.

1.2.1 The retinothalamic pathway

At E12, axons from retinal ganglion cells leave the retina and grow along the ventral margin of the optic stalk pioneering the optic nerve. The optic nerve enters the brain at the optic chiasm where most axons grow straight across to the opposite side of the brain (Bovolenta and Dodd, 1991), while others turn 90° and project to the same side of the brain. Once through the chiasm, the axons form the optic tract and project dorsolaterally to the ventral diencephalon as a tightly fasciculated bundle. As the tract projects into the dorsal diencephalon (in the region of the lateral geniculate nucleus) the fibres fan out and some turn through as much as 90^0, to grow

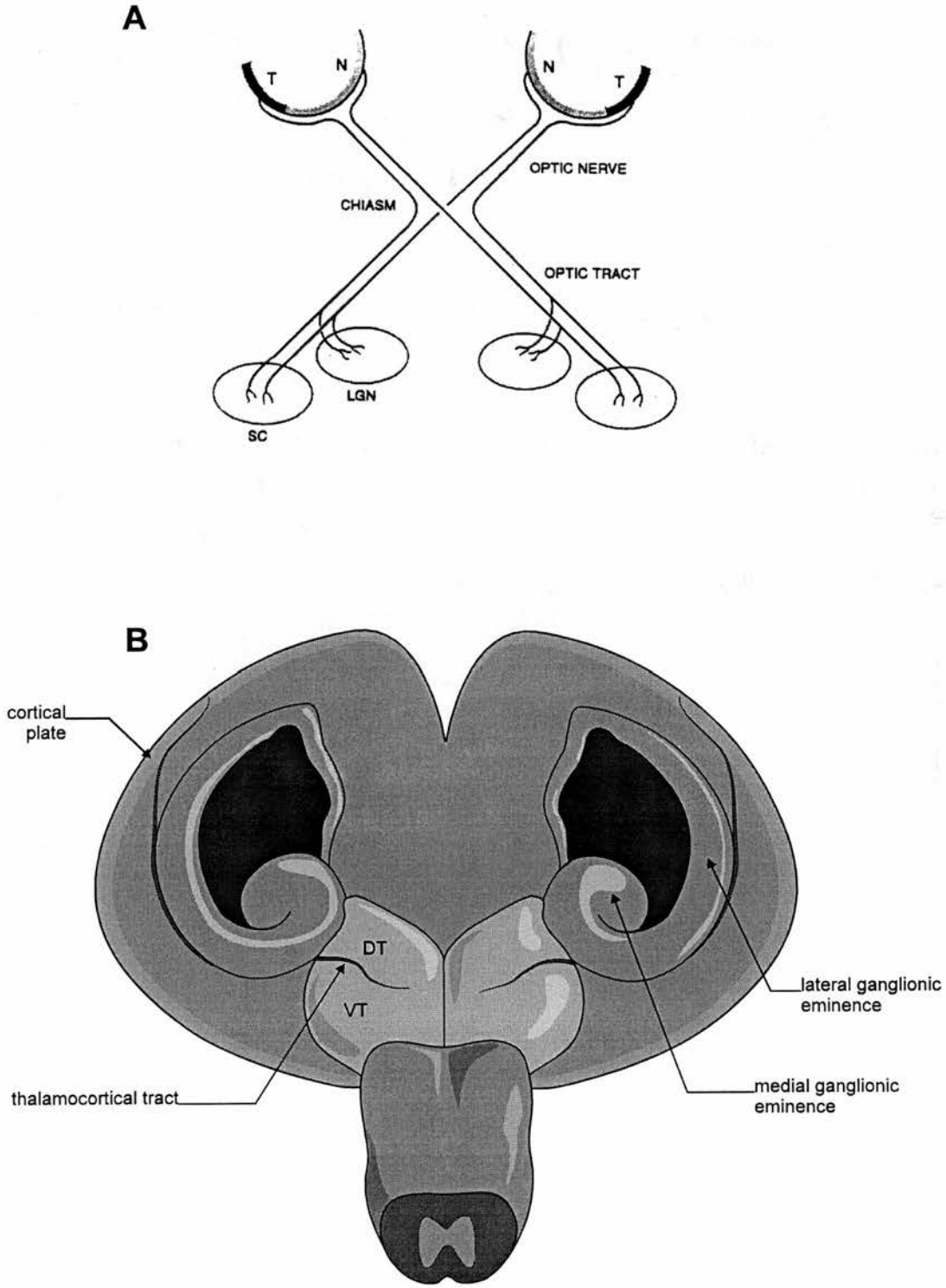
dorsocaudally along the brainstem to the superior colliculus. The remaining retino-thalamic fibers synapse on thalamocortical neurons (Figure 3A).

1.2.2 The thalamocortical pathway

At E15, axons from thalamic cells leave the thalamus and project anterolaterally through the primitive internal capsule, a large fibre bundle that contains the main axons running from and to the cerebral hemispheres, beneath the developing striatum (Fig. 3B). Once through the internal capsule, thalamocortical afferents do not immediately grow into the cortical plate but instead project tangentially in the underlying and more mature subplate (Lund & Mustari, 1977; Miller et al., 1993; Bicknese et al., 1994; De Carlos et al., 1994). In rodents, when thalamocortical afferents reach their appropriate cortical area, their main target cells, layer 4 neurons, are still being generated (Lund & Mustari, 1977; Kageyama & Robertson, 1993). After a brief waiting period in rodents (Lund & Mustari, 1977; Blakemore & Molnar, 1990; Catalano et al., 1991; Kageyama & Robertson, 1993) or a lengthy one in cats and primates (Rakic, 1977, 1988; Shatz & Luskin, 1986; Ghosh et al., 1992), thalamocortical axons extend collaterals into the cortical plate. Between E16 and early postnatal life, these axons progressively invade more superficial parts of the cortical plate, paralleling the deep-to-superficial gradient of maturation of this structure (Lund & Mustari, 1977). Once the mature pattern of connections between the thalamus and the cortex is established cells of the subplate die within the first two postnatal weeks in mice (Price, Aslam, Tasker & Gillies, submitted).

Fig. 3. Schematic diagram of the retinothalamic and pioneering thalamocortical pathways in embryonic mice. (A) Retinothalamic pathway. The diagram shows the position and pattern of decussation of retinal ganglion cell axons at the optic chiasm and their innervation of the lateral geniculate nucleus (LGN) of the thalamus and the superior colliculus. Axons from the nasal retina of each eye project across the optic chiasm, while axons from the temporal retina do not cross the chiasm and project to the LGN on their own side (adapted from Guillery et al., 1995). (B) Thalamocortical pathway. Dorsal regions of the cerebral hemispheres have been peeled away to reveal thalamic structures and the pioneering thalamocortical tract. Pioneering thalamocortical axons project deep into the tissue through the internal capsule which lies beneath the medial ganglionic eminence. Axons emerge from the internal capsule in the ventrolateral telencephalon and project through the subplate to their target cortical areas.

Figure 3


A
T
N
N
T
OPTIC NERVE
CHIASM
OPTIC TRACT
LGN
SC
B
cortical plate
DT
VT
thalamocortical tract
lateral ganglionic eminence
medial ganglionic eminence

1.3 MECHANISM OF AXON GUIDANCE

How does a growth cone on a retinal axon navigate to the thalamus and how does a growth cone on a thalamic axon navigate to the cortex?

Axons need a substrate to grow on. Pioneering retinothalamic and thalamocortical axons project through a terrain of cells (both neuronal and glial) and extracellular matrix (a complex association of extracellular glycoproteins organised into aggregates and polymers: Letourneau et al., 1994) to reach their target areas. Although none of the molecular guidance signals for axonal growth cones have been identified with certainty, it is likely that growth cones are guided by a combination of substrate-bound and diffusible factors distributed in their pathway (Heffner et al., 1990; Jessell & Dodd, 1990; Lander, 1990; Bixby & Harris, 1991; Mason & Godement, 1991; Yaginuma & Oppenheim, 1991; Gotz et al., 1992; Kuwada, 1992). These guidance cues can be adhesive or repulsive or act as both to different growth cones and may guide axons by mechanisms of differential adhesion (Letourneau, 1975; Gunderson, 1987), chemorepulsion (Kapfhammer et al., 1986; Kapfhammer & Raper, 1987; Walter et al., 1987a, 1987b; Patterson, 1988; Keynes & Cook, 1992; Baier & Bonhoeffer, 1992; Fitzgerald et al., 1993; Pini 1993, 1994) or chemotropism (Lumsden & Davies, 1983, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990). After synapse formation, connections may be refined and remodelled by a process that is more dependent on patterns of electrical activity (Goodman and Shatz, 1993).

1.3.1 Differential adhesion

The filopodia of growth cones can detect differences in the local adhesivity of substrates and turn to grow along the substrate of greatest adhesivity. Cell adhesion molecules on the surfaces of neurons mediate these adhesive interactions between neurons and other non-neuronal cells, including glia. (Rutishauser, 1984; Grumet et al., 1983). Integrins mediate the adhesive interactions between neurons and molecules in the extracellular matrix (Letourneau, 1994).

The most adhesive substrate is often earlier projecting axons. Neurites have been shown to grow along previously extended fiber bundles, a process called selective fasciculation (Nakai, 1960; Nakajima, 1965; Bray et al., 1980; Wessells et al., 1980). Selective fasciculation has been implicated in the formation of the thalamocortical and corticothalamic tracts in mouse. The 'Handshake Hypothesis' (Blakemore & Molnar, 1990; Molnar & Blakemore, 1991) suggests that pioneering thalamocortical and corticothalamic axons meet in the internal capsule, and fasciculate on each other to reach their appropriate target areas. Several neuronal tracing studies contradict this hypothesis. Double injections of DiI and DiA show that the corticothalamic and thalamocortical pathways are physically separate during development (Miller et al.,1993; Bicknese et al., 1994). Thalamocortical axons project in the subplate underlying the cortical plate at a distance from the corticothalamic tract, which projects in the white matter. Selective fasciculation is unlikely to occur between thalamocortical and corticothalamic axons but will almost certainly occur between pioneering and later projecting thalamocortical axons and between pioneering and later projecting corticothalamic axons. In addition, cells of the reticular nucleus, that lies just lateral to the thalamus, send axons into the thalamus as early as E13 (Mitrofanis & Baker, 1993). This projection is among the earliest the thalamus

receives, preceding afferents from the retina or cortex (Lund & Bunt, 1976; De Carlos & O'Leary, 1992; Molnar & Blakemore, 1990). One hypothesis is that pioneering thalamocortical axons may be guided to the internal capsule by selective fasciculation on reticulothalamic axons (Mitrofanis & Guillery, 1993).

1.3.2 Chemorepulsion

Growth cones respond not only to attractive cues, but also to contact-mediated repulsive signals. For example when chick ganglion cells from either temporal or nasal retina are confronted with stripes of membranes prepared from either anterior or posterior tectum, nasal retinal axons will grow on both types of membrane whereas temporal retinal axons only grow on membranes from anterior tectum (Walter et al., 1987a). The response of the temporal axons is based on avoidance of the posterior tectum, rather than attraction to the anterior tectum, since prior treatment of the posterior membranes with heat, protease, or phospholipase C renders them as good a substrate for temporal axons as anterior tectal membranes.

1.3.3 Chemotropism

Growth cones may be oriented by gradients of chemoattractant molecules that are released selectively by intermediate or final cellular targets. For example, trigeminal sensory neurons extend axons *in vitro* in response to a diffusible factor secreted by one of their normal target tissues, the maxillary epithelium (Lumsden & Davies, 1983, 1986). A similar *in vitro* analysis has suggested that the axons of corticospinal projection neurons extend collateral branches toward one of their final targets, the basilar pons, in response to a difffusible factor secreted by pontine tissue (Heffner et al., 1990). The most convincing evidence to date that chemotropism plays a role in

axon guidance is from studies where rat floor plate cells from the ventral midline of the spinal cord have been shown to promote the outgrowth of commissural axons from rat dorsal spinal cord explants and attract these axons in collagen gel cocultures (Tessier-Lavigne et al., 1988). Two membrane-associated proteins isolated from chick brain, netrin-1 and netrin-2, possess commissural axon outgrowth-promoting activity (Kennedy et al., 1994). Netrin-1 mRNA is expressed by floor plate cells, whereas netrin-2 mRNA is detected at lower levels in the ventral two-thirds of the spinal cord, but not the floor plate. Heterologous cells transfected with netrin-1 and netrin-2 secrete diffusible forms of the proteins and can attract commissural axons at a distance. These results show that netrin-1 is a chemotropic factor expressed by floor plate cells and suggest that the two netrin proteins guide commissural axons in the developing spinal cord (Kennedy et al., 1994). A role for the netrins has now been demonstrated *in vivo*. Both netrins and netrin receptors have been knocked out and there are major defects in axonal pathways that normally cross the midline (Tessier-Lavigne, unpublished). The sequences of netrins are highly related (50% homologous) to unc-6, a laminin-related protein, which plays a role in growth cone guidance in C. elegans (Hedgecock et al., 1990; Ishii et al., 1992). In unc-6 mutants, axons that normally travel circumferentially around the worm, from dorsal to ventral or ventral to dorsal, get misrouted (Hedgecock et al., 1990). These studies suggest that molecules involved in growth cone guidance are conserved across widely diverging species.

There is now increasing evidence for the existence of diffusible chemorepulsive molecules. The semaphorins are a family of transmembrane and secreted molecules that have been strongly implicated in repulsive neuronal growth cone guidance (Kolodkin et al., 1992; Luo et al., 1993; Messersmith et al., 1995; Kolodkin et al.,

1993; Luo et al., 1995). For example, chick collapsin, a secreted semaphorin, mediates sensory neuron growth cone collapse *in vitro* (Luo et al., 1993) and Semaphorin III, the rat homologue of collapsin (Coll-1), probably acts as a target-derived chemorepellant for specific populations of sensory afferents in the developing rat spinal cord (Messersmith et al., 1995; Puschel et al.,1995).

1.4 ARE RETINOTHALAMIC AND THALAMOCORTICAL AXONS GUIDED BY DIFFERENTIAL ADHESION?

If retinothalamic and thalamocortical axons are guided by differential adhesion we would expect to see restricted expression of cell adhesion molecules (CAMs) and extracellular matrix (ECM) molecules extending from the retina to the thalamus and then to the cortex at the time of axon tract formation. We would also expect to see restricted expression of CAMs or receptors for these ECM molecules (integrins) on the navigating axons. We would expect these molecules to promote neurite outgrowth *in vitro* and growth cones to show a preference for specific molecules in 'choice' *in vitro* experiments. Finally if these molecules are disrupted we would expect to see disruption of corresponding axon tracts.

Is there evidence for restricted spatiotemporal expression of CAMs and ECM molecules in the developing retinothalamic and thalamocortical tracts?

Immunoreactivity for the ECM molecules, tenascin (Bartsch et al., 1992) and laminin (Liesi & Silver, 1988) in mouse, and chondroitin-6-sulphate, chondroitin-4-sulphate and keratan sulphate in chick colocalize with retinal axons during development of the retinothalamic pathway (McAdams & McLoon, 1995). Cell

adhesion molecules, TAG-1/axonin-1 in mouse (Wolfer et al., 1994) and SC1/DMGRASP in chick (Pollerberg & Mack, 1994) are expressed in retinothalamic axons. Immunostaining for the ECM molecules CSPG, neurocan, phosphocan and cytotactin (tenascin) and the CAMs, L1, NCAM and TAG-1 reveal upregulation of expression in the neocortex (Yamamoto et al., 1990; Chung et al., 1991; Sheppard et al., 1991; Miller et al., 1992; Bicknese et al., 1994; Oohira et al., 1994; Wolfer et al., 1994) during thalamic innervation and cortico-cortical projection (Lund & Mustari, 1977; Coogan & Burkhalter, 1988; Catalano et al., 1991; Kageyama & Robertson, 1993).

Do retinothalamic and thalamocortical axons express receptors for these ECM molecules or CAMs?

In chick, laminin receptors (members of the β1-integrin family) have been identified on developing retinotectal axons (Hall et al., 1987; Cohen et al., 1989; de Curtis et al., 1991). Receptors for other ECM molecules on the growth cones of navigating retinothalamic and thalamocortical axons have yet to be identified. So far, NCAM, L1 and TAG-1 are the only CAMs to be identified on developing retinothalamic axons in chick (Lemmon & McLoon, 1986) and developing thalamocortical axons in rodents (Fushiki & Schachner, 1986; Wolfer et al., 1994).

Do these CAM and ECM molecules promote neurite outgrowth *in vitro*?

The ECM molecules laminin, chondroitin-6-sulphate, chondroitin-4-sulphate, keratan sulphate, CSPG, neurocan, phosphocan and cytotactin (tenascin) and CAMS TAG-1/axonin-1, SC1/DMGRASP, L1 and NCAM affect CNS neurite outgrowth *in vitro* (Laugenauer & Lemmon, 1987; Doherty et al., 1990, 1991; Furley et al., 1990;

Iijima et al.,1990 1991; Lochter et al., 1991; Snow & Letourneau, 1992; Friedlander et al., 1994; Wolfer et al., 1994; Pollerberg & Mack, 1994). In addition, Tuttle et al., (1995) show that maturation-dependent upregulation of growth-promoting molecules in the developing cortical plate controls thalamic and cortical neurite growth *in vitro*. In support of this Emerling & Lander (1994) have shown that thalamic cells placed on vibratome sections of brain exhibit an age-dependent increase in adhesion to cortical plate.

<u>If CAMs and ECM molecules are disrupted, are corresponding axon tracts also disrupted?</u>

The function of CAMs, integrins and ECM molecules in growth cone guidance has been directly tested in only a few cases either by antibody or enzymatic pertubation (eg Harrelson et al., 1988; Tang et al., 1992; Kolodkin et al., 1992) or genetic mutation (eg Elkins et al., 1990; Hedgecock et al., 1990; Grenningloh et al., 1991; McIntire et al., 1992). For example, if antibodies against NCAM are injected into the eye of a chick, the ordered progression of retinal ganglion cell axons into the optic nerve and through the optic tract to the tectum is disrupted (Thanos et al., 1984), suggesting a role for NCAM in axon fasciculation and in establishing or maintaining the ordered arrangement of axons in the chick visual pathway. Antibodies against the CAM L1 also result in disruption in axon fasciculation in the optic nerve of the chick (Chang et al., 1987).

However, genetically engineered mice lacking the tenascin gene develop normally without obvious defects (Saga et al., 1992) implying that tenascin is not essential for normal development (Erickson, 1993). It is possible that in mutant mice the essential functions of tenascin, if any, are replaced by another member of the tenascin family.

These studies suggest that although CAMs and ECM molecules certainly play a role in growth cone guidance other mechanisms are also involved since axons are still largely able to find their targets in the few studies where several key CAMs and ECM molecules have been disrupted.

1.5 DO CORTEX-DERIVED GROWTH FACTORS PROMOTE AND/OR INFLUENCE THE GROWTH OF THALAMIC AXONS TO THE CORTEX?

Although there is increasing evidence of a role for CAMs and ECM molecules in guiding thalamic axons through the neocortex, the question remains as to how pioneering thalamic axons are initiated to grow and guided out of the thalamus. It is possible that they are initiated and/or guided by a diffusible factor released by the cortex (Molnar & Blakemore, 1995). Evidence from other systems reflects a role for target-derived diffusible factors in axon growth and guidance (see chemotropism section above).

In recent years, interactions between the developing cortex and thalamus have been studied in organotypic and dissociated cocultures (Yamamoto et al., 1989, 1992; Bolz et al., 1990, 1992; Hisanaga & Sharp, 1990; Molnar & Blakemore, 1991; Novak & Bolz, 1993; Emerling & Lander, 1994; Tuttle et al., 1995). Many of these studies investigated the processes by which thalamic axons grow through the neocortex and recognize their target cells in layer 4. The possibility of trophic and growth-promoting interactions between these structures *in vitro* has received less attention. Some experiments have failed to demonstrate such interactions, but this may have been because serum was used in the culture medium (Bolz et al., 1992). Serum contains many growth factors that may mask growth-promoting or trophic

interactions (Esber et al., 1973; Honn et al., 1975; Annis et al., 1990). Other studies, using serum-free medium, have indicated that postnatal cortical slices do release as yet unidentified diffusible factors that enhance neurite outgrowth from embryonic thalamic explants (Rennie et al., 1994; Lotto & Price, 1994). The evidence for a release of cortex-derived growth factors *in vitro* is consistent with *in vivo* observations. For example, thalamocortical and corticothalamic neurons can be rescued from axotomy-induced death by the addition of a macromolecular fraction of medium preconditioned with cultured neocortical slices to the sites of the lesions (Cunningham et al., 1987; Haun and Cunningham, 1993). In addition the infusion of high levels of nerve growth factor (NGF) into the visual cortex can prevent the deleterious effects of visual deprivation on thalamocortical afferents (Domenici et al., 1991, 1992; Maffei et al., 1992). Finally, identified growth factors, such as fibroblast growth factor (FGF), NGF and brain-derived neurotrophic factor (BDNF), are produced by pre- and postnatal cortical cells (Large et al., 1986; Whittemore et al., 1986; Maisonpierre et al., 1990; Castren et al., 1992; Matsuyama et al., 1992).

Do *embryonic* cortical slices release these or other diffusible growth factors and enhance neurite outgrowth from embryonic thalamic explants?

I first examined the hypothesis that the *embryonic* cortex releases a diffusible factor that initiates, accelerates and/or guides thalamic neurite outgrowth using an *in vitro* coculture system (Chapter 2).

1.6 BORDERS OF GENE EXPRESSION MAY GUIDE AXONS

It has also been suggested that cues generated around the borders between domains of regulatory gene expression may guide developing axons to their correct targets (Wilson et al., 1993). For example, during development of the chick hindbrain eight transient segmental swellings in the neuroepithelium called rhombomeres are visible (Lumsden & Keynes, 1989, and see Fig.1C). Labelling for neurofilament reveals that reticular axons are associated with the boundaries between the rhombomeres (Lumsden & Keynes, 1989; Trevarrow et al., 1990). Several transcription factor-encoding genes (*Hox* family and *Krox-20*), a receptor tyrosine kinase (*Sek*) and a member of the *Wnt* family of secreted factors (*Wnt-8c*) have borders of expression that coincide with the rhombomere boundaries and hence the developing tracts (see review by Wilson et al., 1993). In transplantation experiments where an ectopic boundary is formed by apposition of different rhombomeres that are not normally contiguous, axons appear to grow along the new boundary. When transplants fail to form a boundary, axons do not accumulate in the region where the transplanted and host tissue meet (Kuratani & Eichele, 1993).

In developing zebrafish embryos, the early axonal tracts of the postoptic (TPOC) and posterior commissures (TPC) project along the border (within a few cell diameters-Macdonald & Wilson, unpublished observations) of Pax-6 gene expression (Krauss et al., 1991). It had been suggested that the TPOC guides later-growing retinal axons to the optic tectum. However, if retinal axons are forced to grow into the diencephalon before the TPOC has arisen, they are able to navigate correctly to the optic tectum (Holt & Harris, 1993). This suggests they are guided by signals in the

neuroepithelial cells that lie along the pathway; this pathway coincides with the borders between transcription factor gene expression.

There is evidence that transcription factors may directly regulate the expression of molecules that could guide developing axons. For example, in vitro binding studies suggest that neural cell adhesion molcule (NCAM), a molecule implicated in axon pathfinding, is regulated by *Hox 2.5* and *2.4* gene proteins (Jones et al., 1992a) and the ECM molecule, cytotactin is regulated by the homeobox-containing gene *Evx-1* (Jones et al., 1992b). In Drosophila, the *Ubx* gene directly regulates tissue-specific expression of connectin, a cell adhesion molecule (Gould & White, 1992).

At least four mechanisms have been proposed to explain how developing axons might derive guidance information from the borders between domains of regulatory gene expression (Wilson et al., 1993).

1. If regulatory genes control the expression of cell adhesion molecules or extracellular matrix molecules, then they may pattern the tissue into areas with different adhesive properties. It is possible that growth cones detect the differences in adhesive properties between adjacent populations of cells and preferentially grow along the border between them (Burmeister & Goldberg, 1988).
2. Cells at the boundary may express unique guidance cues, different from those in adjacent domains, that provide a substrate pathway for navigating axons. For example, the border cells of segments in insects have physical and molecular properties diifferent from other cells in the same segment (Nubler-Jung, 1979). In addition, the rhombomere boundaries of the chick hindbrain express high levels of

laminin and peanut-agglutin-binding glycoproteins, and low levels of NCAM compared to cells within the rhombomeres (Lumsden & Keynes, 1989; Layer & Alber, 1990).

3. Boundary cells may influence growth cone guidance by secreting diffusible chemotropic molecules which navigating growth cones detect and grow towards. Alternatively boundary cells may indirectly regulate the expression of CAMs in adjacent cells through interactions with other genes. In both cases growth cones of pioneering axons may respond to the graded distribution of the cue but their axon trajectories would not necessarily be parallel to the border of gene expression. For example the homeobox gene engrailed (*en*) is expressed in a graded manner by cells of the chick tectum (Gardner & Barald, 1991; Martinez et al., 1991) that correlates with, and precedes the graded expression of a 33kDa cell surface glycoprotein (Itasaki et al., 1991; Itasaki & Nakamura, 1992). *In vitro* studies show that this molecule can influence retinal growth cone direction (Baier & Bonhoeffer, 1992).

4. Boundaries of regulatory gene expression may indirectly influence growth cone guidance by directly or indirectly specifying cell phenotype. For example a regulatory gene may directly, or indirectly through secretion of a diffusible morphogen, (Ingham & Martinez Arias, 1992; Nubler-Jung, 1979), instruct cells to differentiate into neurons with specific molecular properties that enable their axons to select correct pathways. In situ hybridization and immunocytochemistry studies have shown that the earliest neurons in the zebrafish forebrain differentiate along boundaries of regulatory gene expression (Macdonald et al., 1994). For example, the nuclei of the tract of the posterior commissure (nTPOC) and of the

medial longitudinal fasciculus (nMLF) both develop and extend axons along the ventral boundary of *Pax-6* and *rtk-1* expression.

Various studies have shown that morphological constrictions in the tissue (Fig.1C), and underlying domains of regulatory gene expression (including *Pax-6, Dlx-2* and *Wnt-3*) pattern the neuroepithelium of the developing forebrain into a regional structure (Puelles & Rubenstein, 1993; Rubenstein & Puelles, 1994; Bulfone et al., 1993; Figdor & Stern, 1993; Salinas & Nusse, 1992; Zimmer & Zimmer, 1992 and Stoykova & Gruss, 1994). The borders of many of these domains of gene expression may guide developing axons in the forebrain (Salinas & Nusse, 1992; Bulfone et al., 1993). For example the caudal boundary of *Pax-6* expression in the diencephalon may coincide with the tract of the posterior commissure in the mouse (Walther & Gruss, 1991).

At E15, *Pax-6*, a homeobox gene, is expressed in the murine ventral thalamus in an overlapping domain with another homeobox gene, *Dlx-2*. Both genes form sharp borders of expression with *Wnt-3*, a secreted factor, in the dorsal thalamus at the zona limitans intrathalamica. *Dlx-2* is also expressed in the ventral diencephalon in the region of the optic stalk. One of my main aims was to examine the hypothesis that the developing retinothalamic and thalamocortical tracts are guided by borders of *Pax-6* and *Dlx-2* gene expression in the forebrain. I used the neuronal tracer DiI to label the axon tracts and whole-mount in situ hybridization to study the expression of *Pax-6* and *Dlx-2* (Chapter 4).

If a boundary of gene expression determines the location of a specific tract then certain predictions can be made. For example, ablation of the gene should result in disruption of the associated tract, and ectopic expression of the gene should result

in rerouting of the axon tract along the new boundary of gene expression. Several methods have been employed to disrupt gene expression. These include injection of antisense DNA/RNA of the gene of interest (Christine Holt's personal communication); injection of antibodies raised against the protein product of the gene of interest (Krauss et al., 1991) and the use of mutant species where the gene of interest is altered or completely absent.

In this study I used the *Pax-6* mutant mouse Small eye (Sey), in which a point mutation in the *Pax-6* locus results in a dysfunctional *PAX-6* protein (Hill et al., 1991), to test the hypothesis that disruption of *Pax-6* disrupts axon tract formation. I first characterized embryonic diencephalic anatomy (morphological constrictions in the tissue and cell densities) and expression of regulatory genes (*Dlx-2, Wnt-3* and *Pax-6* itself) in Sey embryos (Chapter 3). Having characterized the Sey forebrain I then studied the development of the retinothalamic tract in Sey brains (Chapter 5).

CHAPTER 2

DO CORTEX-DERIVED FACTORS PROMOTE AND/OR INFLUENCE THE GROWTH OF THALAMIC AXONS TO THE CORTEX?

2.1 ABSTRACT

In this chapter I investigated whether embryonic cortex-derived diffusible factors initiate and/or influence the growth of axons from embryonic thalamus *in vitro*. Slices of embryonic day 15 (E15) thalamus were cultured either alone or with embryonic or postnatal anterior or posterior cortex and the effects on neurite density and neurite length from dorsal and ventral thalamus were measured. The results indicate that explants of both anterior or posterior embryonic cortex do not stimulate increased outgrowth from either dorsal or ventral thalamus, whereas postnatal cortex does stimulate significantly more outgrowth from dorsal and ventral thalamus compared to control levels. It is therefore unlikely that an embryonic cortex-derived diffusible factor initiates neurite ougrowth from the thalamus *in vivo*. Neurite length measurements suggest the embryonic cortex releases diffusible factors that inhibit the growth of most embryonic thalamic neurites whereas postnatal cortex releases factors that stimulate the growth of most thalamic neurites. The *in vivo* relevance of these *in vitro* findings must be treated with caution but it is possible that diffusible growth inhibitory and stimulatory molecules secreted by the embryonic and postnatal cortex respectively may influence the growth of thalamic axons once they have reached the cortical plate.

2.2 INTRODUCTION

Recent *in vitro* coculture experiments have provided useful insights into the development of connections between the thalamus and the cortex. Many of these coculture studies have focused on the growth of thalamic axons *into* the visual cortex and their recognition of target cells in layer 4 (Yamamoto et al., 1989, 1992; Bolz et al., 1990, 1992; Hisanaga & Sharp, 1990; Molnar & Blakemore, 1991; Novak & Bolz, 1993; Emerling & Lander, 1994; Tuttle et al., 1995). Coculture techniques have also been used to investigate trophic and growth-promoting interactions between these structures. For example, growth factors released from cortical cells enhance the survival of dissociated postnatal thalamic cells *in vitro* (Hisanaga & Sharp 1990). In addition, developing neocortical explants promote the growth of neurites from embryonic thalamic explants cultured in serum-free medium (Rennie et al., 1994; Lotto & Price, 1994, 1995). Conditioned medium and conditioned substrate experiments have shown that these interactions are mediated by diffusible growth factors (Rennie et al., 1994; Lotto & Price, 1994). The molecule involved has been partially characterized and is between 10-30kd in size. Cross-species co-cultures have indicated that it/they are highly conserved in divergent mammalian species (Lotto & Price,1994). These cortex-derived diffusible growth factors (CDGFs) are thought to play a role in early postnatal growth and refinement of neocortical connections. Do CDGFs play a role in prenatal thalamocortical development?

I tested the hypothesis that diffusible factors released from developing neocortex initiate, accelerate and/or guide the prenatal growth of axons from the thalamus to the

neocortex. Embryonic day 15 (E15) thalamic slices were cultured in liquid medium either alone or with embryonic or postnatal slices of anterior or posterior cortex. E15 thalamic explants were chosen because the birth of the posterior thalamus is completed between E14 and E15 (Rennie et al., 1992) and the development of thalamocortical afferents is well-advanced by E16 (Lotto & Price, 1995). Thus, if diffusible factors released by the neocortex initiate, accelerate and/or guide the growth of thalamocortical afferents *in vivo*, such effects should be detectable on E15 thalamic tissue *in vitro*.

2.3. MATERIALS AND METHODS

BALB/c mice from an isolated breeding colony were mated overnight. Plugged females were separated from the male the following day, embryonic day 1 (E1). On E15 (gestation in the mouse is 20-21 days) embryos were removed from anaesthetized (0.3 ml urethane in isotonic saline i.p.) mothers and their brains quickly dissected in oxygenated ice cold Earle's balanced salt solution (EBSS). The tissue was sliced coronally at 350 μm with a McIlwain tissue chopper. Slices containing both dorsal and ventral regions of thalamus were microdissected and placed on collagen-coated membranes in preincubated (for at least 12 hours at 37°C, in 5%CO_2) chemically defined serum-free medium. Explants were 'nicked' in such a way to easily identify the dorsoventral orientation.

Cortical explants were isolated from mice ranging from E16 to postnatal day 7 (P7), with the day of birth deemed P0. Embryonic brains were dissected in ice cold EBSS, sliced coronally at 350μm and slices from both anterior and posterior cortex were microdisssected and stored separately in preincubated culture medium. Mice aged

between P0 and P7 were deeply anaesthetized by inducing hypothermia (animals were surrounded with ice for 7 minutes). The brains were then quickly removed and sliced coronally at 350μm. Slices from both anterior and posterior cortex were collected and stored separately as described above.

Identification of dorsal and ventral thalamus.

Coronal sections of tissue containing dorsal and ventral thalamus were identified by studying (i) the anatomical features of the slices and comparing them to an atlas of the embryonic mouse brain and (ii) the expression pattern of the homeobox-containing gene *Dlx-2* which is expressed in the ventral but not the dorsal thalamus (Bulfone et al., 1993) using in situ hybridization on paraffin-embedded sectioned tissue with digoxigenin-labelled riboprobes (Appendix 1).

Culture method.

Two slices of E15 thalamus and two slices of either anterior or posterior cortex ranging from E16-P7 were plated at a distance from one another (Fig.1.) on collagen-treated membranes (Transwell-COL; Costar U.K., 3μm pores) suspended in chemically defined serum-free medium (Romijn et al., 1984). The serum-free medium was essential since serum contains many growth factors in variable amounts that may prevent the detection of factors released by the cultured tissues. 700μl of culture medium was placed in the lower chamber of each culture-well and 200-300μl of the same mediun was placed in the upper chamber. This was sufficient to just cover the explants when they were plated. In some wells either 2 or 4 thalamic explants were cultured alone (ie with no other tissue in the well). Previous studies show that cultured brain explants maintain their characteristic organisation (Gahwiler,

Fig. 1. Schematic diagram of a typical coculture. The parallel horizontal lines indicate the orientation of the collagen grooves on the filter. Two reference points on the thalamic slice were used for quantification: D (midway between the top and middle of the thalamic slice), and V (midway between the middle and bottom of the thalamic slice). Opposite each point I counted the number of neurites that fell within a 500μm window at the edge of the tissue (short vertical lines), and the length of the ten longest neurites in the window.

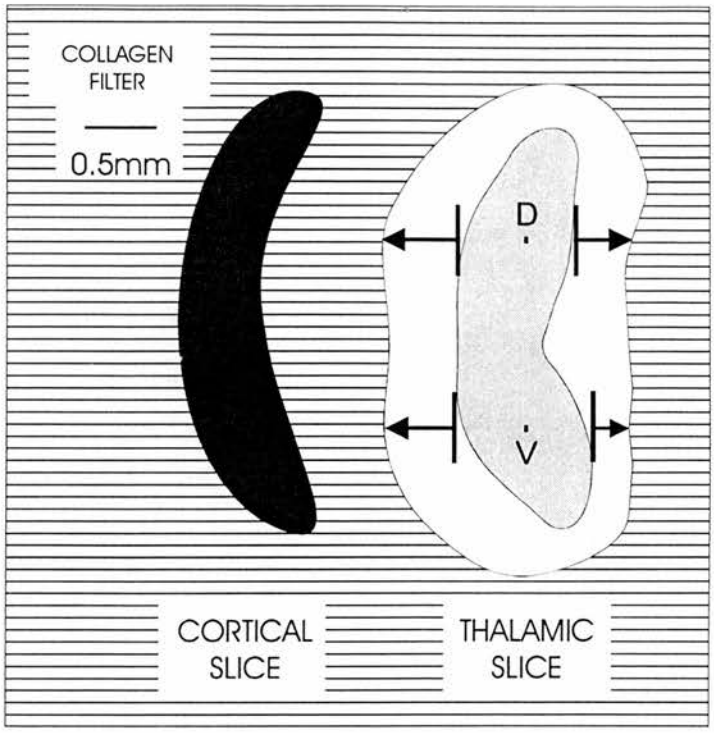


Figure 1

1988; Yamamoto et al., 1989, 1992; Bolz et al., 1990, 1992; Molnar & Blakemore, 1991). In all experiments the volumes of cortical explants were kept constant as were the volumes of thalamic explants. The explants were cultured for three days at 37°C in 5% CO_2 and then fixed in 4% paraformaldehyde in 0.1% phosphate buffer for a minimum of two hours.

Analysis

During the manufacture of Transwell-COL membranes, most of the collagen fibres orient themselves in one direction and, as a result, they generate parallel equally spaced narrow grooves (35μm wide). The explants were plated on the collagen membranes with their dorso-ventral axis perpendicular to the direction of the grooves. Individual neurites and fascicles tended to grow along the grooves which facilitated accurate measurements of neurite length and density. The dorsoventral length of each explant was measured and outgrowth was quantified from 4 regions whose centres were opposite a point (1) halfway between the midpoint and the dorsal edge of the explant and (2) halfway between the midpoint and the ventral edge of the explant (Figure 1). Density and length of neurite outgrowth were measured under phase-contrast microscopy (x20 objective). For each explant, neurite density was calculated by counting the number of neurites that fell within the four 500μm windows. When fascicles were encountered and could not be resolved into individual neurites, the number of neurites within a fascicle was estimated by comparing the width of the fascicle to the average width of a single neurite. To quantify neurite length the 10 longest neurites in each window were measured. Data on neurite density and neurite length from dorsal and ventral thalamus were kept separate. This was to test for any specificity of interaction between ventral thalamus and anterior

cortex and between dorsal thalamus and posterior cortex (the target regions of these thalamic areas *in vivo*).

In all wells the cortical and thalamic explants were placed at a distance to prevent neurite contact. Explants where neurites from cortex and thalamus had touched each other were not quantified. In these cases the possibility that fibres may have received some contact-mediated trophic support could not be excluded. The results were tested for statistical significance using a Students-T test.

Crystals of the carbocyanine dyes, DiI and DiA, were placed at each end of every explant to ensure that outgrowth emerging from the dorsal thalamus originated in that area, and that the same was true for the ventral thalamus.

2.4 RESULTS

The results were from 64 cultures. In situ hybridization with digoxigenin-labelled antisense *Dlx-2* riboprobes confirmed that slices taken for culture contained unlabelled dorsal and labelled ventral thalamus (Fig 2). Figure 3 shows the mean neurite density from dorsal and ventral thalamus when cultured alone or with anterior (Fig.3A) or posterior (Fig.3B) cortex. The results indicate that the anterior *embryonic* (E16-E20) cortex did not stimulate an increased density of outgrowth from dorsal or ventral thalamus, nor did the posterior *embryonic* cortex stimulate an increased density of outgrowth from dorsal or ventral thalamus compared to thalamus cultured alone. On the other hand, *postnatal* day 4 (P4) anterior cortex stimulated denser outgrowth from dorsal thalamus ($p<0.003$), and P2 and

Fig. 2. *Dlx-2* expression in the diencephalon of E14.5 embryos revealed by RNA in situ hybridization on coronal sections. The broken line shows the location of the zli. (A) *Dlx-2* expression in the mge and in ventricular cells of the ventral but not the dorsal thalamus. (B) Detail of A showing *Dlx-2* expression in the ventral but not dorsal thalamus. (C) Control showing no *Dlx-2* expression in a section hybridized with a *Dlx-2* sense riboprobe. zli, zona limitans intrathalamica; mge, medial ganglionic eminence. Scale bars=100μm

Figure 2

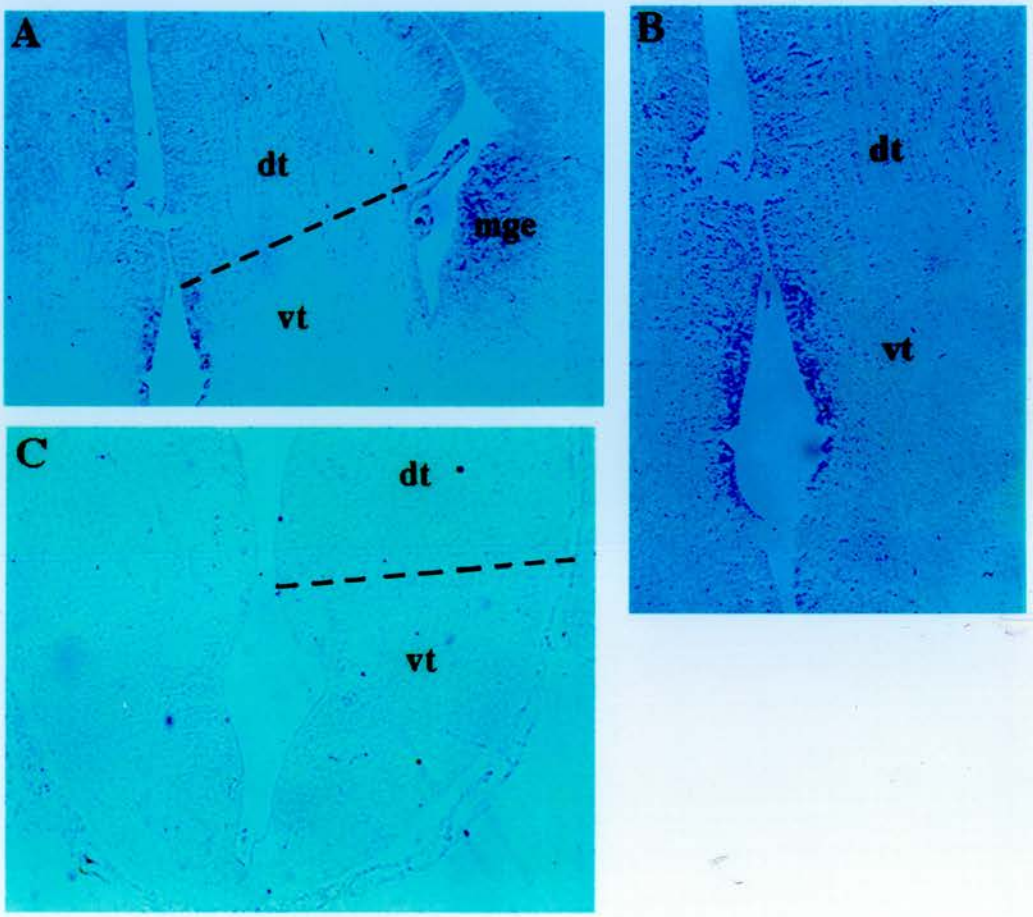
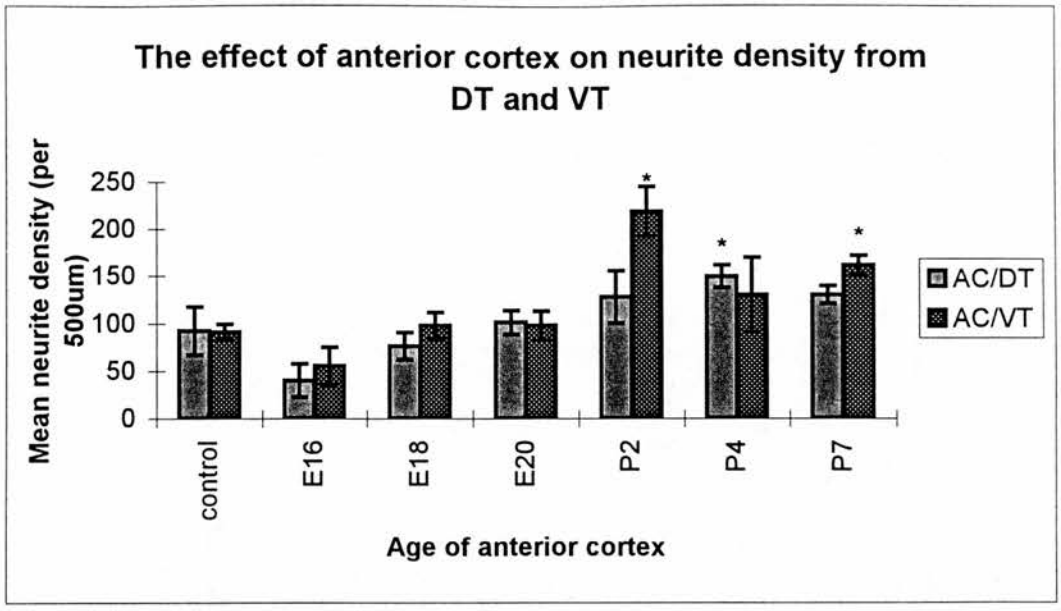


Fig. 3. Histograms show the density of neurite outgrowth from E14.5 thalamic slices cultured alone or with embryonic or postnatal cortical slices (each value is a mean +/- sem). Filled bars are data on outgrowth from the dorsal thalamus, cultured alone or with anterior cortex (A) or posterior cortex (B). Stippled bars are data on outgrowth from the ventral thalamus cultured alone or with anterior cortex (A) or posterior cortex (B). The only significant effects (indicated by *) were with postnatal cortex. Interestingly the anterior cortex stimulated significant outgrowth from the ventral thalamus a day or two earlier than the posterior cortex stimulated significant outgrowth from the dorsal thalamus.

Figure 3

A


The effect of anterior cortex on neurite density from DT and VT
Mean neurite density (per 500um)
250
200
150
100
50
0
control
E16
E18
E20
P2
P4
P7
Age of anterior cortex
AC/DT
AC/VT

B

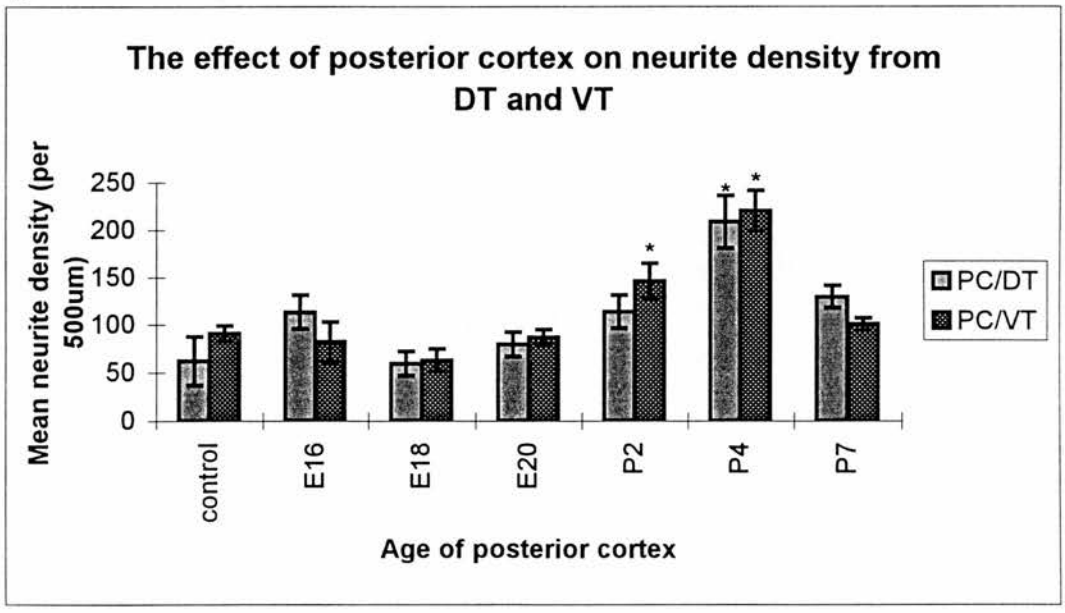
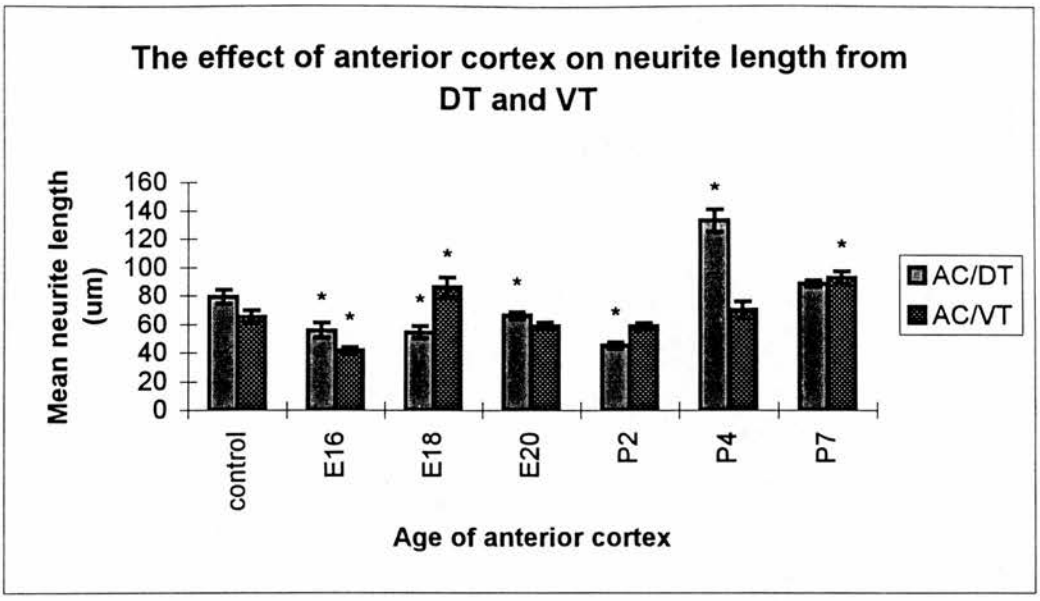

The effect of posterior cortex on neurite density from DT and VT
Mean neurite density (per 500um)
250
200
150
100
50
0
control
E16
E18
E20
P2
P4
P7
Age of posterior cortex
PC/DT
PC/VT

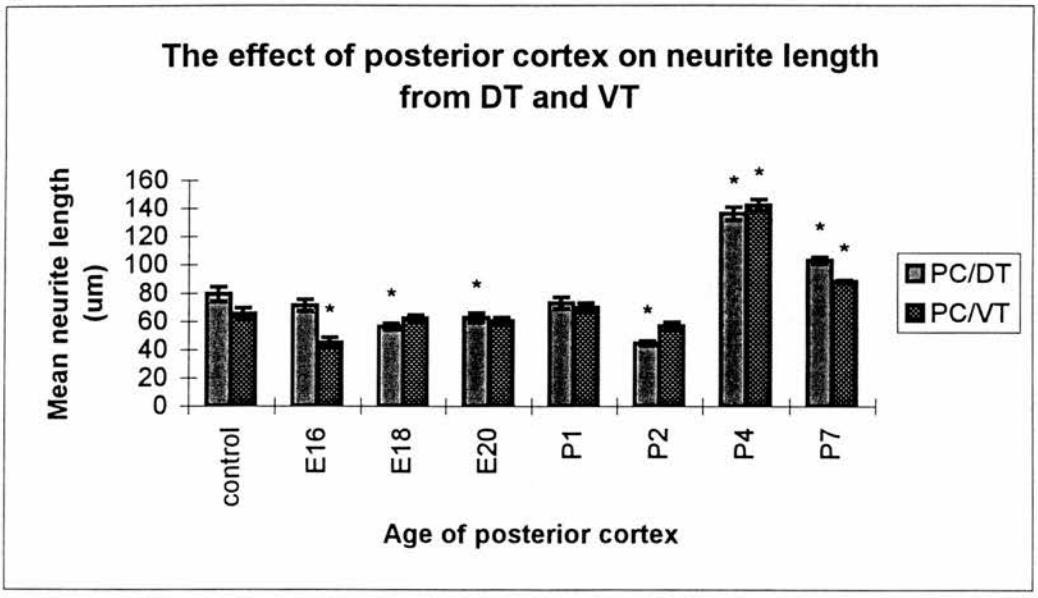
Fig. 4. Histograms show the average length of neurites from E14.5 thalamic slices cultured either alone or with embryonic or postnatal cortical slices (each value is a mean +/- sem). Filled bars are data on neurite length from the dorsal thalamus cultured alone, or with anterior (A) or posterior (B) cortex. Stippled bars are data on neurite length from ventral thalamus cultured alone or with anterior (A) or posterior (B) cortex. (* marks significant effects).

Figure 4

A


The effect of anterior cortex on neurite length from DT and VT
Mean neurite length (um)
160
140
120
100
80
60
40
20
0
control
E16
E18
E20
P2
P4
P7
AC/DT
AC/VT
Age of anterior cortex

B


The effect of posterior cortex on neurite length from DT and VT
Mean neurite length (um)
160
140
120
100
80
60
40
20
0
control
E16
E18
E20
P1
P2
P4
P7
PC/DT
PC/VT
Age of posterior cortex

P7 anterior cortex stimulated denser outgrowth from ventral thalamus compared to control levels ($p<0.002$) (Fig. 3A). P4 posterior cortex stimulated denser outgrowth from dorsal thalamus ($p<0.004$), and P2 and P4 posterior cortex stimulated denser outgrowth from ventral thalamus compared to control levels ($p<0.02$ and $p<0.002$ respectively) (Fig. 3B). The results also showed that E16 posterior cortex stimulated denser outgrowth from dorsal thalamus than E16 anterior cortex ($p<0.01$), and P7 anterior cortex stimulated denser outgrowth from ventral thalamus than P7 posterior cortex ($p<0.006$). Figure 4 shows the mean neurite length from explants of dorsal and ventral thalamus when cultured alone or with anterior (Fig.4A) or posterior (Fig.4B) cortex. The results show there was a reduction in mean neurite length from dorsal thalamus when cultured with embryonic (E16, $p<0.002$; E18, $p<0.0006$; E20, $p<0.03$), and P2 anterior cortex ($p<4.7\times10^{-7}$). There was a reduction in mean neurite length from ventral thalamus when cultured with E16 anterior cortex ($p<6.46\times10^{-6}$) and an increase in mean neurite length when cultured with E18 anterior cortex ($p<0.012$). E20, P2 and P4 anterior cortex had no effect on mean neurite length from ventral thalamus above control levels. There was an increase in mean neurite length from dorsal and ventral thalamus when cultured with P4 ($p<2.11\times10^{-7}$), and P7 ($p<1.27\times10^{-5}$) anterior cortex respectively. The mean neurite length from dorsal thalamus was significantly reduced when cultured with explants of E18 ($p<0.0002$), E20 ($p<0.008$), and P2 ($p<4.02\times10^{-7}$) posterior cortex but was no different from control levels when cultured with E16, and P1 posterior cortex. There was a reduction in mean neurite length from ventral thalamus when cultured with E16 posterior cortex ($p<0.0003$), but no difference when cultured with explants of E18, E20, P1 and P2 posterior cortex. Both P4 and P7 explants of posterior cortex stimulated increased neurite length from dorsal and ventral thalamus ($p<0.0006$).

In summary, the mean neurite length from dorsal and ventral thalamus was reduced or no different when cultured with E16-P2 anterior or posterior cortex (apart from a very small but significant increase in neurite length from ventral thalamus cultured with E18 anterior cortex), and increased or no different when cultured with P4 and P7 anterior or posterior cortex.

Were there any differences in mean neurite length from dorsal or ventral thalamus when cultured with anterior or posterior cortex?

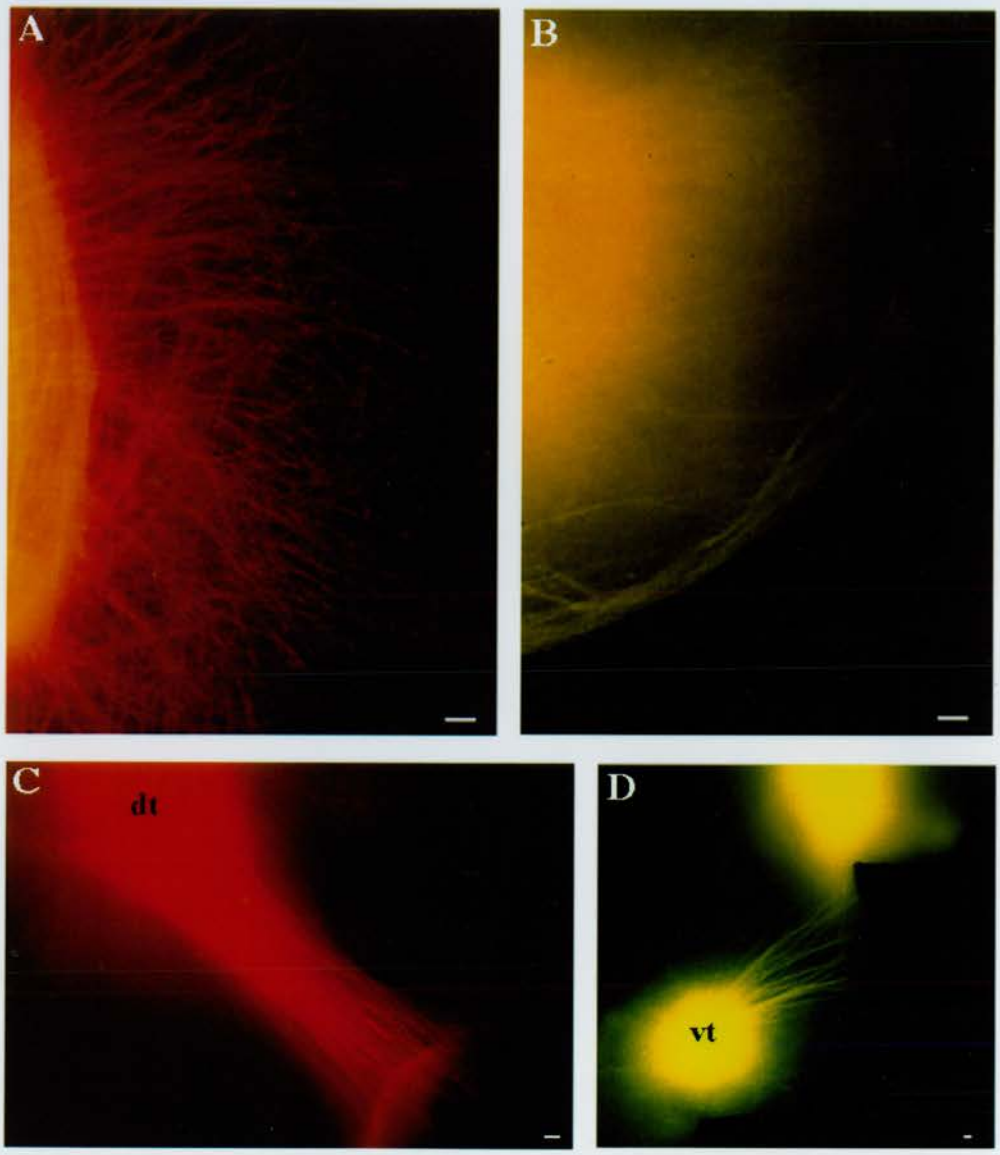
The mean neurite length from dorsal thalamus was significantly greater when cultured with E16 posterior cortex compared to E16 anterior cortex ($p<0.01$) and with P7 posterior cortex compared to P7 anterior cortex ($p<1.25E-5$). At all other ages there was no difference in the mean neurite length from dorsal thalamus when cultured with anterior or posterior cortex.

The mean neurite length from ventral thalamus was significantly greater when cultured with E18 anterior cortex compared to E18 posterior cortex ($p<0.002$) and with P4 posterior cortex compared to P4 anterior cortex ($p<8.09E-17$). At all other ages there was no significant difference in mean neurite length when cultured with anterior or posterior cortex.

Crystals of the carbocyanine dyes, DiI and DiA, were placed at each end of every explant, and the labelling they produced confirmed that neurite outgrowth emerging from the dorsal thalamus *originated* in that area (Fig. 5A) and that the same was true for the ventral thalamus (Fig. 5B). In some cases the DiI labelled tracts projected to the lateral surface of the tissue. This is the direction they would normally take *in vivo* (Fig. 5C and 5D).

Fig. 5. Slices of E14.5 thalamus after three days in culture. (A) DiI-labelled neurites emerging from the dorsal thalamus. (B) DiA-labelled neurites projecting from a crystal of DiA in the ventral thalamus to the ventral edge of the slice. (C) DiI-labelled axons that have fasciculated and grown towards the lateral edge of the thalamic slice. (D) DiA labelled axons projecting from a crystal of DiA in the ventral thalamus to the lateral edge of the thalamic slice. Scale bars=100μm

Figure 5



2.5 DISCUSSION

The first set of results concern the effects of embryonic and postnatal cortex on neurite outgrowth (density) from slices of embryonic thalamus.

The results indicate that explants of both anterior and posterior *embryonic* cortex do not stimulate increased outgrowth from either dorsal or ventral thalamus, whereas *postnatal* cortex does stimulate significantly more outgrowth from dorsal and ventral thalamus compared to control levels. This *in vitro* result contradicts the findings of Molnar and Blakemore (1995) who state that explants of rat prenatal cortex stimulate increased neurite outgrowth from embryonic thalamic explants. Their results remain unpublished, and it is therefore difficult to compare the two opposing findings. The results in this experiment indicate it is unlikely that an embryonic cortex-derived diffusible factor initiates neurite outgrowth from the thalamus *in vivo.* This is perhaps not surprising as the route a diffusible factor would have to take to affect thalamic cells *in vivo* is convoluted (see Fig.3B in Chapter 1). It is possible that thalamic cells are stimulated to grow by local cues in the thalamus. The results showed considerable neurite outgrowth from thalamic slices cultured alone (Figs. 3 and 4), suggesting that thalamic cells are stimulated to grow by mechanisms intrinsic to the thalamus. To ensure that these cells had not received signals to grow from regions other than the thalamus before the tissue was plated, it will be important to culture younger thalamic explants alone and assess the amount of neurite outgrowth. Intrinsic cues within the thalamus may not only stimulate thalamic cells to grow but may also guide thalamic axons out of the thalamus. When crystals of DiI and DiA were placed into dorsal and ventral thalamus respectively, axons were labelled

projecting to the lateral surface of the tissue in the direction they would normally take *in vivo* (Fig 5C & 5D) (see Chapter 3).

It is also possible that diffusible factors, released from intermediary targets or regions through which the developing thalamocortical tract projects, may initiate and/or guide thalamic axons to the internal capsule. Candidate structures include (i) the perireticular nucleus, a population of cells surrounding the internal capsule, (ii) the reticular nucleus, which lies just lateral to the thalamus, and (iii) the medial ganglionic eminence (MGE). For example, DiI-labelled early thalamocortical axons turn through a sharp angle when they reach the perireticular cells on their way to the cortical subplate (Mitrofanis & Guillery, 1993). It is possible that they are deviated by chemorepulsive cues on, or secreted by, the perireticular cells. It would be interesting to coculture slices of thalamus with explants of perireticular nuclues, reticular nucleus or MGE to investigate the possibility of diffusible growth-promoting and chemotropic interactions between the thalamus and these structures.

The second set of results concern the effects of embryonic and postnatal cortex on mean neurite length from slices of thalamus. The mean neurite length from dorsal thalamus was significantly reduced in the presence of embryonic (E16-E20) and P2 anterior cortex and significantly increased when cultured with P4 anterior cortex. In a similar vein the mean neurite length from ventral thalamus was significantly reduced when cultured with E16 posterior cortex and significantly increased when cultured with P4 and P7 posterior cortex.

A number of studies show that extrinsic cues including CAMs, ECM molecules, diffusible chemorepulsive and chemotropic molecules, neurotrophins, neurotransmitters and certain ions, promote or inhibit neurite outgrowth *in vitro*

(Laugenauer & Lemmon, 1987; Mattson et al., 1988a,b,c; McCobb et al., 1988a,b; McCobb & Kater, 1988; Doherty et al., 1990; Furley et al., 1990; Iijima et al., 1991; Lochter et al., 1991; Snow & Letourneau, 1992; Friedlander et al., 1994; Wolfer et al., 1994; Pollerberg & Mack, 1994; Lotto & Price, 1995). The effects are mediated by intracellular 2nd messengers including calcium, cyclic-AMP and protein kinase C, which regulate the polymerization or depolymerization of actin microfilaments and tubulin microtubules in the filopodia, lamellapodia and within the body of growth cones. For example, calcium influx into growth cones mediates the growth inhibiting actions of several neurotransmitters (Mattson et al., 1988d,e; McCobb et al., 1988c; McCobb & Kater, 1988) and the stimulatory effects of several CAMs including NCAM (Doherty, 1991).

It is possible that CAMs, ECM molecules, chemorepulsive or chemotropic molecules, neurotransmitters and ions released from dying or viable embryonic cortex may directly or indirectly influence intracellular 2nd messengers in thalamic cells. Although I did not assess tissue viability in the coculture system with vital stains such as acridine orange or propidium iodide, there was no disintegration of cortical tissue, an indicator of cell death. In addition, previous studies have demonstrated that cocultures of embryonic and postnatal cortex and thalamus remain healthy for up to 3 weeks in serum-free medium (Gahwiler, 1981; Blakemore & Molnar, 1990). It therefore seems unlikely that any reduction or increase in mean neurite length from dorsal or ventral thalamus is due to death of the cortex and subsequent release of molecules that inhibit or promote neurite growth. An acridine orange study is essential to confirm this.

Assuming that the cortical slices are healthy it is possible that thalamic neurite length is inhibited by the release of ECM molecules, diffusible chemorepulsive molecules

or inhibitory neurotransmitters from the cortex. Candidate ECM molecules include tenascin and neurocan. Both molecules are expressed in embryonic subplate and cortical plate (Miller et al., 1992; Oohira et al., 1994; Tuttle et al., 1995; Sheppard et al., 1994), and both exist in soluble forms which have growth inhibiting properties *in vitro* (Friedlander et al., 1994; Lochter et al., 1991). Candidate neurotransmitters include glutamate and nitric oxide. Both are found in the embryonic cortex (Terada et al., 1996) and both have growth inhibiting properties *in vitro* (Dawson et al., 1991). In addition nitic oxide synthase (NOS), the enzyme responsible for production of nitric oxide (Bredt & Synder, 1990; Dawson et al., 1991; Hope et al., 1991), is transiently expressed by cells of the developing chick optic tectum and appears to be regulated by retinal axons (Williams et al., 1994). The stimulatory effects of P4 and P7 anterior cortex on dorsal and ventral embryonic thalamus respectively, and of P4 and P7 posterior cortex on both dorsal and ventral thalamus, may be due to the release of CAMs, ECM molecules, diffusible chemotropic factors, stimulatory neurotransmitters or neurotrophins from postnatal cortical cells. Candidate CAMs include NCAM, L1 and TAG-1 all of which are expressed in postnatal cortex (Fushiki & Schachner, 1986; Chung et al., 1991; Tuttle et al., 1995), and exist in soluble forms with growth-promoting properties *in vitro* (Lagenaur & Lemmon, 1987; Doherty et al., 1990, 1991). Candidate neurotrophin molecules include FGF, NGF and BDNF. FGF, BDNF and NGF are synthesized in the cortex (Large et al., 1986; Thomas et al., 1991; Maisonpierre et al., 1990; Castren et al., 1992) and FGF and NGF stimulate neurite outgrowth from thalamic explants cultured alone (Lotto & Price, 1995). Experiments are in progress in the lab to isolate and characterize the factor(s) released from postnatal cortex. It would be interesting to extend these studies to embryonic cortex to look for inhibitory molecules.

In conclusion, it is unlikely that a cortex-derived diffusible factor is involved in stimulation of thalamic neurite outgrowth. It is more likely that thalamic cells are stimulated to grow by intrinsic cues or by signals from nearby structures. These studies are the first to show that embryonic cortex releases diffusible factors that inhibit the growth of embryonic thalamic neurites *in vitro*. The *in vivo* relevance of these *in vitro* findings must be treated with caution. However, it is possible that diffusible growth inhibitory and stimulatory molecules secreted by embryonic and postnatal cortex respectively may influence the growth of thalamic axons once they have reached the cortical plate.

CHAPTER 3

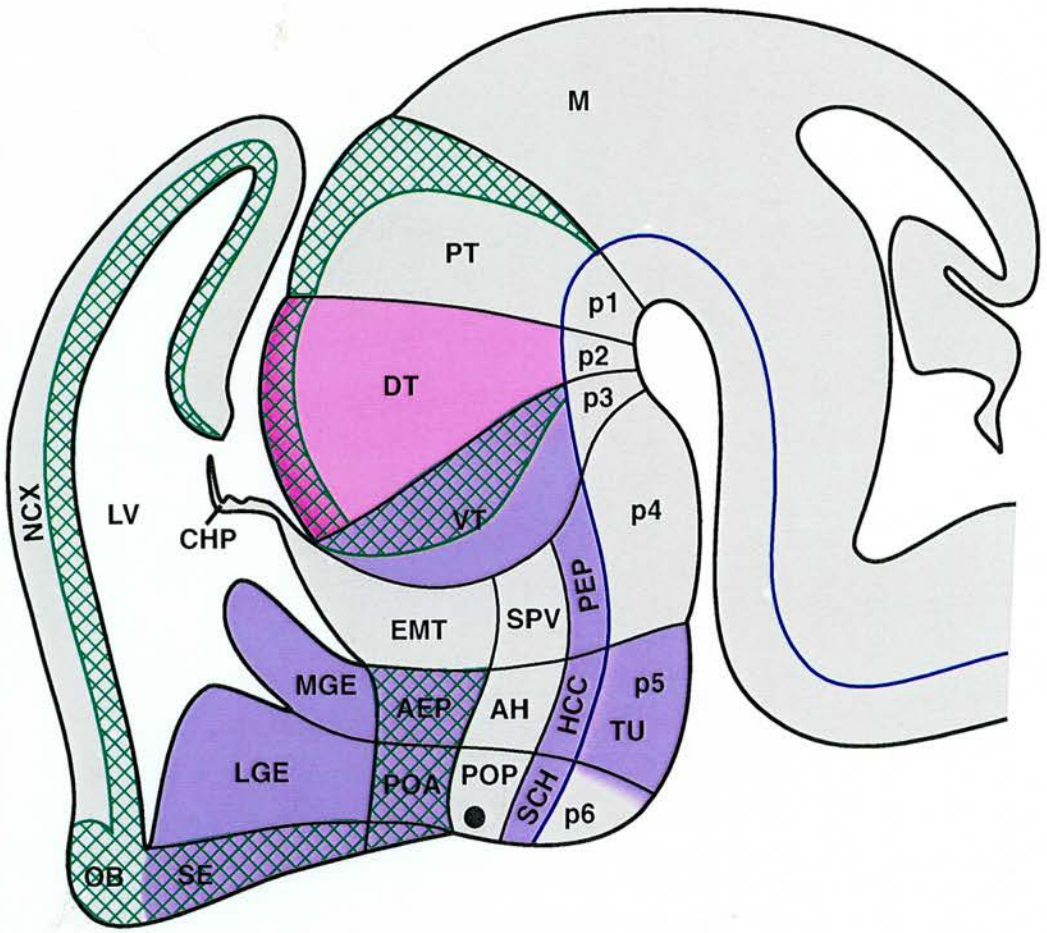
A ROLE FOR PAX-6 IN THE DEVELOPMENT OF THE MURINE DIENCEPHALON

3.1 ABSTRACT

The embryonic murine diencephalon is subdivided into 3 prosomeres (p1-3) based on differences in anatomy and gene expression. *Pax-6* is one of the earliest regulatory genes to be expressed in a regionally restricted manner in the diencephalon. Here I tested whether normal PAX-6 protein is required for diencephalic regionalization by examining morphological features and patterns of regulatory gene expression (*Dlx-2* and *Wnt-3*) in the embryonic diencephalon of Small-eye mice (*Pax-6* mutants). In the mutants, I could identify all the prosomeres, although they were clearly abnormal. Their morphology was distorted, they contained fewer cells than normal, the normal difference in the density of cells between p2 and p3 was lost and the p1/mesencephalon boundary was unclear. Although *Pax-6* and *Dlx-2* were still expressed in p3 and *Wnt-3* was still expressed in p2, as in wild-types, the patterns of staining for these genes were abnormal. In wild-types embryos, staining for *Dlx-2* expression was intense in p3 and decreased abruptly at the p2/p3 border; in the mutants, staining for this gene was very weak in p3 and faded at the p2/p3 border. Expression of *Wnt-3* in the dorsal midline of p2, where it is normally co-expressed with *Pax-6*, was lost in the mutants. In conclusion, a degree of diencephalic regionalization occurs in the absence of normal PAX-6 protein, but it is severely impoverished both cellularly and molecularly. These data provide evidence that the normal expression of *Pax-6* makes a crucial contribution to the control of diencephalic cell numbers and prosomeric development.

Fig. 1. Schematic map of the expression patterns of *Pax-6* (green), *Dlx-2* (purple) and *Wnt-3* (pink) in the E14.5 +/+ mouse forebrain (medial view, adapted from Bulfone et al., 1993, and Stoykova and Gruss, 1994). Abbreviations: AEP, anterior entopeduncular area; AH, anterior hypothalamus; CHP, choroid plexus; DT, dorsal thalamus; EMT, eminentia thalami; HCC, hypothalamic cell cord; LGE, lateral ganglionic eminence; LV, lateral ventricle; M, mesenecephalon; MGE, medial ganglionic eminence; NCX, neocortex; OB, olfactory bulb; p1-p6, prosomeres1-6; PEP, posterior entopeduncular area; POA, posterior preoptic area; POP, preoptic recess; PT, pretectum; SE, septum; SCH, suprachiasmatic area; SPV, supraoptic/paraventricular area; TU, tuberal hypothalamus; VT, ventral thalamus.

Figure 1


M
PT
p1
p2
p3
DT
NCX
LV
CHP
VT
p4
PEP
EMT
SPV
MGE
AEP
AH
HCC
p5
TU
LGE
POA
POP
SCH
p6
OB
SE

3.2 INTRODUCTION

Recent studies of the temporally and spatially restricted patterns of expression of regulatory genes in the murine forebrain (prosencephalon), together with earlier anatomical descriptions of this complex structure, have generated a convincing framework for understanding the morphological development of the prosencephalon (the Prosomeric Model: Puelles & Rubenstein, 1993; Rubenstein et al., 1994). The early prosencephalon (primary prosencephalon) divides into the secondary prosencephalon and the diencephalon, the subject of this study. The diencephalon is subdivided into three prosomeres (p1-p3) identified by morphological constrictions in the tissue and the expression domains of regulatory genes including *Pax-6*, *Dlx-2* and *Wnt-3* (Fig. 1; Bulfone et al., 1993; Puelles & Rubenstein, 1993; Stoykova & Gruss, 1994). The pretectum forms in p1, the dorsal thalamus in p2 and the ventral thalamus in p3. As shown in Fig. 1, *Pax-6* is expressed in p3 with a caudal expression boundary where p2 meets p3, although in the roof plate its expression continues caudally to end at the junction of p1 and the mesencephalon (Stoykova & Gruss, 1994). *Pax-6* is also expressed at more rostral sites (Fig. 1; Stoykova & Gruss, 1994). *Dlx-2* (a homeobox-containing gene homologous to Drosophila *distal-less*: Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Bulfone et al., 1993) is also expressed in p3 with a caudal expression boundary at the junction of p2 and p3, and in more rostral prosomeres (Fig. 1: Bulfone et al., 1993). *Wnt-3* (that encodes a secreted peptide) is expressed in p2 and also further caudally in the mesencephalon and rhombencephalon (not shown in Fig. 1), with an anterior expression boundary where p2 meets p3 (Salinas & Nusse, 1992); its expression is highest dorsally, as indicated by the depth of shading in Fig. 1 (Bulfone et al., 1993).

As yet the molecular mechanisms that establish the regionalization and specification of the forebrain are unclear. Here, I examined the role of *Pax-6* in the development of the diencephalon. *Pax-6*, independently isolated by homology to *gooseberry-distal* (Walther & Gruss, 1991) and from positional cloning at the aniridia locus (Ton et al., 1991), encodes two DNA-binding motifs, a paired domain (Bopp et al., 1986; Treisman et al., 1991) and a paired-like homeodomain (Frigerio et al., 1986). Its mRNA is first detected on embryonic day 8.5 (E8.5) in the mouse and is restricted to p3 before morphological constrictions form in the diencephalon. *Dlx-2* and *Wnt-3* are expressed on E9.5 (Bulfone et al., 1993). Therefore, *Pax-6* is a prime candidate for a regulator of diencephalic regionalization, differentiation and/or maintenance (Mansouri et al., 1994; Stoykova & Gruss, 1994).

I used a mouse with a mutation of *Pax-6* (Small-eye) to study the role of this gene in the development of the diencephalon. There are several alleles of Small eye: the one used here was the original spontaneous mutation designated Sey, in which a point mutation in the *Pax-6* locus results in a dysfunctional PAX-6 protein (Hill et al., 1991). I tested the hypothesis that disruption of *Pax-6* disrupts embryonic diencephalic anatomy (morphological constrictions in the tissue and cell densities) and expression of regulatory genes (*Dlx-2*, *Wnt-3* and *Pax-6* itself).

3.3 MATERIALS AND METHODS

Embryonic development was assumed to have begun at midnight of the night of mating. Sey/Sey mice die at birth (Hogan et al., 1986), and Sey/Sey embryos (easily distinguished by the absence of eyes and a shortened snout) were obtained from Sey/+ x Sey/+ matings (heterozygotes were readily distinguished by their smaller

than normal eyes: Hill et al., 1991). Age-matched controls were +/+ littermates from these matings and +/+ embryos from +/+ x +/+ matings.

Anatomical Study

E14.5 embryos were removed from anaesthetized mothers (0.3ml urethane in sterile saline, i.p.) by Caesarian section. The brains were removed in phosphate buffered saline (PBS) at 4°C, and fixed in 4% paraformaldehyde for 2-3 hours at room temperature. The brains were washed briefly in PBS, dehydrated in alcohol, placed in choloroform overnight and embedded in wax. 10μm parasagittal serial sections were cut and Nissl-stained.

The borders between the prosomeres were identified using several criteria, including the presence of ridges of low cell density, tissue constrictions and borders of gene expression (see Results). The cell densities in the mesencephalon and p1-3, and the volumes of p2 and p3 (the diencephalic prosomeres whose borders could be accurately delineated in both +/+ and Sey/Sey mice, see Results) were analysed. To estimate densities, 4-5 sections taken at equally-spaced mediolateral positions from each of 3 brains were selected. For each section, a 10x10 square graticule was placed over the mesencephalon, p1, p2 and p3 in +/+ and Sey/Sey brains. All cells with a visible nucleus covered by the grid were counted and the average cell density (mm^{-3}) was calculated for each prosomere. Corrections for double cell counts were not made because relative rather than absolute cell densities and numbers were of concern here. To estimate volumes, ten sections taken at equally-spaced mediolateral positions through the prosomeres in each of 3 brains were selected. The area of each prosomere in each section was measured using NIH IMAGE 1.58 VDM software. For each brain, volumes were obtained by multiplying each area by the thickness of tissue

between sections and summing the values. An estimate of total cell number in each prosomere was obtained by multiplying its volume by its corresponding average cell density.

In situ hybridizations

Embryonic day 14.5 (E14.5) mouse brains were dissected and fixed overnight in 4% paraformaldehyde at 4°C. Whole-mount in situ hybridizations were performed as described in Conlon & Rossant (1992) (See Appendix 3). The *Dlx-2* antisense riboprobe was transcribed from a 1.7kb fragment derived from the 3' untranslated region of the *Dlx-2* cDNA clone (a gift from J.L.R.Rubenstein). The *Wnt-3* antisense riboprobe was transcribed from a 0.6kb fragment derived from a noncoding and nonconserved region of the *Wnt-3* cDNA clone (a gift from R.Nusse). The *Pax-6* antisense riboprobe was transcribed from a 1.7 kb fragment derived from the *Pax-6* cDNA clone (a gift from R.Hill). Sense probes were generated from these plasmids for controls (see Appendix 1). Stained embryos were cleared in glycerol and photographed. Some embryos were embedded in wax as described above, and sectioned at 10μm.

3.4 RESULTS

Gross morphology

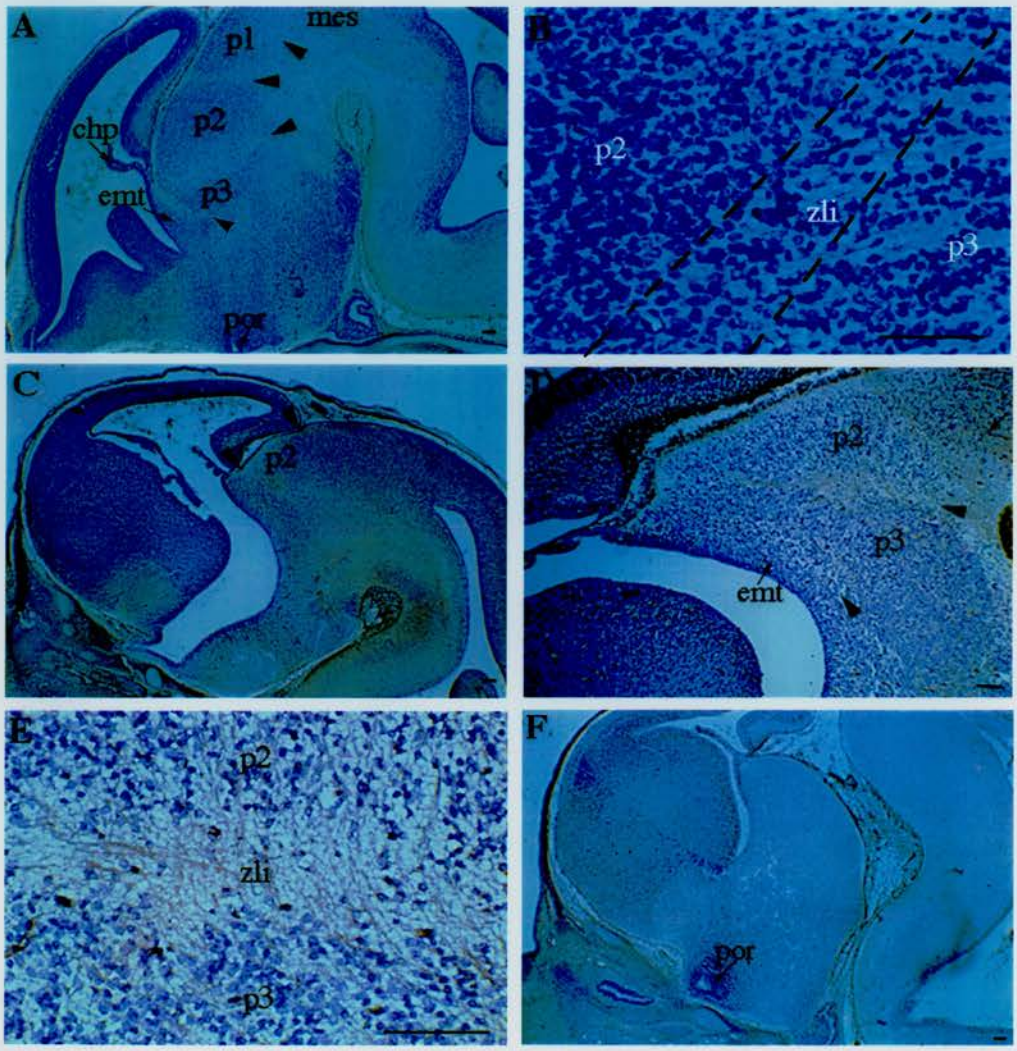
In this study, the Prosomeric Model of Rubenstein et al. (1994) was followed for the subdivision of the forebrain into distinct neuromeres on the basis of anatomical landmarks and patterns of regulatory gene expression. As shown in Fig. 2A (arrowheads), in E14.5 +/+ embryos four ridges of low cell density in the tissue mark

the borders between the mesencephalon and p1, between p1 and p2 (fasciculus retroflexus, rf), between p2 and p3 (zona limitans intrathalamica, zli: Fig. 2B) and between the eminentia thalamus (emt, in alar p4) and p3. Associated with the ridges are morphological constrictions in the tissue (e.g. the p1/p2 constriction is clear in Fig. 2A), but these are less accurate markers of the prosomeric borders at E14.5. As the neuroepithelium increases in size, the morphological constrictions can move out of register with the ridges (Northcutt & Butler, 1993). In recent studies, the ridges, which are in some cases the sites of developing axonal tracts, have been shown to be the true markers of prosomeric borders (Bulfone et al., 1993). The ridges between the emt and p3 and between p2 and p3 (the zli) converge on the base of the choroid plexus (chp in Fig. 2A), where a small constriction is seen in intact forebrain and in some sections.

In E14.5 Sey/Sey embryos the rostral diencephalon is highly abnormal. Rostral to the choroid plexus, the dorsal surface of the diencephalon, which normally has a smooth convexity, plunges ventrally and the ventricle is enlarged (compare Fig. 2A and 2C). In Sey/Sey diencephalon only three ridges of low cell density are visible, rather than four in +/+ diencephalon. The two most rostral ridges are seen in Fig. 2D (arrowheads). They converge on the base of the choroid plexus, where a tissue constriction is seen. These ridges almost certainly correspond to the emt/p3 ridge and the zli ridge (Fig. 2E) in +/+ embryos. The third, more caudal ridge is less well defined. It coincides with a striking tissue constriction that lies under the caudal pole of the neocortex (Fig. 2C), and probably corresponds to the rf (p1/p2 border) in +/+ embryos. (These conclusions from morphological examination are supported by data on gene expression, as described below.) In Sey/Sey embryos, there is neither a ridge

Fig. 2. Parasagittal sections through the heads of E14.5 embryos. **A** +/+ control: arrowheads mark four ridges of low cell density corresponding to the borders between mesencephalon (mes)/p1, p1/p2, p2/p3 and p3/p4 (emt). **B** Detail of **A** showing high cell density in p2 and lower cell density in p3. zli marks the p2/p3 border. **C** Sey/Sey embryo: arrowheads mark the constrictions between p1 and p2, and between p2 and p3. Note the absence of the constriction marking the p1/mesencephalon border. **D, E** Details of **C.** In **D**, arrowheads indicate the p2/p3 and p3/emt borders and the little arrow indicates dorsoventral axons which probably correspond to the circumferential descending axons in +/+ embryos (Mastick and Easter, 1995). **F** Sey/Sey embryo: lateral section showing maintenance of the preoptic recess (por) in basal p6. Scale bars, 100μm.

Figure 2



of low cell density nor a constriction between p1 and the mesencephalon. The neuroepithelium of p4, p5 and p6 appears a little thinner than normal in the dorsoventral direction (compare Fig. 2A and 2C). The position of the preoptic recess (por) of the diencephalon is maintained and serves as a landmark for identifying basal p6 (compare Fig. 2A and 2F) (Bulfone et al., 1993). The striatum (anterior to the enlarged ventricle) is much larger than normal and the cortex is smaller than normal in Sey/Sey embryos (Fig. 2C), as described previously by Schmahl et al. (1993). The mesencephalon appears normal.

Cellular analysis

Regional differences in cell densities may be an indicator of segmentation, since diencephalic neuromeres appear to develop as independent units separated by cell lineage restriction boundaries (Figdor & Stern, 1993). In the diencephalon of +/+ mice, average cell density was significantly higher in p2 than in both p3 (data in Table 1: $p<0.001$, Student's t-test) and p1 (density in p1 = 8.6×10^5 cells mm^{-3}) ($p<0.05$, Student's t-test). Cell density in the mesencephalon was not significantly different to that in p1. These differences in cell density between p1, p2 and p3 have not been reported previously. There were no such differences in Sey/Sey mice (densities for p2 and p3 are in Table 1; density for p1 = 7.2×10^5 cells mm^{-3}).

Table 1 shows the total numbers of cells in p2 and p3 in +/+ and Sey/Sey embryos. Whereas there was no difference in the densities of cells between p2 and p3 in the Sey/Sey mice, p2 (which is larger than p3 in +/+ and Sey/Sey mice: Table 1, $p<0.05$; Student's t-test) contained significantly more cells than p3 in both +/+ and Sey/Sey embryos. These data indicate that a major defect in the Sey/Sey embryos was a loss of variation in cell density between p2 and p3. Diencephalic cells contributed to these

two prosomeres in normal rank order (i.e. more cells contributed to p2 than to p3 in both +/+ and Sey/Sey embryos), although there was a tendency for cell number to equilibrate between the two (p2:p3 ratio was 2.0:1 in +/+ embryos, but 1.4:1 in Sey/Sey embryos). In Sey/Sey embryos, there were generally fewer cells in this region of the diencephalon (p2 and p3) and the volumes of p2 and p3 were smaller (Table 1). I do not yet know whether this was a consequence of a reduced proliferative rate or an accelerated rate of cell death.

Table 1.

	p3 +/+	*p3* *Sey/Sey*	*p2* +/+	*p2* *Sey/Sey*
Mean cell density ($x10^5$ mm^{-3})	6.2	8.1	10.0	7.9
Mean volume (mm^3)	0.16	0.06	0.21	0.09
Mean cell number $\pm$ s.e.m. ($x\ 10^4$)	9.8	4.9	20.3	7.0
(n=3)	$\pm 1.0^{a,c}$	$\pm 0.3^{b,c}$	$\pm 1.5^{a,d}$	$\pm 0.1^{b,d}$

Significant differences: [a] ($p<0.003$); [b] ($p<0.03$); [c] ($p<0.025$); [d] ($p<0.01$)

Table 1. Cell densities, tissue volumes and cell numbers in p2 and p3 in +/+ and Sey/Sey E14.5 embryos. Data are from 4-5 sections from each of 3 brains. Means for cell number are compared with Student's t-test (two-tailed).

Molecular analysis

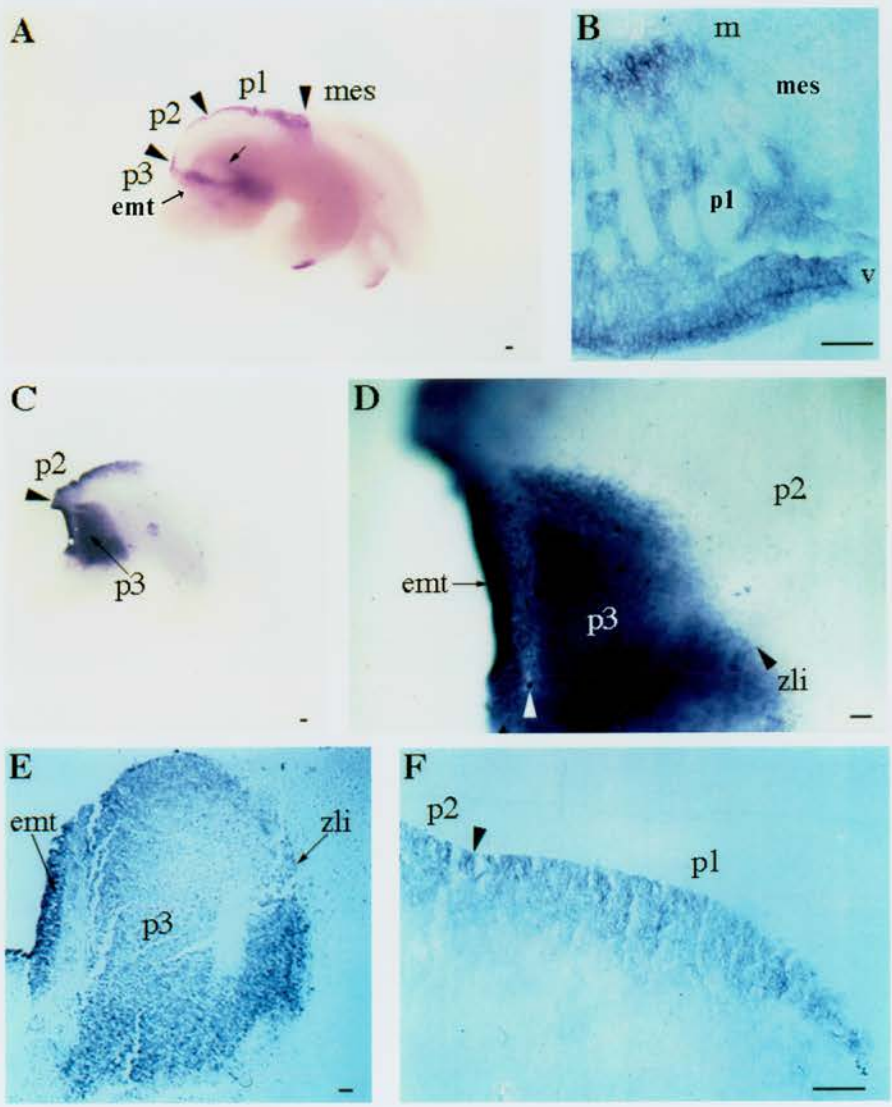
Pax-6 expression

In agreement with previous accounts of expression in E13 wild-type mice (Stoykova & Gruss, 1994), whole mount in situ hybridizations on E14.5 embryos showed *Pax-6* expressed to roughly equivalent extents on both ventricular (medial) and mantle (lateral) surfaces of caudal p3 with a sharp boundary of expression at the zli (Fig. 3A) (n=10 embryos). *Pax-6* expression also extended caudally from p3 through the roof plate of p2 and p1 and expanded mediolaterally on the dorsal surface of p1, forming a sharp caudal boundary that coincided with the p1/mesencephalon border (Fig. 3A,B). Sections confirmed labelled cells in the ventricular and mantle layers and radially-aligned *Pax-6* positive cells between the two (Fig. 3B).

In all E14.5 Sey/Sey embryos (n=6), *Pax-6* expression was abnormal although two main features of the +/+ pattern were seen, i.e. the expanded rostral region and the caudally-extending roofplate expression (Fig. 3C). *Pax-6* expression was strong in the emt of Sey/Sey embryos (Fig. 3D,E), whereas it was very low or absent in the emt of +/+ embryos (Fig. 3A). In Sey/Sey embryos, as in +/+ embryos, p3 expressed *Pax-6*. This expression extended from the zli (the position of the zli was confirmed in counterstained sections of in situ hybridizations) to the emt/p3 border (arrowheads in Fig. 3D; note how the borders of gene expression follow the emt/p3 border and the zli as they converge dorsally on the site of the choroid plexus). The expression in p3 was slightly lower than that in the emt (Fig. 3E), although it was higher than in p3 of +/+ embryos. The domain of expression in p3 was abnormally broad in the Sey/Sey embryos, and appeared to fill all of alar p3 rather than just its caudal part (as was the case in +/+ embryos). *Pax-6* mRNA was still detected along the dorsal midline of the diencephalon in Sey/Sey embryos, although in contrast to +/+ embryos the

Fig. 3. *Pax-6* expression in E14.5 embryos revealed by whole-mount in situ hybridization. **A** +/+ embryo: arrowheads mark the mesencephalon (mes)/p1, p1/p2, and p2/p3 borders. The photograph is focussed on the medial (ventricular) surface and label on the lateral (mantle) surface (small arrow) is out of focus. *Pax-6* expression is in ventricular and mantle layers of p3 and along the dorsal midline of p2 and p1, forming a sharp transverse caudal boundary at the p1/mesencephalon border. Label in emt is weak or absent. **B** Section through the p1/mesencephalon border in a whole-mount in situ hybridization such as in **A**: *Pax-6* expression is in ventricular (v) and mantle (m) cells of p1. Note expression in differentiated cells of the mantle zone. **C** Sey/Sey embryo: there is a larger domain of *Pax-6* expression in p3, loss of the transverse expression at the p1/mesencephalon border, and extension of *Pax-6* expression into the alar plate from the dorsal midline of p2 and p1. **D** Detail of **C**: arrowheads mark the p2/p3 and the emt/p3 borders. **E, F** Sections through whole-mount in situ hybridizations from Sey/Sey embryos, showing label in p3 (**E**) and in p1 (**F**). Scale bars, 100μm.

Figure 3


A
p1
mes
p2
p3
emt
B
m
mes
p1
v
C
p2
p3
D
p2
emt
p3
zli
E
emt
zli
p3
F
p2
p1

expression extended further ventrally such that it probably encroached on the alar plate (compare Fig. 3A and C). The caudal boundary of *Pax-6* expression in p1 in Sey/Sey embryos was abnormal (Fig. 3C,F): it was restricted to the roof plate and did not form a sharp transverse boundary at the p1/mesencephalon border as in +/+ embryos (compare Fig. 3A,B with Fig. 3C,F).

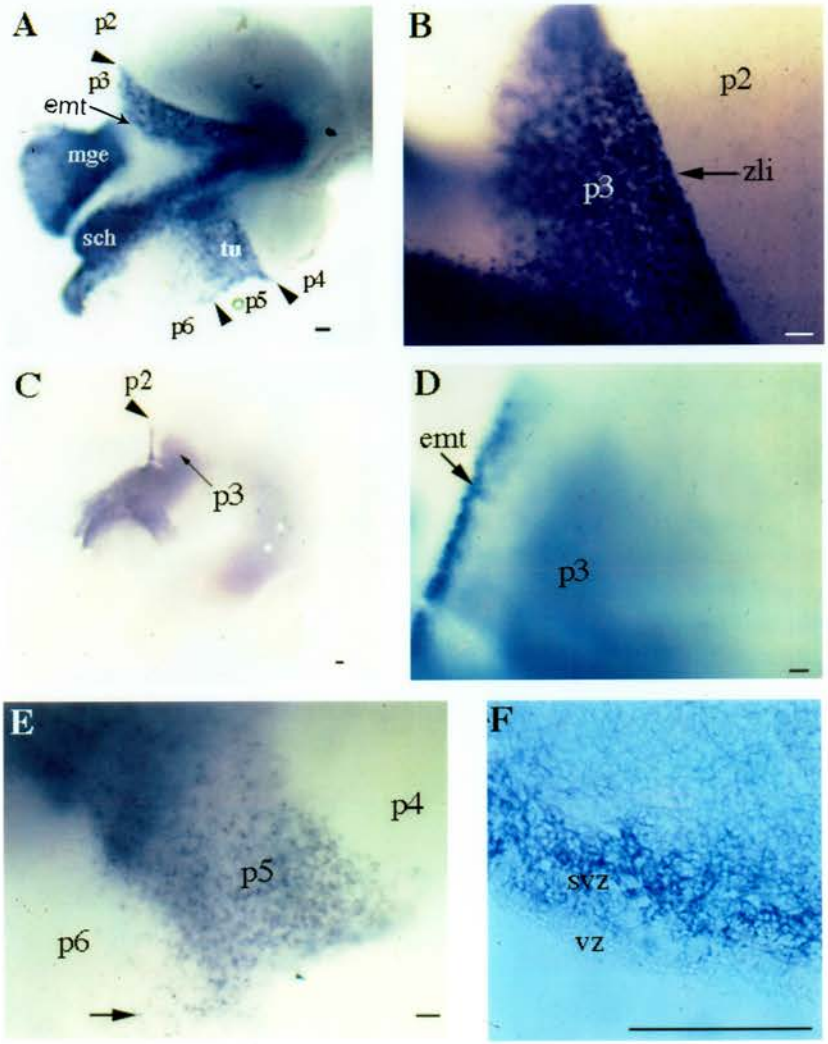
Dlx-2 expression

In agreement with previous accounts of normal expression (Bulfone et al., 1993), *Dlx-2* was expressed in two domains in E14.5 +/+ embryos (n=15 embryos) (Fig. 4A). The anterior domain (part of which is shown in Fig. 4A) coincided with the medial ganglionic eminence (mge), the lateral ganglionic eminence (lge) and the septum of the basal telencephalon with a sharp dorsal boundary of expression at the border between the lge and the cerebral cortex. The posterior domain extended in the alar plate from the area of the suprachiasmatic nucleus (sch) through the hypothalamic cell cord (hcc) and posterior entopeduncular area (pep) into the ventral thalamus (vt), or alar p3, and terminated abruptly at the zli (Fig. 4B). Sectioned material confirmed that *Dlx-2* was expressed by cells of the subventricular layers in the above structures (Fig. 4F; Porteus et al., 1994). *Dlx-2* was also expressed in basal p5, forming a sharp caudal boundary at the p4/p5 border and a more diffuse rostral boundary at the p5/p6 border, with some transcripts detected in basal p6 (Fig. 4A). The anterior and posterior domains of *Dlx-2* expression were separated by a strip of *Dlx-2* negative tissue corresponding to the optoeminential zone, which extends from the emt to the optic stalk (Fig. 4A; Bulfone et al., 1993).

In E14.5 Sey/Sey embryos, *Dlx-2* was also expressed in two domains (n=6 embryos). The anterior domain appeared normal (not shown). The posterior domain of *Dlx-2*

Fig. 4. *Dlx-2* expression in E14.5 embryos revealed by whole mount in situ hybridization. **A** +/+ control showing normal *Dlx-2* expression in alar p3, the posterior entopeduncular area, hypothalamic cell cord, suprachiasmatic area (sch) (alar p4-p6), tuberal hypothalamus (tu) (basal p5) and the medial ganglionic eminence (mge). **B** Detail of **A**, showing the sharp posterior boundary of *Dlx-2* expression at the zli (p2/p3 border). **C** *Dlx-2* expression in Sey/Sey embryo. Arrowhead marks the position of the p2/p3 border. (Shading on the ventral surface of the mesencephalon is not specific labelling). **D** Detail of **C:** strong ectopic *Dlx-2* expression in the emt is separated by a strip of *Dlx-2*-negative tissue from a weaker domain of expression in p3, with a blurred caudal border. The appearance of the staining in p3 results from the superimposition of very weak, low contrast but specific label from different depths. **E** *Dlx-2* expression in p5 of a Sey/Sey embryo. Note the maintenance of a sharp caudal boundary of expression at the p4/p5 border. Arrow indicates *Dlx-2* mRNA transcripts detected in p6. **F** +/+ embryo: section showing Dlx-2 label; svz, subventricular zone; vz, ventricular zone. Scale bars, 100μm.

Figure 4


A
p2
p3
emt
mge
sch
tu
p4
p5
p6
B
p2
p3
zli
C
p2
p3
D
emt
p3
E
p4
p5
p6
F
svz
vz

expression in Sey/Sey embryos appeared normal rostral to the p4/p5 border; transcripts were detected in the alar plate in tissue corresponding to the sch, hcc, pep and in basal p5 (Fig. 4C,E). The sharp caudal boundary of *Dlx-2* expression in basal p5 was maintained at the p4/p5 border, and the rostral p5/p6 boundary was less strict, as in +/+ embryos, with transcripts in basal p6 (Fig. 4E). *Dlx-2* expression was abnormal caudal to the p4/p5 border (Fig. 4C,D). A strong band of ectopic *Dlx-2* expression was seen in the emt (Fig. 4D) (*Dlx-2* is not expressed in the emt in +/+ embryos). *Dlx-2* expression was very weak in p3, and its caudal boundary appeared faded and diffuse in wholemounts (compare Fig. 4B with Fig. 4D: the appearance of the staining in p3 in Fig. 4D results from the superimposition of very weak, low contrast but specific label from different depths). Indeed, the labelling in p3 was so weak that it was not seen clearly in sectioned material.

Wnt-3 expression

In E14.5 +/+ embryos (n = 15 embryos), *Wnt-3* was expressed in a single domain coinciding with p2 (Fig. 5A); expression was strongest in ventricular cells of the dorsal midline (Fig. 5B), from where it extended ventrally. There was a sharp rostral boundary of expression at the p2/p3 border (zli), and a caudal boundary at the p1/p2 border (Fig. 5A; Salinas & Nusse, 1992). In Sey/Sey embryos (n = 6 embryos), *Wnt-3* was also expressed in a single domain with a rostral boundary and a more diffuse caudal boundary of expression. The rostral boundary of *Wnt-3* expression appeared to coincide with the zli but the labelling was so weak in p2 that it was not seen clearly in sectioned material. Strong dorsal midline expression of *Wnt-3,* characteristic in the +/+ embryos (Fig. 5A,B), was absent in Sey/Sey embryos (Fig. 5C).

Fig. 5. *Wnt-3* expression in E14.5 embryos revealed by whole mount in situ hybridization. **A** Medial view of +/+ control: note the strong *Wnt-3* expression in cells of the dorsal midline. Arrow marks the position of the *zli* and the sharp rostral boundary of *Wnt-3* expression. Arrowhead marks the less-distinct caudal boundary of *Wnt-3* expression at the p1/p2 border. **B** Dorsal view of +/+ control: *Wnt-3* expression on the dorsomedial surface of p2. **C** Sey/Sey embryo: very faint *Wnt-3* expression in p2 forming a rostral boundary in the region of the p2/p3 border and a diffuse caudal boundary in the region of the p1/p2 border (borders marked with arrowheads). Scale bars, 100μm.

Figure 5

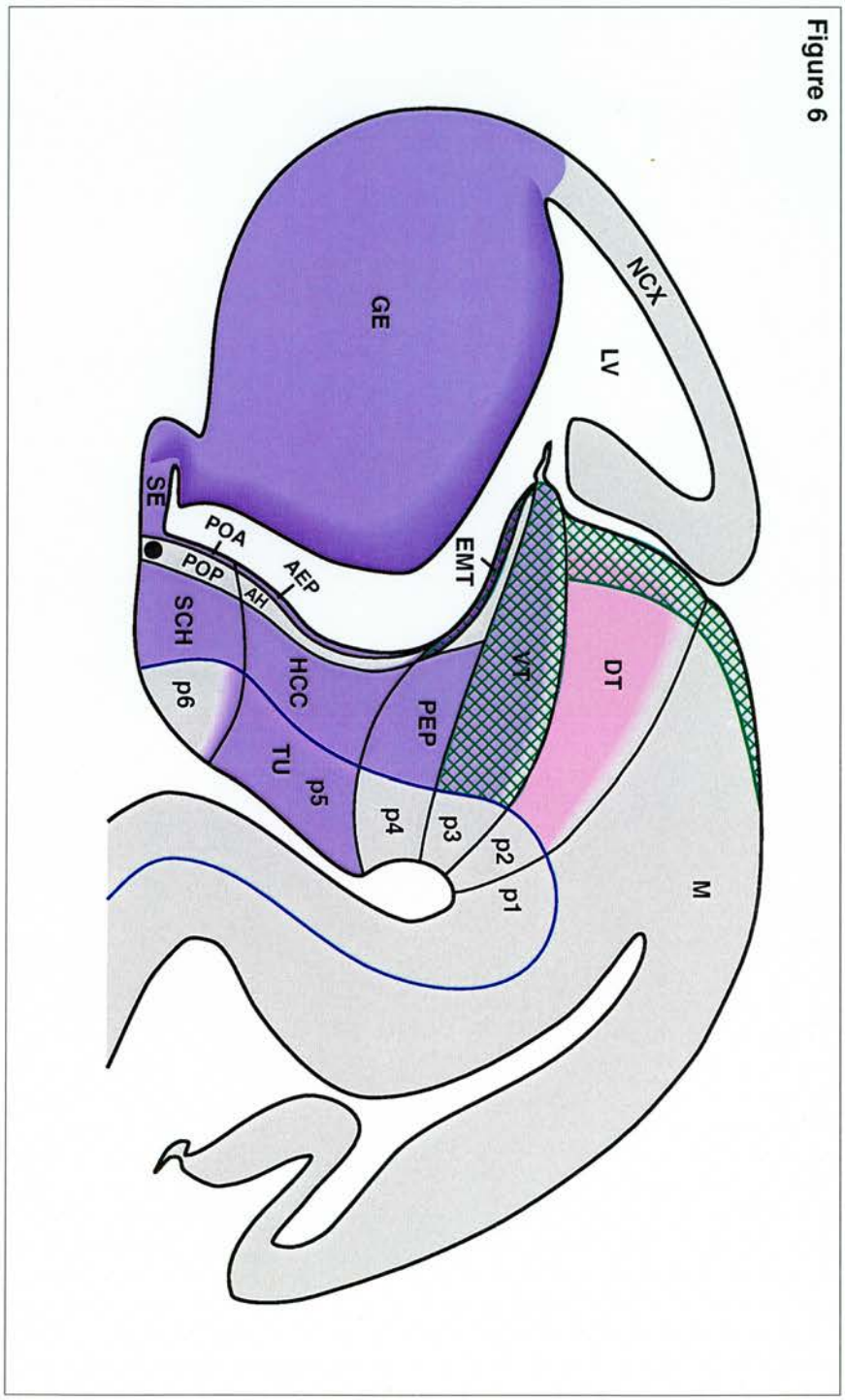
3.5 DISCUSSION

In Sey/Sey embryos, all the prosomeres can be identified from observations of anatomy and patterns of gene expression, and so I concluded that normal PAX-6 protein is not required for some degree of diencephalic regionalization to occur. However, prosomeres 2 and 3 of Sey/Sey embryos are strikingly abnormal: (i) they are smaller and contain fewer cells than normal, (ii) the difference in cell density between p2 and p3 is lost and the difference in cell numbers between them is reduced, (iii) there is reduced expression of *Dlx-2* in p3 and *Wnt-3* in p2, with *Wnt-3* expression being especially low along the dorsal midline where it is normally highest and where *Pax-6* is co-expressed, and (iv) *Dlx-2* expression appears to fade out at the p2/p3 border rather than terminating abruptly as in wild-type embryos. The main molecular findings are summarized in Fig. 6.

A possible explanation for the loss of cell density and, to a lesser extent, cell number differences between p2 and p3 in Sey/Sey embryos is that abnormal cell mixing occurs between p2 and p3. This might contribute to an equilibration of cell density and cell number across this border. There is now considerable evidence from studies of the normal forebrain and hindbrain that migration of cells across neuromeric boundaries becomes restricted as the neuromeres develop their unique identities (Figdor & Stern, 1993; Fraser et al., 1990), although a small percentage of cells may violate the boundaries early or late in development (Birgbauer & Fraser, 1994; Golden & Cepko, 1996). In the hindbrain, it is thought that cell mixing between rhombomeres is inhibited by differences in the cell adhesive properties of adjacent rhombomeres (Guthrie et al.,1993). It is possible that a similar mechanism operates in the forebrain to prevent cell migration across prosomere borders, and *Pax-6* might

Fig. 6. Schematic map of the expression patterns of *Pax-6*, *Dlx-2* and *Wnt-3* in an E14.5 Sey/Sey mouse embryo. Conventions are as in Fig. 1.

Figure 6


NCX
LV
GE
SE
POA
POP
AEP
AH
EMT
SCH
p6
HCC
TU
p5
p4
PEP
VT
p3
p2
p1
DT
M

regulate this process. As yet, there is little direct evidence for this, although several studies suggest that possible downstream targets of homeobox genes include cell adhesion molecules (Gould et al., 1990; Jones et al 1992a, 1992b; Edelman & Jones, 1992). Cell adhesion molecules are known to be expressed in restricted patterns in the prosomeres of the wild-type diencephalon. For example, R-cadherin, a Ca^{2+}-dependent cell-cell adhesion molecule, delineates p3 (Matsunami & Takeichi, 1995) and it has been suggested that homophilic binding mediated by R-cadherin may restrict the migration of R-cadherin-expressing cells across the p2/p3 border (Ganzler & Redies, 1995).

However, it is equally possible that the migration of cells across the p2/p3 border remains restricted in Sey/Sey embryos. The blurring of the border of *Dlx-2* expression at the p2/p3 boundary and reduced *Dlx-2* expression in p3 might reflect the fact that normal *Pax-6* expression is required for normal *Dlx-2* expression in this area. *Pax-6* is normally expressed in caudal p3, and *Dlx-2* expression may decrease in this region in Sey/Sey embryos if *Pax-6* normally directly or indirectly regulates *Dlx-2* expression. The downregulation of *Wnt-3* expression in p2 may be a direct or indirect consequence of the loss of normal *Pax-6* expression in the dorsal part of this prosomere. There is clear evidence for interactions among homeobox genes and between homeobox genes and wingless-type genes in other species, including Drosophila (DiNardo & O'Farrell, 1987; Ingham, et al., 1988; Hidalgo & Ingham, 1990; Cohen, 1990; Heemskerk et al., 1991) and zebrafish (Krauss, et al., 1992), and they have been hypothesized in mouse (McMahon et al., 1992; Bulfone et al., 1993; Iler et al., 1995).

The expression of *Pax-6* itself in Sey/Sey embryos was interesting. In rostral diencephalon, there was an increased domain of *Pax-6* expression in p3. In caudal

diencephalon, there was a loss of the normal transverse stripe of *Pax-6* expression at the p1/mesencephalon border. The alteration in the level of *Pax-6* transcripts in Sey/Sey embryos suggests that a functional PAX-6 protein is required (directly or indirectly) for the expression of its own gene in the forebrain. *In vitro* binding studies have demonstrated that *Pax-6* can recognize sites within its own promoter (Plaza et al., 1993). The abnormal expression of *Pax-6* expression in caudal p1 coincides with a loss of both the ridge of low cell density and the constriction in the tissue that normally mark the p1/mesencephalon border. Thus, *Pax-6* may also be involved in the formation of this border. In a similar vein, injection of antibodies against the PAX-b protein disrupts formation of the constriction at the midbrain/hindbrain border in zebrafish (Krauss et al., 1992).

Another interesting finding was that, in Sey/Sey embryos, expression of *Pax-6* was abnormally high in the emt (the dorsal part of p4) and *Dlx-2* was ectopically expressed here (although not in other regions of p4). The same considerations as those discussed above for the p2/p3 border apply to this observation. Further work will be needed to determine whether this finding is a consequence of high interprosomeric cell migration or changes in gene expression among cells that continue to respect the p3/p4 border.

Clearly, the overall shape of the forebrain of the Sey/Sey embryo is distorted. In particular, the striatum is enlarged and the neocortex is abnormally small, as has been described previously by Schmahl et al. (1993). At least some of the distortions in the shape of the promomeres rostral to the diencephalon in Sey/Sey embryos (represented in Fig. 6) are probably secondary to changes in the shape of the telencephalon, where *Pax-6* is also normally expressed (Fig. 1). For example, the enlarged striatum may restrict the ability of p4, 5 and 6 to expand dorsally. Future

work will evaluate whether the defects in the most rostral parts of the Sey/Sey forebrain are primary or secondary.

The Small-eye phenotype studied here results from a point mutation that introduces a stop codon between the paired box and the homeobox. Since the several alleles of Small-eye have similar phenotypes, and studies with antibodies that recognize epitopes encoded 5' to the site of the mutation have failed to detect protein in Sey/Sey embryos, it is very likely that any truncated PAX-6 protein that is made in the homozygotes is highly unstable and afunctional (Hill et al., 1991). However, at present I cannot exclude absolutely the possibility that the abnormalities in forebrain development that I have described here result from a loss of specifically the PAX-6 homeodomain.

In conclusion, normal *Pax-6* expression is needed for the diencephalic prosomeres to develop some of their important cellular and molecular attributes and specific distinguishing features, although a degree of diencephalic regionalization can occur without it. *Pax-6* may also help regulate the overall number of diencephalic cells.

CHAPTER 4

AXON TRACTS AND REGULATORY GENE EXPRESSION IN THE EMBRYONIC MOUSE FOREBRAIN

4.1 ABSTRACT

In this chapter I investigated whether the pathways of developing tracts correlate with patterns of regulatory gene expression. Tracts were labelled with the fluorescent neuronal tracers DiI and DiA and expression patterns were revealed by whole mount in situ hybridization.

DiI-labelled axons of the retinothalamic tract project from the floor of the forebrain to the thalamus on the *lateral* surface of the diencephalon. *Dlx-2* mRNA expression extends on the *medial* surface of the diencephalon from the hypothalamic cell cord to the ventral thalamus. The trajectory of retinothalamic axons overlaps the pattern of *Dlx-2* expression but does not appear to contact *Dlx-2* expressing cells, which may indicate an indirect effect between *Dlx-2* expressing cells and retinothalamic axons.

DiI labelled axons of the thalamocortical tract project parallel to the longitudinal axis of the embryo. The position of their cell bodies in the ventral thalamus appeared to correlate with a band of *Pax-6* expression on the lateral surface of the ventral thalamus. It is possible that *Pax-6* is expressed in cells of the ventral thalamus whose axons project to the cortex.

In addition, the collateral branch of the corticospinal tract to the superior colliculus was labelled in E14.5 embryos suggesting it forms earlier than previously thought and may correlate with a border of *Pax-6* expression in the tectum.

4.2 INTRODUCTION

In *Drosophila*, zebrafish and chick there is a correlation between pioneering axons and the borders of regulatory gene expression (Krauss et al., 1991; Figdor & Stern, 1993; Wilson et al., 1993) (see Chapter 1). Few studies in mouse have set out directly to study correlations between tracts and boundaries so at best evidence is often circumstantial. However, in E10.5 mouse forebrain the tract of the postoptic commissure (TPOC) correlates with a stripe of expression of the homeobox gene, *Nkx-2.2* suggesting the TPOC may use the border of *Nkx-2.2* for guidance (Shimamura et al., 1995). It is possible that the optic tract of the retinothalamic pathway correlates with the caudal domain of *Dlx-2* expression from the optic stalk to the ventral thalamus (see Fig. 1, Chapter 3). It is also possible that the pathway of thalamocortical axons as they leave the thalamus correlates with *Pax-6* expression in the ventral thalamus.

I labelled the retinothalamic and thalamocortical tracts in E14.5 embryos, and examined if there was a correlation between the tracts and patterns of *Pax-6* and *Dlx-2* mRNA expression.

4.3 METHODS

DiI labelling of retinothalamic and thalamocortical pathways

E14.5 mouse embryos were removed from deeply anaesthetized mothers (0.3ml urethane in isotonic saline i.p.) by Caesarian section. The heads were removed in phoshate buffered saline (PBS) at 4°C, and fixed in fresh 4% paraformaldeyde in 0.1M PBS (pH7.4) overnight. Small crystals of the anterograde and retrograde

fluorescent tracers, DiI and DiA (Honig & Hume, 1986; Godement et al., 1987), were placed into rostral and caudal cortex respectively using a glass micropipette. The embryos were stored in 4% paraformaldehyde and the DiI and DiA were allowed to diffuse for at least 1 year in the dark. After removal of the skin and excess mesenchyme, the cortical hemispheres were removed and separated along the dorsal midline. Each hemisphere was cut in half sagittally. The brains that had their cortical hemispheres removed were also cut along the dorsal midline. The hemisections were cut in half sagittally. The slices were placed in 1:1 glycerol:PBS for 1 hour followed by 4:1 glycerol:PBS to clear the tissue enabling the tracts to be seen clearly. Embryos were examined under epifluorescence microscopy using a rhodamine filter set that revealed DiI (red) and not DiA, and fluorescein that revealed DiA (yellow), and photographed using Fujichrome 400 film. The retinothalmic tract was labelled by removing the eye and placing a small crystal of DiI into the optic cup. The brains were stored in 4% paraformaldeyde and the DiI was allowed to diffuse in the dark for 9 months. In some cases the embryos were stored at 37°C for 2 weeks to accelerate the labelling process. After removal of the skin, excess mesenchyme and cortical hemispheres, the brains were hand-sectioned sagitally and examined under epifluorescence microscopy using a rhodamine filter. Labelled axons were photographed with Fujichrome 400 film. Problems were envisaged visualizing tracts in E14.5 embryos due to the relatively small numbers of thalamocortical axons innervating the cortex at this age. For this reason tracts were labelled in E18.5 embryos (see Appendix IV).

Whole mount in situ hybridization

E14.5 mouse brains were dissected and fixed overnight in 4% paraformaldehyde at 4°C. Whole mount in situ hybridizations with *Dlx-2* and *Pax-6* antisense riboprobes were performed as described in Conlon & Rossant (1992) (see appendix 3).

4.4 RESULTS

Retinothalamic tract

Crystals of DiI placed into both eyes of an E14.5 embryo, labelled two tracts on the ventral surface of the brain (Fig. 1A) (n=8). The tracts crossed each other at the ventral midline and then projected laterally (Fig. 1A). Axons labelled from the eyes to the ventral midline correspond to axons of the optic nerve, the position where the tracts cross each other at the ventral midline corresponds to the optic chiasm, and tracts labelled posterior to the optic chiasm correspond to axons of the optic tract (Taylor & Guillery, 1994). Axons were also labelled on the lateral surface of the diencephalon (Fig. 1B). In the ventral diencephalon the DiI-labelled axons were tightly fasciculated. In dorsal diencephalon, the axons were defasciculated and some had turned through as much as 90° (arrow Fig. 1B), to project dorsocaudally along the brainstem.

In agreement with previous studies (Bulfone et al., 1993), *Dlx-2* was expressed on the *medial* surface of the ventral diencephalon, from the area of the suprachiasmatic nucleus (sch) through the hypothalamic cell cord (hcc) and posterior entopecuncular area (pep) into the ventral thalamus (vt), and terminated abruptly at the zli, a region of low cell density that divides the thalamus into dorsal and ventral regions (Fig. 1C). Cells that expressed *Dlx-2* mRNA were on the medial surface of the diencephalon

(subventricular zone-see chapter 3), and they did not contact retinothalamic axons which grew just deep to the pial edge on the lateral surface of the diencephalon. There were no labelled axons on the medial surface of the diencephalon.

Thalamocortical tract

A crystal of DiI placed in the anterior neocortex labelled axons and two bands of cell bodies on the lateral surface of the diencephalon (Fig. 2A and 2B) (n=8). The axons projected parallel to the longitudinal axis of the embryo and connected the two bands of cell bodies. The posterior band of cell bodies (marked by asterisks in Fig. 2A) lay in the ventral thalamus and probably comprised thalamocortical neurons retrogradelly labelled by the DiI crystal in the anterior neocortex. The posterior limit of these cell bodies lay adjacent to a kink in the tissue corresponding to the boundary between dorsal and ventral thalamus (Fig. 2C). The anterior band of cell bodies lay in the reticular nucleus of the thalamus and probably comprised reticulothalamic neurons that may have been labelled by transneuronal transport of DiI. DiI-labelled axons that connected the two bands of cell bodies, and extended rostromedially were probably thalamocortical axons retrogradely labelled by the DiI. These axons projected deep into the tissue towards the internal capsule (bright label in Fig. 2A). A crystal of DiA placed in posterior neocortex also labelled axons and two bands of cell bodies on the lateral surface of the diencephalon (Fig. 2D). The posterior band of cell bodies lay in the dorsal thalamus just posterior to the kink in the tissue corresponding to the boundary between dorsal and ventral thalamus, and probably comprised thalamocortical neurons retrogradely labelled from the DiA crystal in the posterior neocortex. The anterior band of cell probably comprised reticulothalamic neurons labelled by transneuronal transport of the DiA.

Fig. 1. DiI-labelled tracts and *Dlx-2* mRNA expression in E14.5 embryos. Crystals of DiI were placed in the back of the eye one year before dissection. *Dlx-2* mRNA was revealed by whole mount in situ hybridization. (A) Ventral view of the brain. The DiI has been transported along the optic nerve through the optic chiasm and into the optic tract. (B) Lateral view of the diencephalon. The cortical hemispheres have been removed and the brain has been sliced along the midline and laid on its cut surface. DiI-labelled axons of the optic tract are seen emerging from the floor of the forebrain and projecting through ventral regions of the ventral thalamus as a tightly fasciculated bundle. In the dorsal thalamus the axons are defasciculated and some have turned to project caudally. (C) Medial view of the diencephalon showing *Dlx-2* expression in the sch, hcc, pep, vt and mge. The black circle indicates the position of the optic stalk. on, optic nerve; ot, optic tract; vt, ventral thalamus; dt, dorsal thalamus; zli, zona limitans intrathalamica; emt, eminentia thalami; mge, medial ganglionic eminence; sch, suprachiasmatic area; hcc, hypothalamic cell cord; pep, posterior entopeduncular area; tu, tuberal hypothalamus. Scale bars=100μm

Fig. 2. Axon tracts and *Pax-6* expression in E14.5 embryos. Crystals of DiI were placed in the anterior cortex and crystals of DiA in the posterior cortex, one year before dissection. *Pax-6* mRNA was revealed by whole mount in situ hybridization. (A) Lateral view of the diencephalon. The DiI has labelled cell bodies in the reticular nucleus of the thalamus and in the ventral thalamus. Labelled axons are seen projecting rostrally from the ventral thalamus. (B) Detail of (A). (C) Camera lucida drawing of the same bands of cell bodies and axons labelled in (A). The posterior band of cell bodies lie in the ventral thalamus just anterior to a kink in the tissue (arrowhead) that marks the position of the zli. (D) Camera lucida drawing of DiA-labelled cell bodies and axons on the lateral surface of the diencephalon. The posterior band of cell bodies lies in the dorsal thalamus just posterior to the kink in the tissue (arrowhead) that marks the position of the zli. (E) Lateral view of the diencephalon showing a band of *Pax-6* expression in the ventral thalamus just anterior to the zli. The photograph is focused on the lateral surface and expression on the medial surface (arrowhead) is out of focus. The kink in the tissue marking the position of the zli is not visible because of the way the hemisection was tilted for photography. Asterisks in (A) and (E) mark points that may correspond. rn, reticular nucleus of the thalamus; vt, ventral thalamus; dt, dorsal thalamus; zli, zona limitans intrathalamica. Scale bars =100μm (A, B, E), and 500μm (C & D).

Figure 1

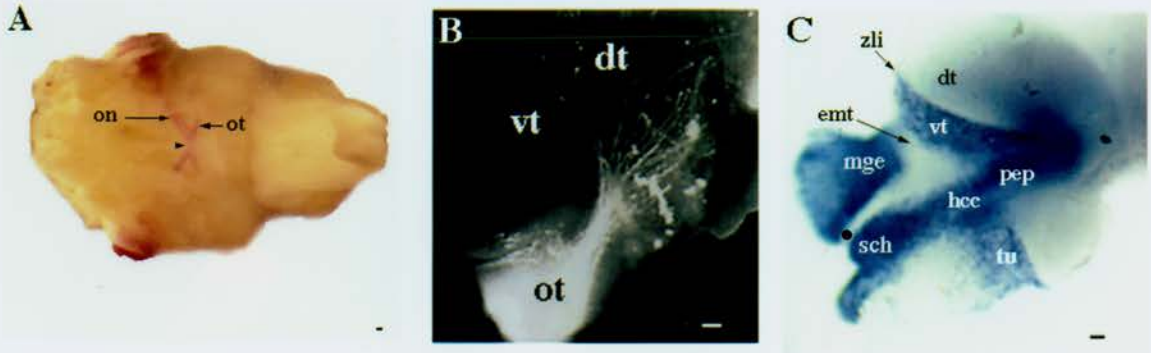


Figure 2

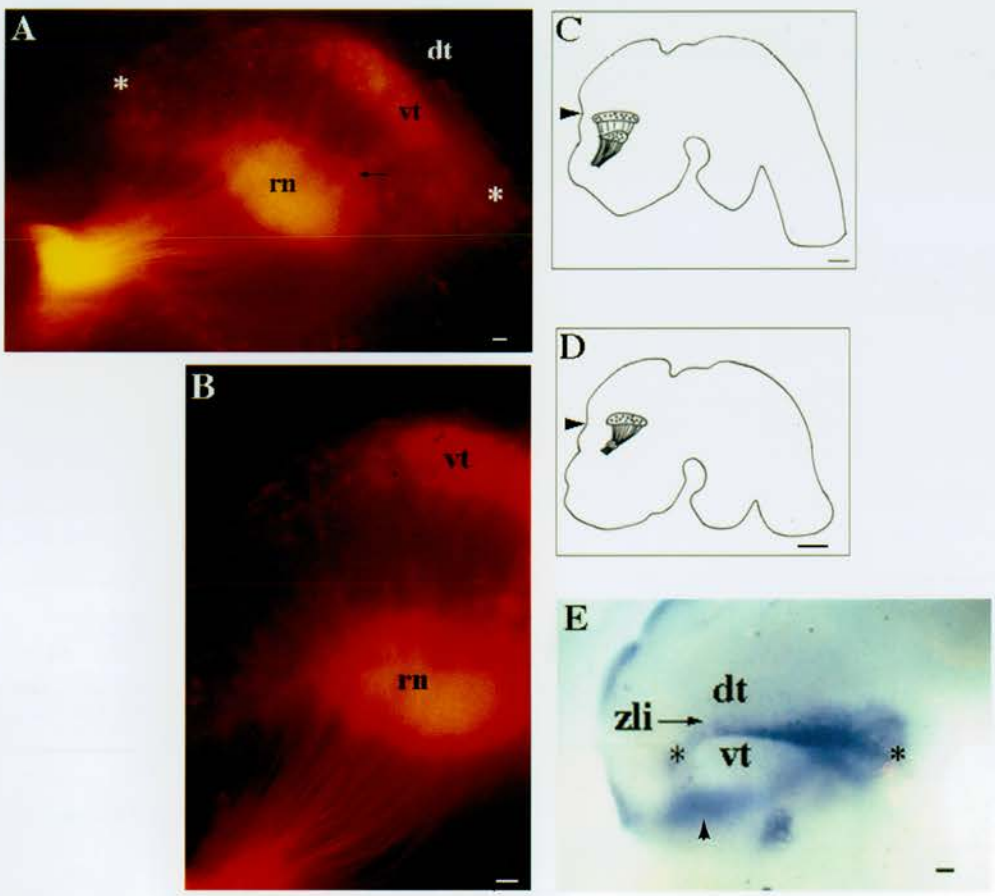
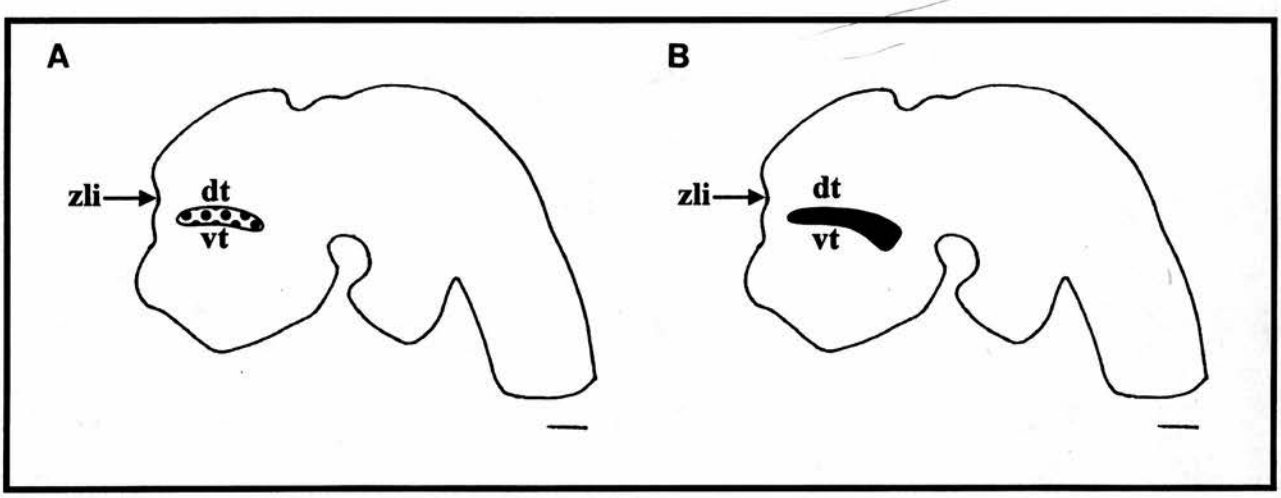


Fig. 3. Summary diagram. DiI labelled cell bodies in the ventral thalamus lie just anterior to the zli (A), and may correlate with Pax-6 expression in the ventral thalamus that also lies just anterior to the zli (B).

Figure 3



DiA-labelled axons connecting the two bands of cell bodies were probably thalamocortical axons retrogradelly labelled by the DiA crystal in the posterior neocortex.

Whole mount in situ hybridization with *Pax-6* riboprobes, in separate embryos revealed a transverse band of *Pax-6* mRNA expression on the lateral surface of the ventral thalamus that terminated abruptly at the boundary between dorsal and ventral thalamus (Fig. 2E, see chapter 3 for details of expression pattern).

There appeared to be a correlation between the band of DiI-labelled cell bodies of thalamocortical axons in the ventral thalamus and the band of *Pax-6* expression on the lateral surface of the ventral thalamus (compare Fig. 2A and 2C with Fig. 2E). Both bands lay just anterior to a kink in the tissue that marks the boundary between dorsal and ventral thalamus. These findings are summarized in Figure 3.

Corticospinal tract

A crystal of DiA placed in the posterior cortex of an E14.5 embryo also labelled a longitudinal tract on the lateral surface of the ventral diencephalon and a dorsoventral (transverse) tract to the tectum (Fig. 4A) (n=8). Growth cones were observed on the tips of axons in the tectum and in the ventral midbrain, suggesting that this tract corresponds to the longitudinal corticospinal tract and its collateral branch to the superior colliculus in the tectum (De Carlos & O'Leary, 1992). Interestingly the transverse band of *Pax-6* expression on the lateral surface of the tectum (see chapter 3, Fig. 3A) may correlate with the collateral branch to the superior colliculus in the tectum.

A summary of the tracts labelled in this study is shown in Figure 5.

Fig. 4. Photomontage of the lateral surface of an E14.5 brain. A crystal of DiA in the posterior cortex has labelled longitudinal axons of the corticospinal tract and their dorsoventral (transverse) collaterals to the superior colliculus. The white arrow points to a consistently present and very distinctive kink in the tissue which marks the border between the tectum and the mesencephalon. sc, superior colliculus; cpe, cerebral peduncle. Scale bar=100μm

Figure 4

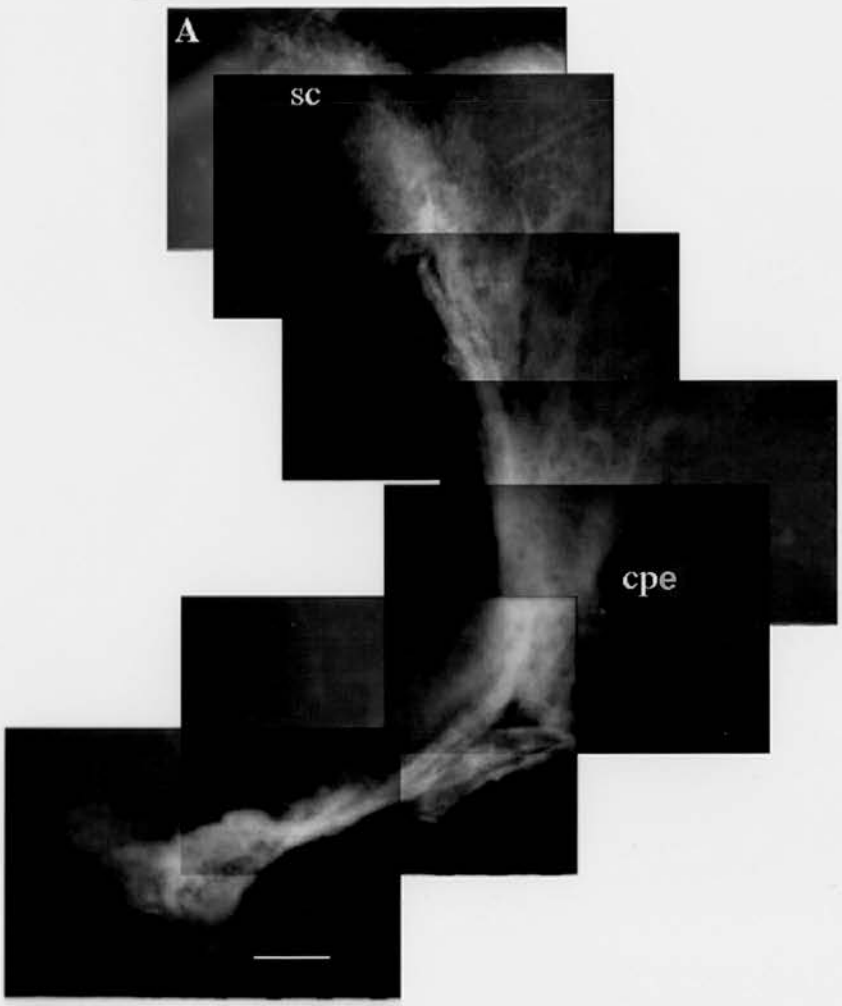
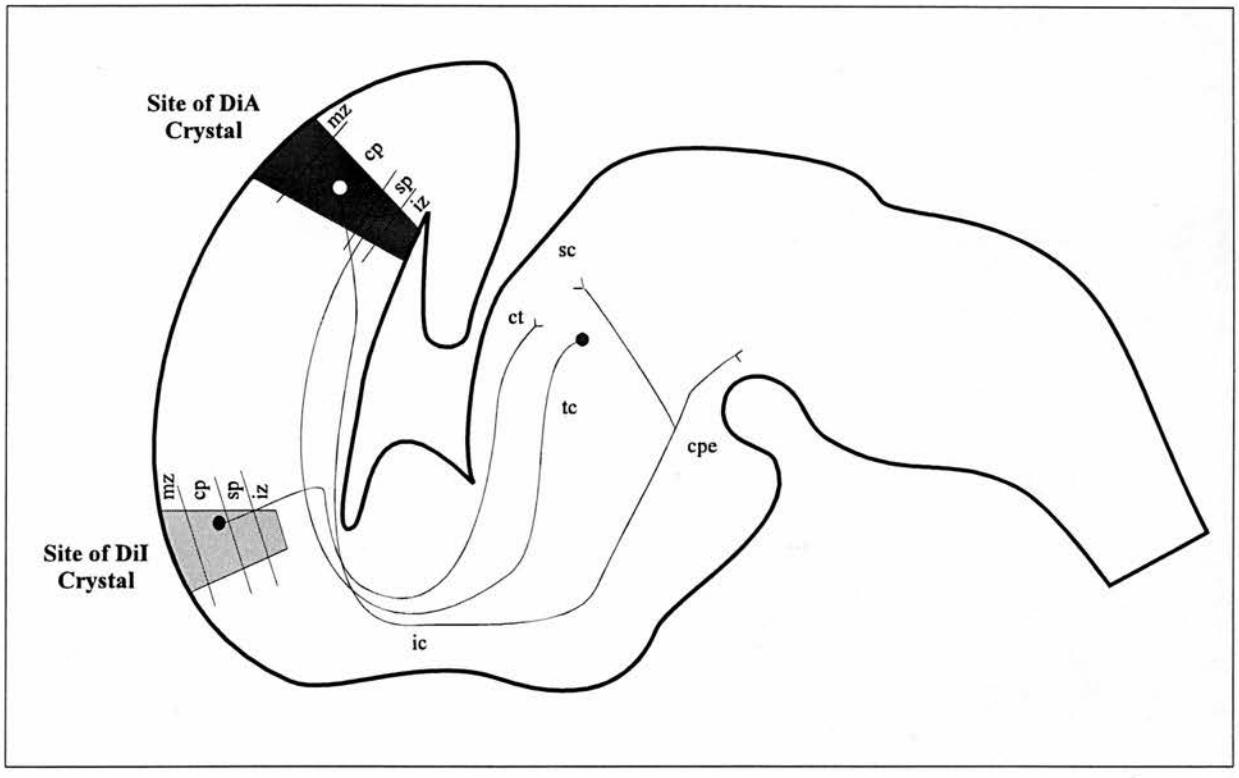


Fig. 5. Summary of cortical efferents and afferents labelled in this study. The drawing is a schematic sagittal section of an E14.5 mouse brain. Dark shading shows the site of the DiA crystal in posterior cortex and light shading shows the site of the DiI crystal in anterior cortex. Thalamic axons project from neurons in the thalamus (grey circle) through the internal capsule and along the subplate. Layer 5 cortical neurons (white circle) project through the intermediate zone to the internal capsule. Once in the region of the midbrain the tract is called the cerebral peduncle and collateral branches project to the superior colliculus in the tectum. Layer 6 cortical neurons (black circle) project through the intermediate zone and internal capsule to the thalamus. cpe, cerebral peduncle; sc, superior colliculus.

Figure 5


Site of DiA Crystal
mz
cp
sp
iz
sc
ct
tc
cpe
mz
cp
sp
iz
Site of DiI Crystal
ic

4.5 DISCUSSION

This study investigated whether pathways of developing tracts correlate with patterns of regulatory gene expression.

Retinothalamic tract

The trajectory of retinothalamic axons overlaps the pattern of *Dlx-2* expression but does not appear to contact *Dlx-2* expressing cells, which may indicate an indirect effect between *Dlx-2* expressing cells and retinothalamic axons.

In agreement with previous studies (Taylor & Guillery, 1994; Lotto & Price, 1995), DiI-labelled axons of the retinothalamic tract projected on the *lateral* surface of the diencephalon to the ventral thalamus as a fasciculated bundle. Whole mount in situ hybridization revealed *Dlx-2* expression on the *medial* surface of the ventral diencephalon from the hypothalamic cell cord to the ventral thalamus. *Dlx-2*-expressing cells did not contact retinothalamic axons and it is unlikely that they provide direct guidance information for pioneering retinothalamic axons. Retinothalamic axons appeared to be labelled on the lateral surface of the sch, hcc and pep whereas *Dlx-2* was expressed in cells on the medial surface of these structures. In spite of the distance between axons and *Dlx-2* expressing cells the trajectory of retinothalamic axons overlapped the pattern of *Dlx-2* expression (compare Fig 1B and 1C). This may be an artefact due to the shape of the brain, or alternatively *Dlx-2* may indirectly affect the trajectory of retinothalamic axons by patterning adjacent tissue in the subpial zone to express guidance cues for navigating growth cones. In insects the boundary regions between segments play an important role in patterning cells in the adjacent environment, either through the release of a

morphogen or through their ability to trigger a cascade of local inductive interactions (Ingham & Martinez Arias, 1992; Nubler-Jung, 1979). It would be interesting to study the retinothalamic pathway in embryos where *Dlx-2* has been disrupted to investigate whether *Dlx-2* indirectly affects guidance of retinothalamic axons.

The location of retinothalamic axons on the superficial surface of the forebrain is consistent with published observations of early tracts in other species (Kevetter & Lasek, 1982; Easter & Taylor, 1989; Wilson et al., 1990; Wilson & Easter, 1991). Since many of these early axons and their cell bodies often lie deep in the adult brain (Sidman et al., 1971), it is unclear why early tracts form superficially among the pial neuroepithelial end-feet, but features of this region suggest several possibilities. According to the 'blueprint hypothesis', preformed channels are present in the subpial zone and provide regions of low resistance to growth (Singer et al., 1979; Silver & Sidman, 1980). Alternatively, cell adhesion molecules (Silver & Rutishauser, 1984) and substrate adhesion molecules (Letourneau et al., 1988) are concentrated here, and may provide an adhesive substrate. For example, the ECM molecule laminin-1 is found at the end feet of neuroepithelial cells along the outer margin of the optic pathway during early stages of development and may serve as an adhesive substrate and possibly as a guidance cue for elongating retinal ganglion cell growth cones in vivo (Morissette & Carbonetto, 1995).

Thalamocortical tract

DiI labelled axons of the thalamocortical tract projected parallel to the longitudinal axis of the embryo. The position of their cell bodies in the ventral thalamus appeared to correlate with a band of *Pax-6* expression on the lateral surface of the ventral thalamus. Crystals of DiI placed in the anterior cortex and DiA in the posterior cortex

of E14.5 embryos labelled two bands of cell bodies in the thalamus. The posterior band of cell bodies lay perpendicular to a kink in the tissue that corresponds to the zli. There appeared to be a correlation between the position of these cell bodies and the domain of *Pax-6* expression on the lateral surface of the ventral thalamus which terminated at the zli. These results must be treated with caution as the in situ hybridizations and tract tracing studies were in separate embryos. It will be essential to combine these techniques in the same embryo to assess a direct correlation between the cell bodies and *Pax-6* expression. However, the preliminary results indicate that *Pax-6* may directly or indirectly regulate neuronal differentiation of thalamocortical neurons in the ventral thalamus.

Wilson et al. (1993) have suggested that boundaries of regulatory gene expression may indirectly influence growth cone guidance by directly or indirectly specifying cell phenotype. A regulatory gene may directly, or indirectly (through secretion of a diffusible morphogen) (Ingham & Martinez Arias, 1992; Nubler-Jung, 1979), instruct cells to differentiate into neurons with specific molecular properties that enable their axons to select correct pathways. The cell bodies of axons would therefore be predicted by the boundary of regulatory gene expression, but their axons would not necessarily project parallel to it.

Combined in situ hybridization and immunocytochemistry studies have shown that the earliest neurons in the zebrafish forebrain differentiate along boundaries of regulatory gene expression (Macdonald et al., 1994). For example, the nuclei of the tract of the posterior commissure (nTPOC) and of the medial longitudinal fasciculus (nMLF) both develop and extend axons along the ventral boundary of *Pax-6* and *rtk-1* expression. In addition, a recent study in the chick shows that the combined expression of *Islet-1*, *Lim-1* and two other LIM homeobox genes *Islet-2* and *Lim-3*,

defines subclasses of motor neurons and may enable these axons to select correct pathways to their peripheral targets (Tsuchida et al., 1994). *In vitro* coculture and transplantation experiments show that *Sonic hedgehog*, (*Shh*), a morphogen that specifies diverse ventral cell fates (Marti et al., 1995; Roelink et al., 1995), is involved in the development of serotonergic neurons of the hindbrain raphe nucleus (Yamada et al., 1991), and dopaminergic neurons of the midbrain substantia nigra (Hynes et al., 1995). It is possible that interactions between *Shh*, which is expressed along the zli of the murine thalamus and in ventral regions of the diencephalon and telencephalon (Ericson et al., 1995), *Pax-6* and *Wnt-3* play a role in specifying the neuronal fate of thalamic cells that project to the cortex. Interactions between the protein product of *Shh* (SHH), and *Pax-6* (PAX-6), have been demonstrated in the zebrafish optic vesicle (Macdonald et al., 1995; Ekker et al., 1995). In addition, interactions between *Shh*, *Pax-1*, *Wnt-1* and *Wnt-3* are important in somite myogenesis (Johnson et al., 1994; Fan & Tessier-Lavigne, 1994), and cell fate determination within the somite has striking parallels to that within the neural tube. It is possible that networks of regulatory genes are conserved not only between species but also in different regions of the same embryo where they may have diverse roles. Preliminary studies in the *Pax-6* mutant mouse Small eye have shown a depletion of thalamocortical axons (Schmahl et al., 1993). It would be interesting to study the expression of *Shh* and the thalamocortical tract in *Pax-6* mutant mice (Sey/Sey) (See Chapter 5).

Corticospinal tract

DiA labelled axons in the tectum originating from sites in the posterior cortex suggested they were collateral branches of the corticospinal tract. This result was

surprising because, studies in the rat suggest that collateral branches of the corticospinal tract to the tectum are absent in E14.5 mouse. In the rat, axons from layer 5 cells destined for the spinal cord are in the midbrain by E17 (De Carlos & O'Leary, unpublished observations) (which corresponds to E16 in the mouse), but collateral branches from these axons do not reach the superior colliculus until postnatal day 1 (P1) (De Carlos & O'Leary, 1992), equivalent to P0 in mice. If axons are present then cell bodies must be present, which is also surprising since birthdating studies suggest that the only cells present in E15 posterior neocortex are those of the subplate, layer6 and marginal zone but not those of layer 5 (Berry & Rogers, 1965; Bayer & Altman, 1991). Layer 6 and subplate cells do not project to the superior colliculus (De Carlos & O'Leary et al., 1992). These conflicting observations more likely reflect a difference in techniques than a difference between the rat and mouse. The De Carlos and O'Leary study injected dyes into different sites and left them for much shorter periods of time (weeks to months compared to one year in this study). It is possible that the tracts were present in E15 rats but were insufficiently labelled. The collateral branch of the corticospinal tract to the superior colliculus in the tectum may correlate with a transverse band of *Pax-6* expression on the lateral surface of the tectum. It will be essential to combine these techniques in the same embryo to assess a direct correlation between the expression and the tract.

CHAPTER 5

AXON TRACTS AND DLX-2 EXPRESSION IN EMBRYONIC SMALL EYE MICE

5.1 ABSTRACT

In this chapter I investigated whether or not the retinothalamic tract formed in Sey/Sey embryos. Although Sey/Sey embryos have no eyes, crystals of DiI placed into the region of the residual optic cup labelled axons in the diencephalon. The tract resembled the retinothalamic tract in wild-type embryos although features of it were clearly abnormal. The optic nerve appeared thinner than normal, and there did not appear to be an optic chiasm in Sey/Sey brains. In wild-type embryos the optic tract is tightly restricted to ventral regions of the diencephalon as it projects through the ventral thalamus. In Sey/Sey embryos the optic tract was broader than normal in the ventral diencephalon but was still restricted to ventral regions of the ventral thalamus.

5.2 INTRODUCTION

In wild-type embryos the retina develops from two layers of neural ectoderm cells in the optic cup. The inner layer forms the neural retina and the outer forms the pigmented retinal epithelium. During normal development, axons from retinal ganglion cells project along the optic stalk, an extension of the ventral diencephalon which connects the retina to the forebrain. The first retinal axons to reach the optic chiasm in the floor of the forebrain pass directly into the optic tract on the same side of the brain, without encountering axons from the other eye (Godement et al., 1990; Marcus & Mason, 1993; Sretavan & Reichardt, 1993; Sretavan et al., 1994). Later projecting retinal axons are segregated into crossed and uncrossed projections at the optic chiasm. Axons from cells in temporal regions of the retina project to ipsilateral diencephalon, whereas axons from cells in nasal regions of the retina project to contralateral diencephalon (Fig. 3A, chapter 1). The position of a ganglion cell in the retina and molecular cues in the optic chiasm determine the ipsilateral or contralateral pathway that is followed at the chiasm (Guillery et al., 1995). Although axons leave the eye with a rough retinotopic order, they intermingle as they pass along the optic nerve (Horton et al., 1979; Naito, 1986, 1989; Reese & Baker, 1993; Colello & Guillery, 1990; Baker & Colello, 1994; Chan & Guillery, 1994). As retinal axon growth cones approach the chiasm and tract, they move along radial glia processes to the pial surface. Once through the optic chiasm, nasal axons disperse across the width of the tract and overlap the distribution of temporal axons in the middle of the tract (Torrealba et al., 1982; Cucchiaro & Guillery, 1984; Chan & Guillery, 1994). It has been suggested that uncrossed (temporal) axons are dependent on crossed (nasal) axons from the other eye for their progression into the

optic tract (Godement et al., 1987b; Guillery, 1989; Chan & Guillery 1993; Taylor & Guillery, 1995a), although this view is not universally accepted (Sretevan & Reichardt, 1993; Sretavan & Shatz, 1987). In contrast, dorsal and ventral retinal axons segregate into the anterior and posterior aspects of the tract, respectively (Torrealba et al., 1982; Simon & O'Leary, 1992a; Reese & Baker, 1993; Chan & Guillery, 1994). This segregation arises in the optic chiasm and is maintained thoroughout the optic tract to the lateral geniculate nucleus (LGN), the visual relay centre in the thalamus.

Sey/Sey embryos have no eyes but structures reminiscent of the optic cup and optic stalk develop (Grindley et al., 1995). In the optic cup two neuroepithelial layers form but they are morphologically similar and their differentiation into distinct layers of the neural retina and pigmented retinal epithelium is disrupted (Grindley et al., 1995).

I investigated whether the optic pathway formed in Sey/Sey embryos.

5.3 MATERIALS AND METHODS

E14.5 embryos were dissected from deeply anaesthetized (0.3ml urethane in 0.1M PBS) mothers in ice-cold PBS. The heads were removed and placed in 4% paraformaldehyde overnight. Sey/Sey embryos were easily identified by the lack of eyes and a shortened snout. Crystals of DiI were placed in the region of the optic cup located at the base of a blood vessel that terminates in the eye in wild-type embryos. This blood vessel is present and easily identified in Sey/Sey embryos. It was assumed that by placing the crystal here it would label the optic nerve. The crystals of DiI

were left to diffuse for 1 year in 4% paraformaldehyde in the dark. In some cases the embryos were placed in the oven at 37°C and left to diffuse for 2 weeks in 4% paraformaldehyde. After appropriate diffusion times, the pial membrane was peeled away, the cortical hemispheres were removed and the brian was sliced along the dorsal midline. The hemisections were cleared in 1:1 glycerol:PBS and then 4:1 glycerol:PBS before viewing under epifluorescence microscopy with a rhodamine filter. Photographs were taken using Fujichrome 400 film.

5.4 RESULTS

In wild-type embryos, crystals of DiI placed in the optic cup clearly labelled the optic nerve, optic chiasm and optic tract on the ventral surface of the brain (Fig. 1A). Axons of the optic tract labelled on the lateral surface of the diencephalon were tightly fasciculated in the ventral thalamus (arrow in Fig. 1B). The fibres were defasciculated in the dorsal thalamus, some had projected dorsally and others had turned through 90° to grow caudally along the brainstem. In Sey/Sey embryos (n=7) crystals of DiI placed in the region of the residual optic cup labelled axons along the ventral margin of the residual optic stalk (Fig. 1C). It is not clear which cells these axons extend from as the retina is severely abnormal in Sey/Sey mice, but this tract probably corresponds to the optic nerve in wild-type embryos. In mutant embryos an optic chiasm was not visible, although axons did invade diencephalic tissue (arrow in Fig. 1C). These axons projected on the lateral surface of the diencephalon as a fasciculated bundle and were in general restricted to ventral regions of the ventral thalamus as in wild-type embryos (compare Fig. 1B and Fig. 2A). The fibres were defasciculated in the dorsal diencephalon, as normal, and the majority projected

dorsally (Fig. 2B). This tract probably corresponds to the optic tract in wild-type embryos. At the dorsal edge of the diencephalon some axons had turned to grow caudally (arrows in Fig. 2B and Fig. 2C).

In Sey/Sey embryos, *Dlx-2* was expressed on the medial surface of the ventral diencephalon from the sch to the pep as normal (Fig. 2D). *Dlx-2* expression was very weak in the ventral thalamus (see Chapter 3 for details of *Dlx-2* expression in Sey/Sey embryos). Retinothalamic axons were labelled on the lateral surface of the ventral diencephalon and they did not contact cells that expressed *Dlx-2* mRNA on the medial surface.

DiI crystals placed in the region of the residual optic cup in Sey/Sey embryos also labelled a longitudinal tract that projected through the tectum and mesencephalon (Fig. 2E). This tract probably corresponds to the mesencephalic tract of the trigeminal nerve (tmesV) in wild-type embryos (see discussion). In addition, DiI crystals placed in the region of the optic cup in Sey/Sey embryos labelled a group of cell bodies and axons just ventral to the optic nerve. The group of cell bodies probably corresponds to the ciliary ganglion in the orbital cavity, and the axons probably correspond to preganglionic and postganglionic fibres of the oculomotor nerve. This tract has not been reported previously in Sey/Sey mice.

Attempts were made to label thalamocortical and corticothalamic axons in Sey/Sey embryos by inserting crystals of DiI and DiA into the cerebral hemispheres. Unfortunately the dyes diffused in the cerebral ventricles making it impossible to see discrete axonal tracts. These experiments are currently being repeated using DiI microinjection rather than insertion of crystals.

Fig. 1. DI-labelled tracts of the optic pathway in E14.5 +/+ (A,B), and Sey/Sey (C) embryos. Crystals of DiI were placed in the back of the eye in +/+ embryos and in the region of the optic cup in Sey/Sey embryos one year before dissection. (A) Ventral view of +/+ brain. The tracer has been transported along the optic nerve and through the optic chiasm into the optic tract. (B) Lateral view of +/+ diencephalon. The cortical hemispheres have been removed and the brain has been sliced along the midline and laid on its cut surface. DiI-labelled axons of the optic tract are seen emerging from the floor of the forebrain and projecting through ventral regions of the ventral thalamus as a tightly fasciculated bundle. In the dorsal thalamus the axons are defasciculated and some have turned to project caudally. (C) Ventrolateral view of Sey/Sey brain showing a thin tract emerging from the DiI crystal (marked by an asterisk). Arrow indicates the point where the tract enters the floor of the forebrain. Scale bars=100μm (A&B) and 200μm in C.

Figure 1

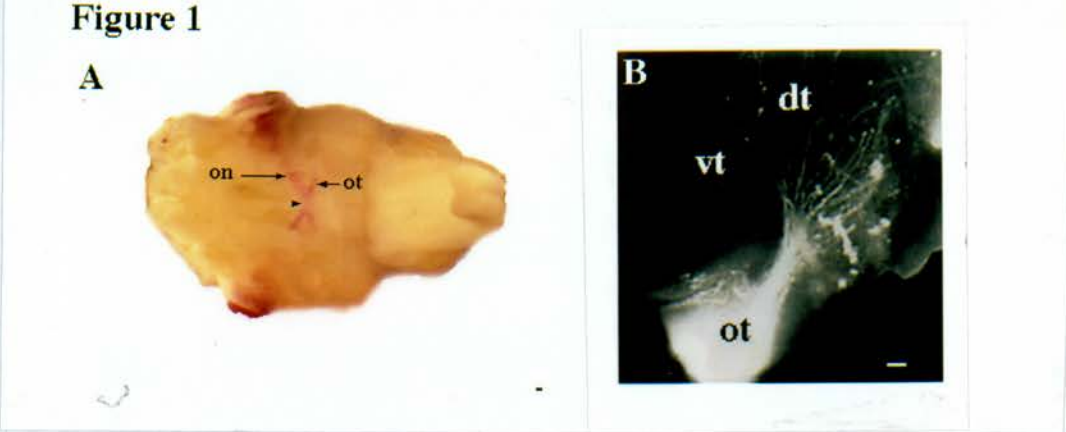
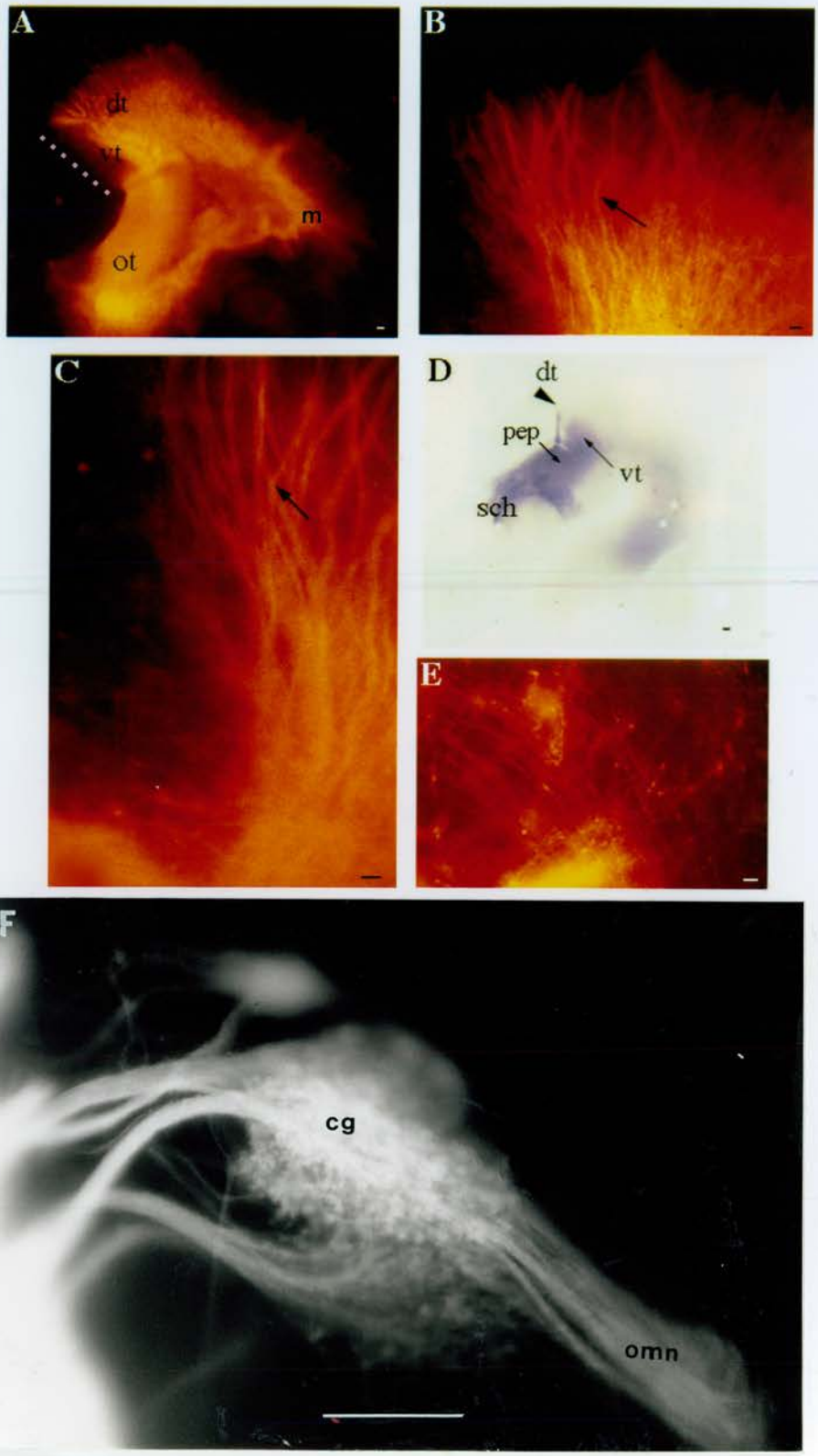


Fig. 2. DiI-labelled axon tracts and Dlx-2 mRNA expression in E14.5 Sey/Sey embryos. Crystals of DiI were placed in the region of the optic cup 1 year before dissection. Dlx-2 expression was revealed by whole mount in situ hybridization. (A-F) show a lateral view of brain. The cortical hemispheres have been removed and the brain has been sliced along the midline and laid on its cut surface. (A) Axons labelled on the lateral surface of the diencephalon are seen projecting from the floor of the forebrain to the dorsal diencephalon as a fasciculated bundle. In the dorsal thalamus the axons are defasciculated. A longitudinal tract is labelled in the mesencephalon. Dotted line marks the edge of the tissue. (B, C) Details of (A) showing defasciculated axons in the dorsal thalamus. Arrows mark axons that have turned to project caudally. (D) Dlx-2 expression on the medial surface of the diencephalon is normal from the sch to the pep but very weak in the ventral thalamus (see chapter 3 Fig. 4. for details). (E) Detail of (A) showing longitudinal axons labelled in the mesencephalon. (F) Axons and cell bodies labelled by the DiI crystal which is just visible in the bottom left hand corner of the picture. The cell bodies lie in the ciliary ganglion, cg, and the axons correspond to the autonomic preganglionic and postganglionic fibres of the oculomotor nerve, omn. Scale bars=100μm (A-F).

Figure 2



5.4 DISCUSSION

In Sey/Sey embryos, the retinothalamic tract formed although some features were clearly abnormal. The main results of this study in Sey/Sey embryos are summarized as follows: (1) the optic nerve formed; (2) the optic chiasm did not appear to form; (3) the optic tract formed.

In wild-type embryos, the optic nerve projects on the ventral surface of the optic stalk to the optic chiasm. This study showed that the optic nerve develops in *Sey/Sey* embryos even though differentiation of the retina is severly disrupted. The optic nerve appeared to contain fewer axons than normal, but those that formed fasciculated with each other. It is unclear which cells these axons originated from in the residual optic cup. Immunohistochemical studies show that although two neuroepithelial layers form in the residual optic cup of Sey/Sey embryos, their differentiation into the RPE in particular, and neural retina is disrupted (Grindley et al., 1995). Their study did not absolutely exclude retinal ganglion cells from the residual optic cup and it is possible that some do develop in Sey/Sey embryos. It will be essential to study the expression of a neural retina specific marker in Sey/Sey embryos. It will also be important to confirm that the tract labelled from the residual optic cup in this study is neuronal by immunostaining with a neuron specific marker.

In Sey/Sey embryos the optic chiasm did not appear to form. There are at least four explanations that may account for this: (1) the molecular properties of axons of the optic nerve are altered, so that they cannot respond to cues in the optic chiasm, that would normally guide them to the same or opposite side of the brain, (2) the cellular and molecular environment of the optic chiasm is altered in Sey/Sey embryos and is not recognized by axons of the optic nerve, (3) the tissue in the region of the optic

stalk and optic chiasm is distorted preventing axons from crossing the ventral midline, (4) the tissue was sectioned dorsal to the optic chiasm

It is possible that the molecular properties of axons in the optic nerve are altered, preventing recognition of guidance cues, and appropriate pathway selection at the optic chiasm. In wild-type embryos the pathway a retinal axon will select at the optic chiasm is determined in the retina, presumably by differential expression of CAMs or integrins on the growth cones of retinal axons. Several candidate molecules for specifying positional information within the retina have been described, but none have yet been shown to have a clear functional role (Kaprielian & Patterson, 1994). Some of these molecules, that include winged helix genes BF-1 and BF-2 (Hatini et al., 1994), and a novel homeobox gene (Deitcher et al., 1994), are expressed along the naso-temporal axis of the retina, and may be linked with the recognition of appropriate pathway cues by nasal and temporal axons (Walter et al., 1987; Godement & Bonhoeffer, 1989; Simon & O'Leary, 1992b). Other molecules that include *Pax-2* (Nornes et al., 1990) have a dorso-ventral differential distribution in the retina (Trisler et al., 1981; Constantine-Paton et al., 1986; McCaffery et al., 1990; Rabbachi et al., 1991), and may underlie the segregation of dorsal and ventral axons in the optic tract. In Sey/Sey embryos, the phenotype of axons projecting from the residual optic cup, may be altered so that they no longer express correct CAMs or integrins that allow for appropriate pathway selection at the optic chiasm and within the optic tract.

It is also possible that the cellular and molecular environment of the optic chiasm is altered in Sey/Sey embryos and is not recognized by axons of the optic nerve. In wild-type embryos, the cellular environment at the optic chiasm is relatively simple at early stages in development. Retinal axons from each eye come into contact with

radial glia of the diencephalon, specialized glia that straddle the midline (McKanna, 1993; Marcus et al., 1995), axons of the other eye, and early developing nerve cells of the anterior hypothalamus that form the tract of the postoptic commissure (tpoc) (Windle, 1935; Windle & Baxter, 1935; Herrick, 1942; Magoun & Hanson, 1942; Taylor, 1991; Easter et al., 1993, 1994; Sretevan et al., 1994). As the growth cones of retinal axons approach the optic chiasm they contact and grow along the radial processes of glia that extend from the ependymal layer of the 3rd ventricle to the pial surface. *In vitro* studies suggest that temporal retinal axons are prevented from crossing the midline by an unidentified inhibitory signal in the optic chiasm, whereas nasal axons grow freely through the chiasmatic midline (Godement et al., 1990; Wizemnann et al., 1993; Wang et al.,1992). It is possible that the midline glia, a specialized subpopulation of diencephalic radial glia that allow nasal retinal axons to project to the opposite side of the brain, are disrupted in Sey/Sey embryos. This may prevent axons from crossing the midline and restrict them to an ipsilateral pathway.

In Sey/Sey embryos, the third ventricle, which is continuous with the lumen of the optic stalk, is enlarged and distorts the floor of the forebrain. It is possible that the enlarged third ventricle prevents axons of the optic nerve from crossing the midline to the opposite side of the brain.

It is also possible that the brains were sliced dorsal to the optic chiasm and the absence of an optic chiasm may be a sectioning artefact. With the sectioning technique used this is unlikely but will be confirmed by studying the trajectory of retinothalamic axons in Sey/Sey brains labelled from a crystal of DiI placed in the optic cup of one side of the brain only. Subsequent detection of DiI-labelled axons in the contralateral diencephalon would suggest that axons can cross the ventral midline in Sey/Sey embryos and that at least some features of the chiasm are retained in

mutant mice. If DiI-labelled axons are only detected in ipsilateral diencephalon, it is unlikely that the optic chiasm forms in Sey/Sey embryos.

Axons were also labelled on the lateral surface of the diencephalon suggesting that an optic tract formed in Sey/Sey embryos. In wild-type embryos, the optic tract projected from the optic chiasm to the lateral surface of the diencephalon as a narrow fasciculated bundle of axons. In the region of the hypothalamic cell cord, the tract broadened and then constricted in the ventral thalamus. In the dorsal thalamus, axons were defasciculated and some projected caudally to the tectum. In Sey/Sey embryos, the optic tract appeared broader than normal, but was still mainly restricted to ventral regions of the ventral thalamus. Previous studies have shown that the first retinal axons in the diencephalon project along the dorsal edge of the tpoc (Easter & Taylor, 1989; Taylor, 1991; Marcus & Mason, 1993; Sretevan et al., 1994). It is possible that axons of the tpoc are also distributed more broadly in Sey/Sey embryos thereby influencing the trajectory of axons of the optic tract. It would be interesting to study the trajectory of the tpoc in Sey/Sey embryos.

One interesting finding was that in Sey/Sey embryos, the trajectory of retinothalamic axons on the lateral surface of the diencephalon appeared to overlap but not contact the altered pattern of *Dlx-2* expression on the medial surface, as in wild-type embryos. It is possible that *Dlx-2* patterns adjacent tissue to express guidance cues that retinothalamic axons use to navigate to the thalamus. In Sey/Sey embryos the pattern of *Dlx-2* expression is disrupted and this may affect the distribution of downstream targets regulated by *Dlx-2*. Putative downstream targets may include CAMs or ECM molecules that guide pioneering retinothalamic axons to their correct

targets. If these cues are disrupted we might expect disruption of the retinothalamic tract.

Crystals of DiI inserted into the optic cup also labelled a longitudinal tract extending from the most anterior prosomere, P6, to the mesencephalon. The tract in the mesencephalon probably corresponds to the descending tract of the mesencephalic nucleus of the trigeminal nerve (dtmesV) in wild-type embryos. In wild-type embryos the early tract of the postoptic commissure (tpoc) projects caudally from the optic chiasm in P6 to merge with the pre-existing dtmesV in p1 and the mesencephalon, and then continues into the rhombencephalon.

Crystals of DiI inserted into the optic cup also labelled axons and a ganglion of cell bodies just ventral to the optic nerve. In wild-type embryos pre- and postganglionic fibres of the oculomotor nerve innervate the muscles of the iris and lens, and their cell bodies lie in the ciliary ganglion in the orbital cavity. In Sey/Sey embryos cell bodies are labelled in the orbital cavity and probably correspond to the ciliary ganglion. The labelled axons probably correspond to pre- and postganglionic fibres of the oculomotor nerve.

CHAPTER 6

SUMMARY AND FUTURE EXPERIMENTS

In this thesis, I investigated possible roles for cortex-derived diffusible growth factors in prenatal thalamocortical development using *in vitro* coculture techniques. Coculture techniques have been used by a number of groups (Yamamoto et al., 1989, 1992; Bolz et al., 1990, 1992; Molnar & Blakemore, 1991; Toyama et al., 1991) to study *contact dependent* interactions between the thalamus and cortex *in vitro*. In this study slices of E14.5 thalamus and embryonic or postnatal cortex were plated *at a distance* on collagen membranes to study interactions between the tissues mediated by diffusible factors from the cortex. The results showed that slices of embryonic cortex did not stimulate increased outgrowth from slices of thalamus, whereas postnatal cortex did stimulate significantly more outgrowth from slices of thalamus compared to control levels. It is therefore unlikely that an embryonic cortex-derived diffusible factor initiates neurite outgrowth from the thalamus *in vivo.* The stimulatory effect of the postnatal cortex on neurite outgrowth from embryonic thalamus confirmed earlier studies (Rennie et al., 1994; Lotto & Price, 1995) and suggested that cortically derived growth factors are more likely to play a role in the growth and refinement of thalamocortical axons once they have innervated the cortex.

The question therefore remains as to what stimulates thalamic cells to extend axons. One possibility suggested was that diffusible factors, released from intermediary targets, or regions through which the developing thalamocortical tract projects, may initiate and/or guide thalamic axons to the internal capsule. Candidate structures include the perireticular nucleus (pn), the reticular nucleus (rn) and the medial ganglionic eminence (mge). Future work might involve coculturing slices of embryonic thalamus with slices of the embryonic pn, rn and mge to investigate the

possibility of diffusible growth-promoting and chemotropic interactions between the thalamus and these structures *in vitro*.

I also studied the effect of embryonic and postnatal cortex on neurite length from slices of embryonic thalamus. The results indicated that the embryonic cortex released diffusible factors that inhibited the growth of most embryonic thalamic neurites, whereas postnatal cortex released factors that stimulated the growth of most thalamic neurites. The *in vivo* relevance of these *in vitro* findings must be treated with caution, but it is possible that diffusible growth inhibitory and stimulatory molecules secreted by embryonic and postnatal cortex respectively may influence the growth of thalamic axons once they have reached the cortical plate. Combined DiI tract tracing and immunohistochemical studies have shown that axons of the early thalamocortical tract project in the chondroitin-rich subplate before turning to innervate their appropriate cortical areas (Bicknese et al., 1994). Inhibitory molecules secreted by the neocortex may act in combination with growth permissive substrate-bound molecules in the subplate to prevent premature innervation of the cortex by thalamic axons. The inhibitory effect described here differs from the findings of Hubener et al., (1995), who concluded that avoidance of the early neocortex by thalamic axons was not due to an inhibitory molecule, but to maturation-dependent upregulation of a growth permissive molecule in the subplate and then later in the cortical plate. In their study, dissociated thalamic cells were plated on membrane preparations of embryonic cortex, and the effects of any diffusible inhibitory factors would have been concealed. Experiments are in progress in the lab to isolate and characterize the factor(s) released from postnatal cortex. It would be interesting to extend these studies to embryonic cortex to look for putative inhibitory molecules. This would involve western blot analysis on conditioned culture medium to compare

the sizes of released factors to those of known inhibitory growth factors. If the factor is novel the next stage would be to isolate it, first roughly by size fractionation and then purify it using one of several techniques: (i) ion-exchange chromatography, (ii) molecular exclusion chromatography or (iii) HPLC.

I also investigated whether normal PAX-6 protein is required for diencephalic regionalization by examining morphological features and patterns of regulatory gene expression (*Dlx-2* and *Wnt-3*) in the embryonic diencephalon of Small-eye mice (*Pax-6* mutants). All prosomeres were identified in Sey/Sey mice, suggesting that normal PAX-6 protein is not required for some degree of diencephlaic regionalization to occur. However, prosomeres 2 and 3 of the diencephalon were strikingly abnormal: (i) they were smaller and contained fewer cells than normal, (ii) the difference in cell density between p2 and p3 was lost and the difference in cell numbers between them was reduced, (iii) there was reduced expression of *Dlx-2* in p3 and *Wnt-3* in p2 and, (iv) *Dlx-2* expression appears to fade out at the p2/p3 border rather than terminating abruptly as in wild-type embryos.

These abnormalities may arise from excessive cell death and/or disruption of cell proliferation, migration and/or differentiation in the diencephalon of Sey/Sey mice. It would be interesting to assess Sey/Sey embryos for signs of cell death using a marker of cell death such as bisbenzamide. I intend to investigate the rate of neuronal proliferation in the diencephalon of E10.5-E18.5 Sey/Sey embryos and compare this to the rate in wild-type embryos using the BrdU labelling technique. Sey/Sey embryos up to age E18.5 (beyond the period of neurogenesis in wild-type embryos) will be included in the study to investigate whether neurogenesis is simply delayed rather than reduced in Sey/Sey embryos. The BrdU labelling technique involves

intraperitoneal injection of pregnant mice with BrdU. After 60 minutes the injected mice are sacrificed, the embryos harvested, fixed and sectioned for BrdU immunohistochemistry. The rate of proliferation is expressed as the proportion of BrdU-labelled cells to the total number of cells.

Previous studies show that migration of cortical cells is disrupted in Sey/Sey mice (Schmahl et al., 1993). The BrdU-labelling technique could be used to investigate migration of diencephalic cells in Sey/Sey embryos. This involves intraperitoneal injection of pregnant mice with BrdU as above, but the injected mice are sacrificed 3 days later by which time the cells should have migrated from the ventricular zone to their final positions. The embryos are then processed for BrdU immunohistochemistry as above. The time and place of 'birth' of each type of neuron and its subsequent movement can thus be reconstructed in both wild-type and Sey/Sey mice.

The reduced expression of *Dlx-2* mRNA in p3 and *Wnt-3* mRNA in p2 may reflect a disruption of differentiation in the diencephalon of Sey/Sey mice. A recent immunohistochemical study (Porteus et al., 1994), revealed coexpression of DLX-2 and MASH-1 (a marker of relatively undifferentiated cells, Guillemot et al., 1993) in cells of the ganglionic eminence and ventral thalamus of wild-type embryos. These cells lay just above BrdU labelled ventricular cells and beneath MAP-2 (a marker of terminal neuronal differentiation, Garner et al., 1988) labelled mantle cells. It is possible that DLX-2 and MASH-1 directly or indirectly regulate the transition of cells from a relatively undifferentiated state to terminal differentiation (Porteus et al., 1994). I intend to study the expression of MASH-1 and MAP-2, in Sey/Sey embryos to investigate the hypothesis that *Pax-6* directly or indirectly regulates the differentiation of diencephalic cells.

The loss of cell density and cell number differences between p2 and p3 in Sey/Sey embryos may reflect abnormal cell mixing between p2 and p3. In wild-type embryos the cell adhesion molecule R-cadherin delineates p3, and α-catenin delineates p2. It is possible that cells are prevented from crossing the p2/p3 border by local differences in cell adhesion between adjacent prosomeres (Ganzler & Redies, 1995). Classic cell lineage restriction studies where a neuroblast is labelled in the neuroepithelium, and the movement of subsequent progeny are followed, are technically very difficult in the very early embryonic mouse. Therefore, it would be interesting to investigate cell mixing indirectly by studying the expression of N-cadherin and α-catenin in the same Sey/Sey embryo using double-label immunohistochemistry. If the expression patterns are disrupted this may indicate abnormal cell mixing at the p2/p3 border.

I also investigated whether the pathways of developing axonal tracts correlate with patterns of regulatory gene expression. The tract tracing and whole mount in situ hybridizations results suggested a correlation between the cell bodies of thalamocortical neurons in the ventral thalamus and *Pax-6* mRNA expression on the lateral surface of the ventral thalamus. It is possible that *Pax-6* regulates the differentiation of thalamocortical axons. In zebrafish, *Pax-6* mRNA expression correlates with the position at which neurons of the nTPOC and nMLF differentiate and extend axons. In the present study, the tract tracing and in situ hybridizations were in separate embryos. In order to demonstrate a direct correlation it will be essential to develop a double-labelling technique to visualize thalamocortical neurons and their cell bodies, and *Pax-6* expression *in the same embryo*. The developing thalamocortical tract of E14.5 embryos (sectioned down the dorsal midline with the

cerebral hemispheres removed), could be labelled using the TUJ1 antibody to neuron specific class 111 beta-tubulin (Moody et al., 1987). Following this treatment the immunostain is stable and the tissue could be taken through the whole-mount in situ hybridization protocol. This technique, combining immunocytochemistry and ISH, is well established in a number of laboratories and has been successfully used in embryos up until the age of E9. Although it is a relatively straightforward method, and is therefore worth trying, there may be problems distinguishing discrete axonal tracts in brains older than E10.5. An alternative method would be to combine whole-mount ISH with DiI neuronal tracing. A crystal of DiI placed into the cerebral hemisphere or internal capsule, would provide a more specific label than the TUJ1 antibody, which labels all axons indiscrimininately. The embryos are first taken through whole mount in situ hybridization with the removal of harsh permeabilizing agents (that may disrupt the axonal membranes, along which DiI diffuses) and replacement with milder detergents such as RIPA. Following ISH, a small crystal of DiI is placed into the cerebral hemisphere or internal capsule of the same *Pax-6*-labelled brains to visualize thalamocortical axons and cell bodies.

If *Pax-6* does specify the fate of thalamocortical neurons, we would predict an absence, or severe disruption of the tract in *Pax-6* mutant mice (Small eye). Previous studies have shown a depletion of this tract (Schmahl et al., 1993). It would be interesting to study the development of this tract in Sey/Sey embryos.

I also investigated development of the retinothalamic tract in Sey/Sey mice using the fluorescent neuronal tracer DiI. Although Sey/Sey embryos have no eyes, a tract resembling the retinothalamic tract in wild-type embryos developed in Sey/Sey embryos, but it was clearly abnormal: (i) the optic nerve appeared to contain fewer

axons, (ii) an optic chiasm did not appear to form, and (iii) the optic tract formed but was broader than normal. It will be essential to confirm that this is a neuronal tract with the neuron-specific marker such as the TUJ1 antibody or MAP-2. To confirm that an optic chiasm is absent Sey/Sey embryos a crystal of DiI could be inserted into the optic cup on one side of the brain only. If there are no DiI-labelled axons in the contralateral diencephalon it is likely that an optic chiasm does not form in Sey/Sey mice.

APPENDIX

A1 RIBOPROBES

The *Dlx-2* antisense riboprobe was transcribed from a 1.7kb fragment derived from the 3' untranslated region of the *Dlx-2* cDNA clone (a gift from J.L.R. Rubenstein). The *Wnt-3* antisense riboprobe was transcribed from a 0.6kb fragment derived from a non-coding and nonconserved region of the *Wnt-3* cDNA clone (a gift from R. Nusse). The *Pax-6* antisense riboprobe was transcribed from a 1.7 kb fragment derived from the *Pax-6* cDNA clone (a gift from R. Hill). Sense probes were generated from these plasmids for controls.

Digoxigenin-labeled cRNA probes were synthesised by labeling these templates with digoxigenin-rUTP (Boehringer Mannheim). The *in vitro* transcription reaction was carried out as follows: 1μg of phenol chloroform extracted and ethanol precipitated, linearised template was incubated with T3 or T7 RNA polymerase 2U/μl in 40mM Tris-HCl, pH 8.0, 6mM $MgCl_2$, 10mM DDT, 10mM NaCl, 2mM Spermidine, RNase Inhibitor 1U/μl, 1mM each ATP, CTP and GTP, 0.65mM UTP and 0.35mM DIG-11-UTP at 37°C for 2 h. The reaction was completed by an incubation with 2U/μl of RNase-free DNase (Boehringer Mannheim) at 37°C for 15 mins and an ethanol precipitation in 20mM EDTA pH 8.0, 0.1 volumes LiCl at -70°C for 1 hr. Following centrifugation for 5mins at 4°C, samples were washed in 70% ethanol, dried, resuspended in 100μl DEPC-treated water and stored at -70°C. Labeling efficiency was checked by the direct detection method outlined in the Boehringer Mannheim DIG System User's Guide.

A2 IN SITU HYBRIDIZATIONS ON SECTIONED MATERIAL

Fixation and sectioning

1.Fix E15 embryos in 4% paraformaldehyde for 1-2.5 hours.

2. Wash 2 or 3 times in 0.1M phosphate buffer (1 hour at least or overnight at 4°C).
3. 2x 10 min washes in 70% ethanol (EtOH).
4. 2x 10 min washes in 95% EtOH.
5. 2x 20 min washes in 100% EtOH.
6. Wash in chloroform (few hours or overnight).
7. Infiltrate the tissue with paraffin wax. Place the embryos in a beaker of melted wax for 3 hours. Change the wax once.
8. Embed the embryos in plastic moulds and orientate into the correct position. Allow the mould to sit at room temperature until a skin forms then float the moulds in a tub of cold water.

Preparation and cleaning of slides

1. Clean slides in sulphur chromic acid for 24 hours.
2. Wash in running water for 24 hours to remove all acid. Slides are now chemically clean.
3. Rinse in distilled water.
4. Store in alcohol.

Tespa-coated slides

1. Add 1ml TESPA to 49 ml acetone. Dip slides into solution and allow to dry.
2. Dip slides in acetone and dry. Repeat.
3. Rinse in deionized dd water and dry.

Sectioning

1. Section tissue at 10μm.
2. Incubate the slides in a slide holder for 2 hours at 37°C.

Prehybridization treatments

1. Wash in xylene for 20 mins (removes wax).
2. Rehydrate through graded ethanol concentrations (100%-95%-70%) ≅ 15 secs each.
3. 0.2M HCl for 20 mins at room temperature (removes protein).
4. Put Proteinase K Buffer in 37° C water bath.
5. 2x 5 min washes in dd water
6. Add proteinase K to PKB [2μgml proteinase K; 40μl of stock (5mg/ml) to 100mls of PKB]. Incubate slides for 20-30 mins in PK at 37° C (permeabilization step, increases accessibility of probe to mRNA in tissue. Partially digests cross-linked proteins in the tissue. NB Prolonged incubation ie > 30 mins may lead to loss of signal from fixed sections and loss of tissue morphology.)
7. 10 mins in 0.2% glycine (prevents further digestion)
8. 5 min wash in 0.1M TEA, pH8.0
9. 10 min wash in 0.25% (v/v) acetic anhydride in 0.1M TEA. (Decreases the ability of charged probes to bind electrostatically to the tissue section.) NB acetic anhydride degrades quickly, add 250μl acetic anhydride to dish, add slides, add 100mls TEA buffer, remove air bubbles, leave for 10mins.
10. Remove slides from acetic anhydride. Remove excess moisture with tissue.
11. Place slides in hybridization tray. Add 200μl of prehybridization buffer (or enough to cover each section) to each slide. Cover with gel bond film. Put filter

paper soaked with 50% formamide and 4X STE in the bottom and lid of the tray. Seal the tray with nescofilm and incubate for 2 hours at 59° C.

Hybridization

1. Remove excess moisture from the slides. Add 100µl of DIG labelled probe and hybridization solution to each slide. [probe] = 150ng/ml. Cover each slide with a strip of gel bond film.
2. Incubate slides for 15 mins at 95° C.
3. Reduce temperature to 59° C and incubate for a further 2 hours.
4. Preincubate RNAse buffer at 37° C.

Posthybridization treatments

1. Remove coverslips with as little damage to the sections as possible. Put forceps under one edge and flip off.
2. 2x 5 min washes in 2x SSC
3. 30 min incubation at 37° C in 20µg/ml RNAse A. (For detecting less abundant RNAs it may be possible to reduce backgrounds further by including 1 unit RNAse T1 per ml, because this enzyme hydrolyzes some sequences that are resistant to RNAse A.)
4. Preincubate 0.1x SSC/30% formamide at 40 °C.
5. 2x 5min washes in 2X SSC.
6. 15 min wash in 0.1X SSC/30% formamide at 40 °C.
7. 30 min incubation in 4% Blocking Solution (diluted in Buffer 1)
8. 2 hour incubation at room temperature with alkaline-phosphatase conjugated with polyclonal sheep anti-DIG antibody diluted 1:1000.
9. 2x 15 min washes in 2x TBS.

10. 3 min wash in TBS (pH9.5) containing 50mM $MgCl_2$

11. Colour reaction. Solution for 10 slides (100µl/slide): 4.5ml NBT, 3.5ml X-Phosphate, 400ml levamisole, 50ml MgCl2, 500ml 0.2M TBS pH9.5, 42ml dd water. Incubate in humid light-proof box for 20 mins- 1 hour.

12. Stop reaction by placing slides in 100mM TRIS-HCL pH 8.0 1mM EDTA.

13. Dehydrate in pure absolute ethanol (methanol contaminants can decrease staining by dissolving NBT.)

14. Clear in xylene. Keep slides covered with xylene until ready to mount.

15. Mount in DPX. and coverslip.

A3 WHOLE MOUNT IN SITU HYBRIDIZATION

Embryonic day 14.5 (E14.5) mouse brains were dissected and fixed overnight in 4% paraformaldehyde at 4 C. The embryos were dehydrated through an ascending series of methanol, bleached for 6 hours in 5:1 methanol/30% hydrogen peroxide and treated with 10μg/ml proteinase K. They were then washed in 2mg/ml glycine in PBT (PBS containing 0.1% Tween-20) and refixed in 0.2% glutaraldehyde/4% paraformaldehyde. The embryos were treated with 0.1% sodium borohydride, washed in PBT, washed twice in hybridization buffer (50% formamide, 0.75M NaCl, 1X PE, 100μg/ml tRNA, 0.05% heparin, 1% SDS) and incubated at 63°C for 1 hour in hybridization buffer. They were then hybridized overnight at 63°C in hybridization buffer containing 1μg/ml digoxigenin(DIG)-labelled riboprobe, washed in Wash buffer 1 (WB1; 300mM NaCl, 1XPE at 63°C, WB 1.5 (50mM NaCl, 1X PE, 0.1% SDS) at 50°C and treated with 100μg/ml RNase A in RNase buffer (10mM Tris-Cl, 1mM EDTA, 500mM NaCl, 0.1% Tween-20) for 1 hour at 37°C. They were then washed in RNAse buffer and then in WB2 (50% formamide, 300mM NaCl, 1X PE, 0.1% Tween-20) for 30 minutes at 50°C. There followed a 30 minute wash in WB3 (50% formamide, 150mM NaCl, 1A PE, 0.1% Tween-20) at 50°C and then a 20 minute wash at 70°C in WB4 (500mM NaCl, 1X PE, 0.1% Tween-20). The embryos were then rocked in TBST containing 2mM levamisole and 10% heat-inactivated goat serum for at least 1 hour at room temperature. At this point the antibody was diluted to a concentration of 1/5000 in TBST containing 2mM levamisole, 1% freshly heat-inactivated goat serum and 4mg of heat inactivated embryo powder, rocked for 30 min at 4°C and centrifuged at 10,000G for 10 min at 4°C. The embryos

were incubated with the preabsorbed antibody overnight at 4°C. They were then washed 6 times for 1 hour each in TBST containing 2mM levanisole at room temperature, followed by two 20 min washes in freshly prepared NTMT (100mM Tris-Cl pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 0.1% Tween-20) containing 2mM levamisole at room temperature with rocking. The colour reaction was then started (4.5µl NBT; 3.5µl BCIP and 2mM levamisole in NTMT). The tubes were rocked for the first 5 minutes and then allowed to develop without rocking in the dark. The colour reaction was stopped by washing twice with NTMT, then extensive washing with 3 changes of PBT(pH5.5) containing 1mM EDTA. The embryos were postfixed in 4% paraformaldehyde/0.1% glutaraldehyde in PBS for 1 hour, washed several times in PBT and cleared by passing them through 1:1 glycerol/PBT and then into 4:1 glycerol/PBT for 1 hour each with rocking.

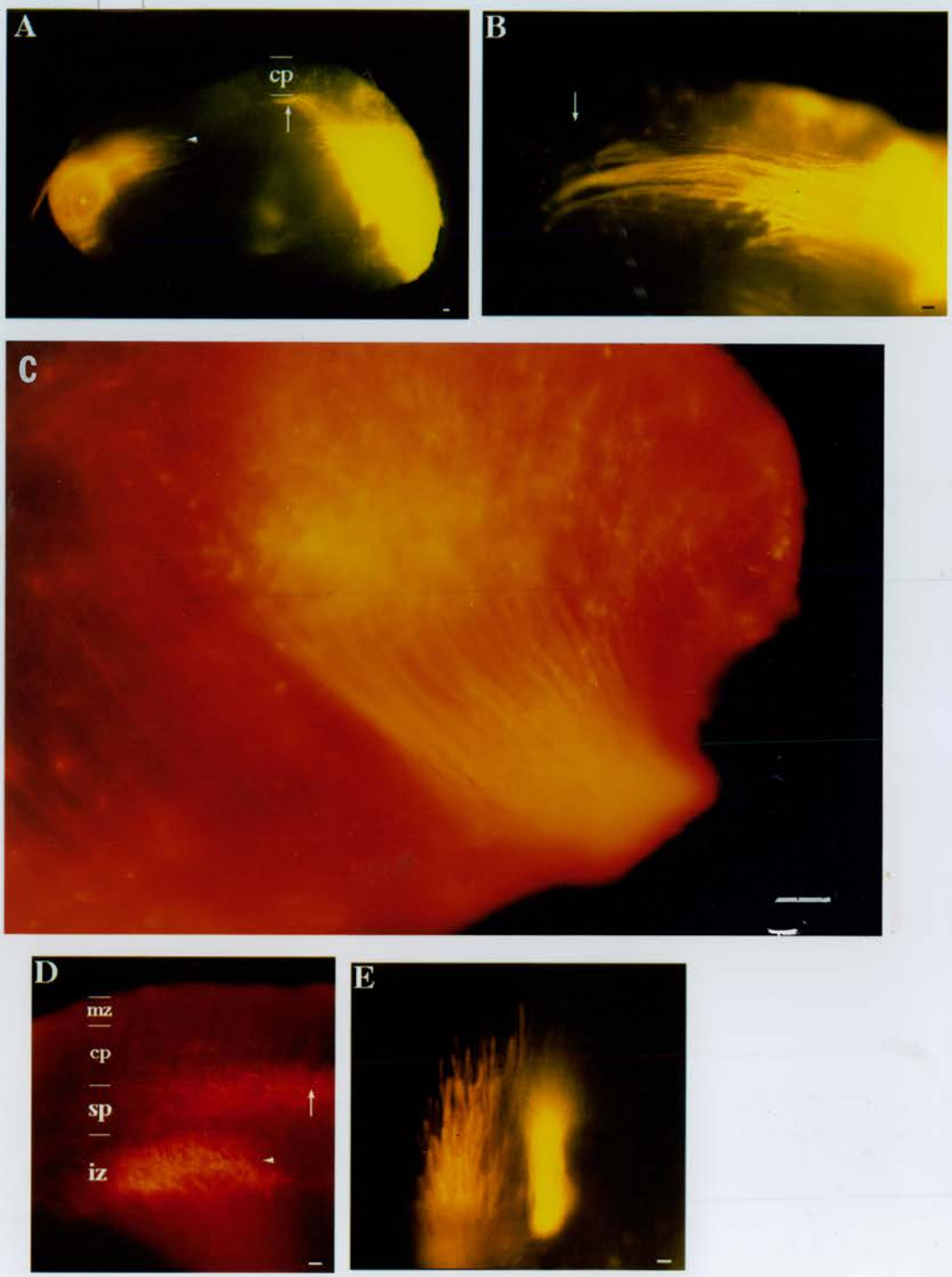
A 4 DiI AND DiA LABELLED TRACTS IN E18.5 MOUSE EMBRYOS

In agreement with previous studies (Bicknese et al., 1994) crystals of DiI placed in the anterior cortex and DiA in the posterior cortex of E18.5 embryos, labelled tracts that could be traced to the thalamus. In general, crystals of DiI in anterior cortex labelled cell bodies in the ventral thalamus and crystals of DiA in posterior cortex labelled cell bodies in the dorsal thalamus. Figure 1A shows the site of a DiI crystal in the anterior cortex and a DiA crystal in the posterior cortex. In this particular brain, the DiA crystal labelled axons in the subplate (arrow in Fig. 1A), and cell bodies in the cortical plate (arrow in Fig. 1B), and the DiI crystal labelled axons in the intermediate zone (arrowhead in Fig. 1A). The DiA labelled cell bodies in the cortical plate probably correspond to corticocortical axons retrogradelly labelled from the DiA crystal. In all brains examined, axons labelled in the subplate, either from crystals of DiA in posterior cortex or DiI in anterior cortex, could be followed through the basal telencephalon to cell bodies in the thalamus (Fig. 1C). The slice of diencephalon in Fig. 1C is from a brain, where a DiI crystal in the anterior cortex, labelled axons in the subplate that could be traced through the basal telencephalon to cell bodies in the thalamus. These cell bodies are probably thalamocortical neurons retrogradelly labelled from DiI in the anterior cortex. The DiI labelled axons in the intermediate zone (arrowhead Fig. 1A), were traced to the thalamus and probably correspond to corticothalamic axons which are known to project in the intermediate zone (Bicknese et al., 1994). In some brains a crystal of DiI in the anterior cortex also labelled three populations of cells in the cerebral hemisphere (Fig. 1D). Cells that lay adjacent to the pial edge were horizontally aligned and probably correspond to cells in the marginal zone. Beneath this population of cells lay radially aligned cells with

pyriform-shaped cell bodies and tall superficially directed apical dendrites. These cells probably correspond to neurons of the deep layers of the cortical plate (Bicknese et al., 1994). Horizontally oriented cells lay just beneath the cortical plate and probably correspond to cells of the subplate (arrow Fig. 1D). The DiI and DiA labelled tracts in Fig. 1A were traced into the basal telencephalon and were maintained as separate bundles of axons in the internal capsule (Fig. 1E).

Fig. 1. Axon tracts in E18.5 embryos. Crystals of DiI were placed in the anterior cortex and crystals of DiA in the posterior cortex one year before dissection. (A) Medial slice of cortex. DiI in the anterior cortex has labelled axons in the intermediate zone (arrowhead). DiA in the posterior cortex has labelled axons in the subplate (arrow) and cell bodies in the cortical plate. (B) Detail of DiA labelled region in (A): arrow indicates cell bodies in the cortical plate. (C) Lateral view of the diencephalon. The DiI has labelled one large band of cell bodies in the ventral thalamus and axons that project rostrally before dipping out of the plane of focus. (D) Anterolateral slice of cortex. The DiI has labelled horizontally aligned cells in the subplate (arrow), radially aligned cells in the cortical plate, horizontally aligned cells in the marginal zone (out of the plane of focus) and axons in the intermediate zone (arrowhead). (E) Medial section of the brain in the region of the internal capsule. Axons labelled from DiI crystals in the anterior cortex (orange) do not intermingle with axons labelled from DiA crystals in the posterior cortex (yellow). cp, cortical plate; mz, marginal zone; sp, subplate; iz, intermediate zone. Scale bars = 100μm (A-E).

Figure 1



BIBLIOGRAPHY

ANGEVINE, J.B. & SIDMAN, R.L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* **192**: 766-768.

ANNIS, C.M., EDMOND, J., & ROBERTSON, R.T. (1990). A chemically-defined medium for organotypic slice cultures. *Journal of Neuroscience Methods* **32**: 63-70.

APKARIAN, P., BOUR, L., & BARTH, P.G. (1994). A Unique Achiasmatic Anomaly Detected In Non-Albinos With Misrouted Retinal-Fugal Projections. *European Journal of Neuroscience* **6**: 501-507.

BAIER, H. & BONHOEFFER, F. (1992). Axon guidance by gradients of a target-derived component. *Science* **255**: 472-475.

BAKER, G.E. & COLLELLO, R.J. (1994) The origin and course of retinofugal axons during normal development of the ferret. *Soc. Neurosci Abstr* **20**: 449.9

BARTSCH, U., BARTSCH, S., DORRIES, U., & SCHACHNER, M. (1992). Immunohistological localization of tenascin in the developing and lesioned adult mouse optic nerve. *European Journal of Neuroscience* **4**: 338-352.

BAYER, S.A. & ALTMAN, J. (1990). Development of layer I and the subplate in the rat neocortex. *Experimental Neurology* **107**: 48-62.

BAYER, S.A. & ALTMAN, J. (1991). Development of the endopiriform nucleus and the claustrum in the rat brain. *Neuroscience* **45**: 391-412.

BERRY, M. & ROGERS, A.W. (1965). The migration of neuroblasts in the developing cerebral cortex. *J Anat* **99**: 691-709.

BICKNESE, A.R., SHEPPARD, A.M., OLEARY, D.D.M., & PEARLMAN, A.L. (1994). Thalamocortical axons extend along a chondroitin sulfate proteoglycan-enriched pathway coincident with the neocortical subplate and distinct from the efferent path. *Journal of Neuroscience* **14**: 3500-3510.

BIRGBAUER, E. AND FRASER, S. (1994). Violation of cell lineage restriction compartments in the chick hindbrain. *Development* **120**: 1347-1356.

BIXBY, J.L. & HARRIS, W.A. (1991). Molecular mechanisms of axon growth and guidance. *Annual Review of Cell Biology* **7**: 117-159.

BLAKEMORE, C. & MOLNAR, Z. (1990). Factors involved in the establishment of specific interconnections between thalamus and cerebral cortex. *Cold Spring Harbor Symposia on Quantitative Biology* **55**: 491-504.

BOLZ, J., NOVAK, N., GOTZ, M., & BONHOEFFER, T. (1990). Formation of target-specific neuronal projections in organotypic slice cultures from rat visual cortex. *Nature* **346**: 359-362.

BOLZ, J., NOVAK, N., & STAIGER, V. (1992). Formation of specific afferent connections in organotypic slice cultures from rat visual cortex cocultured with lateral geniculate nucleus. *Journal of Neuroscience* **12**: 3054-3070.

BOPP, D., BURRI, M., BAUMGARTNER, S., FRIGERIO, G., AND NOLL, M. (1986). Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. *Cell* **47**: 1033-1040.

BOVOLENTA, P. & DODD, J. (1991). Perturbation of neuronal differentiation and axon guidance in the spinal cord of mouse embryos lacking a floor plate: Analysis of Danforth's short-tail mutation. *Development* **113**: 625-639.

BRAY, D., WOOD, P., BUNGE, R.P. (1980). Selective fasciculation of nerve fibers in culture. *Exp Cell Res* **130**: 241-250.

BREDT, D.S. & SNYDER, S.H. (1994). Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron* **13**: 301-313.

BULFONE, A., PUELLES, L., PORTEUS, M.H., FROHMAN, M.A., AND RUBENSTEIN, J.L.R. (1993). Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2 and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J Neuroscience* **13**: 3155-3172.

BURMEISTER, D. & GOLDBERG, D. (1988). *J Neurosci* **8**: 3151-3159.

CAMERON, R.S. & RAKIC, P. (1994). Identification of membrane proteins that comprise the plasmalemmal junction between migrating neurons and radial glial cells. *Journal of Neuroscience* **14**: 3139-3155.

CASTREN, E., ZAFRA, F., THOENEN, H., & LINDHOLM, D. (1992). Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 9444-9448.

CATALANO, S.M., ROBERTSON, R.T., & KILLACKEY, H.P. (1991). Early ingrowth of thalamocortical afferents to the neocortex of the prenatal rat. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 2999-3003.

CHAN, S.O. & GUILLERY, R.W. (1993). Developmental-Changes Produced In the Retinofugal Pathways of Rats and Ferrets By Early Monocular Enucleations - the Effects of Age and the Differences Between Normal and Albino Animals. *Journal of Neuroscience* **13**: 5277-5293.

CHAN, S.O. & GUILLERY, R.W. (1994). Changes In Fiber Order In the Optic-Nerve and Tract of Rat Embryos. *Journal of Comparative Neurology* **344**: 20-32.

CHANG, S., RATHJEN, F.G., RAPER, J.A. (1987). Extension of neurites on axons is impaired by antibodiees against specific nerve cell glycoproteins. *J of Cell Biol* **104**: 355-362.

CHUNG, W-W., LAGENAUR, C.F., UAN, Y., LUND, J.S. (1991). Developmental expression of neural cell adhesion molecules in the mouse neocortex and olfactory bulb. *J Comp Neurol* **314**: 290-305.

COHEN, J., NURCOMBE, V., JEFFREY, P., & EDGAR, D. (1989). Developmental loss of functional laminin receptors on retinal ganglion cells is regulated by their target tissue, the optic tectum. *Development* **107**: 381-387.

COHEN, S. (1990). Specification of limb development in the Drosophila embryo by positional cues from segmentation genes. *Nature* **343**: 173-177.

COLELLO, R.J. & GUILLERY, R.W. (1990). The Early Development of Retinal Ganglion-Cells With Uncrossed Axons In the Mouse - Retinal Position and Axonal Course. *Development* **108**: 515-523.

CONLON, R.A. AND ROSSANT, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116**: 357-368.

CONSTANTINE-PATON, M., BLUM, A.S., MENDEZOTERO, R., & BARNSTABLE, C.J. (1986). A Cell-Surface Molecule Distributed In A Dorsoventral Gradient In the Perinatal Rat Retina. *Nature* **324**: 459-462.

COOGAN, T.A., BURKJATTER, A. (1988). Sequential development of connections between striate and extrastriate visual cortical areas in the rat. *J Comp Neuron* **278**: 242-252.

CUCCHIARO, J. & GUILLERY, R.W. (1984). The Development of the Retinogeniculate Pathways In Normal and Albino Ferrets. *Proceedings of the Royal Society of London Series Biological Sciences* **223**: 141

CUNNINGHAM, B.A., HEMPERLY, J.J., MURRAY, B.A., & ET AL. (1987). Neural cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* **236**: 799-806.

DAWSON, V.L., DAWSON, T.M., LONDON, E.D., BRADT, D.S., SNYDER, S.H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* **88**: 6368-6371.

DE CARLOS, J.A. & OLEARY, D.D.M. (1992). Growth and Targeting of Subplate Axons and Establishment of Major Cortical Pathways. *Journal of Neuroscience* **12**: 1194-1211.

DE CARLOS, J.A., SCHLAGGAR, B.L. & O'LEARY, D.D.M. (1995) Develolpment of acetylcholinesterase-positive thalamocortical and basal forebrain afferents to embryonic rat cortex. *Exp Brain Res* **104(3)**: 385-401.

DE CURTIS, I., QUARANTA, V., TAMURA, R.N., REICHARDT, L.F. (1991). Laminin receptors in the retina: sequence analysis of the chick integrin alpha-6 subunit. Evidence for transcriptional and posttranslational regulation. *J Cell Biol* **113**: 405-416.

DEITCHER, D.L., FEKETE, D.M., & CEPKO, C.L. (1994). Asymmetric Expression of A Novel Homeobox Gene In Vertebrate Sensory Organs. *Journal of Neuroscience* **14**: 486-498.

DINARDO, S. AND O'FARRELL, P.H. (1987). Establishment and refinement of segmental pattern in the Drosophila embryo: spatial control of engrailed expression by pair rule genes. *Genes Dev* **1**: 1212-1225.

DOHERTY, P., FRUNS, M., SEATON, P., DICKSON, G., BARTON, C.H., SEARS, T.A., & WALSH, F.S. (1990). A Threshold Effect of the Major Isoforms of NCAM On Neurite Outgrowth. *Nature* **343**: 464-466.

DOHERTY, P., ROWETT, L.H., MOORE, S.E., MANN, D.A., & WALSH, F.S. (1991). Neurite outgrowth in response to transfected N-CAM and N-cadherin reveals fundamental differences in neuronal responsiveness to CAMs. *Neuron* **6**: 247-258.

DOMENICI, L., BERARDI, N., CARMIGNOTO, G., VANTINI, G., & MAFFEI, L. (1991). Nerve growth factor prevents the amblyopic effects of monocular deprivation. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 8811-8815.

DOMEINICI, L., PARISI, V., MAFFEI, L. (1992). Exogenous supply of nerve growth factor prevents the effects of strabismus in the rat. *Neuroscience* **51**: 19-24.

EASTER, S.S. & TAYLOR, J.S.H. (1989). The Development of the Xenopus Retinofugal Pathway - Optic Fibers Join A Pre-Existing Tract. *Development* **107**: 553-573.

EASTER, S.S., RUSOFF, A.C., KISH, P.E. (1993). Initial tract formation in the mouse brain. *J. Neurosci.* **13**: 285-299.

EASTER, S.S., BURRILL, J., MARCUS, R.C., ROSS, L.S., TAYLOR, J.S.H., & WILSON, S.W. (1994). Initial Tract Formation In the Vertebrate Brain. *Progress In Brain Research* **102**: 79-93.

EDELMAN, G.M. (1988). Modulation of cell adhesion during induction, histogenesis and perinatal development of the nervous system. *Annu Rev Neurosci* **7**: 339-377.

EDELMAN, G.M. AND JONES, F.S. (1992) Outside and downstream of the homeobox. *J. Biol. Chem.* **268**: 20683-20686.

EKKER, S.C., UNGAR, A.R., GREENSTEIN, P., VONKESSLER, D.P., PORTER, J.A., MOON, R.T., & BEACHY, P.A. (1995). Patterning Activities of Vertebrate Hedgehog Proteins In the Developing Eye and Brain. *Current Biology* **5**: 944-955.

ELKINS, T., HORTSCH, M., BIEBER, A.J., SNOW, P.M., & GOODMAN, C.S. (1990). Drosophila fasciclin I is a novel homophilic adhesion molecule that along with fasciclin III can mediate cell sorting. *Journal of Cell Biology* **110**: 1825-1832.

EMERLING, D.E. & LANDER, A.D. (1994). Laminar specific attachment and neurite outgrowth of thalamic neurons on cultured slices of developing cerebral neocortex. *Development* **120**: 2811-2822.

ERICKSON, H.P. (1993). Gene knockouts of c-src, transforming growth factor beta1, and tenascin suggest superfluous, nonfunctional expression of proteins. *Journal of Cell Biology* **120**: 1079-1082.

ERICKSON, J., MUHR, J., PLACZEK, M., LINTS, T., JESSELL, T.M., & EDLUND, T. (1995). Sonic Hedgehog Induces the Differentiation of Ventral Forebrain Neurons - A Common Signal For Ventral Patterning Within the Neural Tube. *Cell* **81**: 747-756.

ESBER, H.J., PAYNE, I.J., BOGDEN, A.E. (1973). Variability of hormone concentrations and ratios in commercial sera used for tissue culture. *J Natl Cancer Instit* **50**: 559-562.

FAN, C.M. & TESSIER LAVIGNE, M. (1994). Patterning of Mammalian Somites By Surface Ectoderm and Notochord - Evidence For Sclerotome Induction By A Hedgehog Homolog. *Cell* **79**: 1175-1186.

FIGDOR, M.C. & STERN, C.D. (1993). Segmental Organization of Embryonic Diencephalon. *Nature* **363**: 630-634.

FRASER, S., KEYNES, R., AND LUMSDEN, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restriction. *Nature* **344**: 431-435.

FRIEDLANDER, D.R., MILEV, P., KARTHIKEYAN, L., MARGOLIS, R.K., MARGOLIS, R.U., & GRUMET, M. (1994). The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. *Journal of Cell Biology* **125**: 669-680.

FRIGERIO, G., BURRI, M., BOPP, D., BAUMGARTNER, S., AND NOLL, M. (1986). Structure of the segmentation gene paired and the Drosophila PRD gene set as part of a gene network. *Cell* **47**: 735-746.

FURLEY, A.J., MORTON, S.B., MANALO, D., KARAGOGEOS, D., DODD, J., & JESSELL, T.M. (1990). The Axonal Glycoprotein TAG-1 Is An Immunoglobulin Superfamily Member With Neurite Outgrowth Promoting Activity. *Cell* **61**: 157-170.

FUSHIKI, S. & SCHACHNER, M. (1986). Immunocytological Localization of Cell-Adhesion Molecules L1 and N-CAM and the Shared Carbohydrate Epitope L2 During Development of the Mouse Neocortex. *Developmental Brain Research* **24**: 153-167.

GAHWILER, B.H. (1981). Morphological-Differentiation of Nerve-Cells In Thin Organotypic Cultures Derived From Rat Hippocampus and Cerebellum. *Proceedings of the Royal Society of London Series B-Biological Sciences* **211**: 287

GAHWILER, B.H. (1981). Nerve-Cells In Organotypic Cultures. *Jama-Journal of the American Medical Association* **245**: 1858-1859.

GAHWILER, B.H. (1981). Organotypic Monolayer-Cultures of Nervous-Tissue. *Journal of Neuroscience Methods* **4**: 329-342.

GAHWILER, B.H. (1988). Organotypic Cultures of Neural Tissue. *Trends In Neurosciences* **11**: 484-489.

GANZLER, S.I.I. AND REDIES, C. (1995). R-cadherin expression during nucleus formation in chicken forebrain neuromeres. *J Neuroscience* **15**: 4157-4172.

GARDNER, C.A. & BARALD, K.F. (1991). The cellular environment controls the expression of engrailed-like protein in the cranial neuroepithelium of quail-chick chimeric embryos. *Development* **113**: 1037-1048.

GARNER, C.G., BRUGG, B., MATUS, A. (1988). A 70-kilodalton microtubule-associated protein (MAP2c), related to MAP2. *J Neurochem* **50**: 609-615.

GASSER, U.E. & HATTEN, M.E. (1990). Central nervous system neurons migrate on astroglial fibers from heterotypic brain regions in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 4543-4547.

GHOSH, A.& SHATZ, C.J. (1992). Pathfinding and target selection by develping geniculocortical axons. *J Neuroscience* **12**: 39-55.

GODEMENT, P., SALAUN, J., & IMBErt, M. (1984). Prenatal and Postnatal-Development of Retinogeniculate and Retinocollicular Projections In the Mouse. *Journal of Comparative Neurology* **230**: 552-575.

GODEMENT, P., VANSELOW, J., THANOS, S., & BONHOEFFER, F. (1987a). A Study In Developing Visual Systems With A New Method of Staining Neurons and their Processes In Fixed Tissue. *Development* **101**: 697-713.

GODEMENT, P., SALAUN, J., & METIN, C. (1987b). Fate of Uncrossed Retinal Projections Following Early Or Late Prenatal Monocular Enucleation In the Mouse. *Journal of Comparative Neurology* **255**: 97-109.

GODEMENT, P. & BONHOEFFER, F. (1989). Cross-Species Recognition of Tectal Cues By Retinal Fibers Invitro. *Development* **106**: 313-320.

GODEMENT, P., SALAUN, J., & MASON, C.A. (1990). Retinal Axon Pathfinding In the Optic Chiasm - Divergence of Crossed and Uncrossed Fibers. *Neuron* **5**: 173-186.

GOLDBERG, D.J. & BURMEISTER, D.W. (1986). Stages in axon formation: Observations of growth of Aplysia axons in culture using video-enhanced contrast-differential interference contrast microscopy. *Journal of Cell Biology* **103**: 1921-1931.

GOLDEN, J.A. & CEPKO, C.L. (1996). Clones in the chick diencephalon contain multiple cell types and siblings are widely dispersed. *Development* **122**: 65-78.

GOODMAN, C.S. & SHATZ, C.J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. *Neuron* **10**: 77-98.

GOTZ, M., NOVAK, N., BASTMEYER, M., & BOLZ, J. (1992). Membrane-bound molecules in rat cerebral cortex regulate thalamic innervation. *Development* **116**: 507-519.

GOULD, A.P., BROOKMAN, J.J., STRUTT, D.I., AND WHITE, R.A.H. (1990). Target of homeotic gene control in Drosophila. *Nature* **348**: 308-312.

GOULD, A.P. & WHITE, R.A.H. (1992). Connectin, A Target of Homeotic Gene-Control In Drosophila. *Development* **116**: 1163-1174.

GRAVINA, A., DOMENICI, L., BERARDI, N., GALLI, L., & MAFFEI, L. (1990). Transplant of embryonal nervous tissue preserves the responses of rat retinal ganglion cells after section of the optic nerve. *Experimental Brain Research* **80**: 631-634.

GRENNINGLOH, G., REHM, E.J., & GOODMAN, C.S. (1991). Genetic analysis of growth cone guidance in Drosophila: Fasciclin II functions as a neuronal recognition molecule. *Cell* **67**: 45-57.

GRINDLEY, J.C., DAVIDSON, D.R., & HILL, R.E. (1995). the Role of Pax-6 In Eye and Nasal Development. *Development* **121**: 1433-1442.

GROSVELD, F., PAUL, J., BIRNIE, G.D., BUCKINGHAM, M.E., SCHIBLER, U., CLAYTON, P.M., RADBRUCH, A., SUEOKA, N., HARRISON, P.R., MIRSKY, R. (1982). Summary of Discussion On the Molecular-Basis of Differentiation and Competence. *Advances In Experimental Medicine and Biology* **158**: 139-142.

GRUMET, M., HOFFMAN, S., EDELMAN, G.M. (1984). Two antigenically related neuronal cell adhesion molecules of different specificites mediate neuron-neuron and neuron-glia adhesion. *Proc Natl Acad Sci USA***81**: 267-271.

GUILLEMOT, F., LO, L-C., JOHNSON, J.E., AUERBACH, A., ANDERSON, D.J., JOYNER, A.L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**: 463-476.

GUILLERY, R.W. (1989). Early Monocular Enucleations In Fetal Ferrets Produce A Decrease of Uncrossed and An Increase of Crossed Retinofugal Components -A Possible Model For the Albino Abnormality. *Journal of Anatomy* **164**: 73-84.

GUILLERY, R.W. (1992) Rules that govern the development of the pathways from the eye to the optic tract in mammals. *In: Development of the visual system (Lam DM, Shatz CS, eds). Cambridge, MA: MIT press.*

GUILLERY, R.W., MASON, C.A., & TAYLOR, J.S.H. (1995). Developmental Determinants At the Mammalian Optic Chiasm. *Journal of Neuroscience* **15**: 4727-4737.

GUNDERSON, R.W. (1987). Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin *in vitro. Dev Biol* **121**: 423-431.

GUTHRIE, S., PRINCE, V. AND LUMSDEN, A. (1993) Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts. *Development* **118**: 527-538.

HALL, D.E., NEUGEBAUER, K.M., & REICHARDT, L.F. (1987). Embryonic neural retinal cell response to extracellular matrix proteins: Developmental changes and effects of the cell substratum attachment antibody (CSAT). *Journal of Cell Biology* **104**: 623-634.

HARRELSON, A.L. & GOODMAN, C.S. (1988). Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily. *Science* **242**: 700-798.

HATINI, V., TAO, W.F., & LAI, E. (1994). Expression of Winged Helix Genes, BF-1 and BF-2, Define Adjacent Domains Within the Developing Forebrain and Retina. *Journal of Neurobiology* **25**: 1293-1309.

HAUN, F. & CUNNINGHAM, T.J. (1993). Recovery of frontal cortex-mediated visual behaviors following neurotrophic rescue of axotomized neurons in medial frontal cortex. *Journal of Neuroscience* **13**: 614-622.

HEDGECOCK, E.M., CULOTTI, J.G., & HALL, D.H. (1990). The Unc-5, Unc-6, and Unc-40 Genes Guide Circumferential Migrations of Pioneer Axons and Mesodermal Cells On the Epidermis In C-Elegans. *Neuron* **4**: 61-85.

HEEMSKERK, J., DINARDO, S., KOSTRIKEN, R., AND O'FARRELL, P.H. (1991). Multiple modes of engrailed regulation in the progression towards cell fate determination. *Nature* **352**: 404-410

HEFFNER, C.D., LUMSDEN, A.G.S., & O'LEARY, D.D.M. (1990). Target control of collateral extension and directional axon growth in the mammalian brain. *Science* **247**: 217-220.

HERRICK, C.J. (1942) Optic and post-optic systems in the brain of *Amblystoma tigrinum. J Comp Neurol* **77**: 191-353.

HIDAGLO, A. AND INGHAM, P.W. (1990). Cell patterning in the Drosophila segment: spatial regulation of the segment polarity gene patched. *Development* **110**: 291-302.

HILL, R.E., FAVOR, J., HOGAN, B.L.M., TON, C.C.T., SAUNDERS, G.F., HANSON, I.M., PROSSER, J., JORDAN, T., HASTIE, N.D., & VANHEYNINGEN, V. (1991). Mouse Small Eye Results From Mutations In A Paired-Like Homeobox-Containing Gene. *Nature* **354**: 522-525.

HISANAGA, K. & SHARP, F.R. (1990). Marked neurotrophic effects of diffusible substances released from non-target cerebellar cells on thalamic neurons in culture. *Developmental Brain Research* **54**: 151-160.

HOGAN, B.L.M., HORSBURGH, G., COHEN, J., HETHERINGTON, C.M., FISCHER, G., AND LYON, M.F. (1986). Small eye (Sey):a homozygous lethal

mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. *J.Embryol.Exp.Morph.* **97**: 95-110.

HOLT, C.E. & HARRIS, W.A. (1993). Position, guidance, and mapping in the developing visual system. *Journal of Neurobiology* **24**: 1400-1422.

HONIG, M.G. & HUME, R.I. (1986). Fluorescent Carbocyanine Dyes Allow Living Neurons of Identified Origin To Be Studied In Long-Term Cultures. *Journal of Cell Biology* **103**: 171-187.

HONN, D.V., SINGLEY, J.A. & CHAVIN, W. (1975). Fetal bovine serum: a multivariate standard. *Proc Soc Exp Biol Med* **149**: 344-347.

HOPE, B.R., MICHAEL, G.J., KNIGGE, K.M., VINCENT, S.R. (1991). Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc Natl Acad Sci USA* **88**: 2811-2814.

HORTON, J.C., GREENWOOD, M.M., HUBEL, D.H. (1979). Non-retinotopic arrangement of fibres in the cat optic nerve. *Nature* **282**: 720-722.

HUBENER, M., GOTZ, M., KLOSTERMANN, S., BOLLS, J. (1995). Guidance of thalamocortical axons by growth-promoting molecules in developing rat cerebral cortex. *Euro J Neurosci* **7 (9)**: 1963-1972.

HYNES, M., PORTER, J.A., CHIANG, C., CHANG, D., TESSIER LAVIGNE, M., BEACHY, P.A., & ROSENTHAL, A. (1995). Induction of Midbrain Dopaminergic-Neurons By Sonic Hedgehog. *Neuron* **15**: 35-44.

IIJIMA, N., OOHIRA, A., MORI, T., KITABATAKE, K., & KOHSAKA, S. (1991). Core protein of chondroitin sulfate proteoglycan promotes neurite outgrowth from cultured neocortical neurons. *Journal of Neurochemistry* **56**: 706-708.

ILER, N., ROWITCH, D.H., ECHELARD, Y., MCMAHON, A.P. AND ABATE-SHAN, C. (1995) A single homeodomain binding site restricts spatial expression of Wnt-1 in the developing brain. *Mech. Development* **53**: 87-96.

INGHAM, P.W., BAKER, N., AND MARTINEZ ARIAS, A. (1988). Regulation of segment polarity genes in the Drosophila blastoderm by fushi tarazu and even skipped. *Nature* **331**: 73-75.

INGHAM, P.W. & MARTINEZ ARIAS, A. (1992). Boundaries and fields in early embryos. *Cell* **68**: 221-235.

ISHII, N., WADSWORTH, W.G., STERN, B.D., CULOTTI, J.G. & HEDGECOCK, E.M. (1992). UNC-6, a laminin-related protein, guides cells and pioneer axon migrations in *C. elegans. Neuron* **9**: 873-881.

ITASAKI, N., ICHIJO,. HAMA, C., MATSUNO, T. & NAKAMURA, H. (1991). Establishment of rostrocaudal polarity in tectal primordium: Engrailed expression and subsequent tectal polarity. *Development* **113**: 1133-1144.

ITASAKI, N., NAKAMURA, H. (1992). Rostrocaudal polarity of the tectum in birds: correlation or *en* gradient and top0ographic order in retinotectal projection. *Neuron* **8**: 787-798.

JESSELL, T.M. & DODD, J. (1990). Floor-plate derived signals and the control of neural cell pattern in vertebrates. *Harvey Lect.* **86**: 87-128.

JOHNSON, R.L., LAUFER, E., RIDDLE, R.D., & TABIN, C. (1994). Ectopic Expression of Sonic Hedgehog Alters Dorsal-Ventral Patterning of Somites. *Cell* **79**: 1165-1173.

JONES, F.S., PREDIGER, E.A., BITTNER, D.A., DEROBERTIS, E.M., AND EDELMAN, G.M. (1992a). Cell adhesion molecules as targets for Hox genes: neural cell adhesion molecule promoter activity is modulated by cotransfection with Hox-2.5 and 2.4. *Proc. Natl. Acad. Sci. USA* **89**: 2086-2091.

JONES, F.S., CHALEPAKIS, G., GRUSS, P., & EDELMAN, G.M. (1992b). Activation of the Cytotactin Promoter By the Homeobox-Containing Gene Evx-1. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 2091-2095.

KAGEYAMA, G.H. & ROBERTSON, R.T. (1993). Development of geniculocortical projections to visual cortex in rat: Evidence for early ingrowth and synaptogenesis. *Journal of Comparative Neurology* **335**: 123-148.

KAPFHAMMER, J.P., GRUNEWALD, B.E., & RAPER, J.A. (1986). The selective inhibition of growth cone extension by specific neurites in culture. *Journal of Neuroscience* **6**: 2527-2534.

KAPFHAMMER, J.P. & RAPER, J.A. (1987). Interactions between growth cones and neurites growing from different neural tissues in culture. *J Neurosci* **7**: 1595-1600.

KAPRIELIAN, Z. & PATTERSON, P.H. (1994). The Molecular-Basis of Retinotectal Topography. *Bioessays* **16**: 1-11.

KENNEDY, T.E., SERAFINI, T., DE LA TORRE, J.R., TESSIER-LAVIGNE, M.(1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**: 425-435.

KEVETTER, G.A. & LASEK, R.J. (1982). Development of the Marginal Zone In the Rhombenecephalon of Xenopus- Laevis. *Developmental Brain Research* **4**: 195-208.

KEYNES, R. & COOK, G.M.W. (1992). Contact inhibition of growth-cone motility during peripheral nerve segmentation. *Biochemical Society Transactions* **20**: 399-401.

KOLODKIN, A.L., MATTHES, D.J., OCONNOR, T.P., PATEL, N.H., ADMON, A., BENTLEY, D., & GOODMAN, C.S. (1992). Fasciclin IV: Sequence, Expression, and Function During Growth Cone Guidance In the Grasshopper Embryo. *Neuron* **9**: 831-845.

KOLODKIN, A.L., MATTHES, D.J., & GOODMAN, C.S. (1993). The Semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* **75**: 1389-1399.

KOMURO, H. & RAKIC, P. (1992). Selective role of N-type calcium channels in neuronal migration. *Science* **257**: 806-809.

KRAUSS, S., JOHANSEN, T., KORZH, V., & FJOSE, A. (1991). Expression Pattern of Zebrafish Pax Genes Suggests A Role In Early Brain Regionalization. *Nature* **353**: 267-270.

KRAUSS, S., JOHANSEN, T., KORZH, V., & FJOSE, A. (1991). Expression of the Zebrafish Paired Box Gene Pax[Zf-B] During Early Neurogenesis. *Development* **113**: 1193-1206.

KRAUSS, S., JOHANSEN, T., KORZH, V., MOENS, U., ERICSON, J.U., & FJOSE, A. (1991). Zebrafish Pax[zf-a] - A Paired Box-Containing Gene Expressed In the Neural-Tube. *Embo Journal* **10**: 3609-3619.

KRAUSS, S., JOHANSEN, T., KORZH, V., & FJOSE, A. (1992). Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353**: 267-270.

KURATINI, S.C. & EICHELE, G. (1993). Rhombomeric transplantation repatterns the segmental organization of cranial nerves and reveals autonomous expression of a homeodomain protein. *Development* **117**: 105-117.

KUWADA, J.Y. (1992) Growth cone guidance in the zebrafish central nervous system. *Curr Opin Neurobiol* **2**: 31-35.

LAGENAUER, C. & LEMMON, V. (1987). An L1-like molcule the 8D9 antigen, is a potent substrate for neurite extension. *Proc Natl Acad Sci USA* **84**: 7753-7757.

LANDER, A.D. (1990). Mechanisms by which molecules guide axons. *Current Opinion in Cell Biology* **2**: 907-913.

LARGE, T.H., BODARY, S.C., CLEGG, D.O., WESKAMP, G., OTTEN, U. REICHARDT, L.F. (1986). Nerve growth factor gene expression in the developing rat brain. *Science* **234**: 352-355.

LAYER, P.G. & ALBER, R. (1990). Patterning of chick brain vesicles as revealed by peanut agglutinin and cholinesterases. *Development* **109**: 613-624.

LEMMON, V. & MCLOON, S.C. (1986). the appearance of an L1-like molecule in the chick primary visual pathway. *Journal of Neuroscience* **6**: 2987-2994.

LETOURNEAU, P.C. (1975). Possible roles for cell-to-substratum adhesion in neuronal morphogenesis. *Dev Biol* **44**: 77-91.

LETOURNEAU, P.C., MADSEN, A.M., PALM, S.L., & FURCHT, L.T. (1988). Immunoreactivity For Laminin In the Developing Ventral Longitudinal Pathway of the Brain. *Developmental Biology* **125**: 135-144.

LETOURNEAU, P.C., PECH, I.V., ROGERS, S.L., PALM, S.L., MCCARTHY, J.B., & FURCHT, L.T. (1988). Growth Cone Migration Across Extracellular-Matrix Components Depends On Integrin, But Migration Across Glioma-Cells Does Not. *Journal of Neuroscience Research* **21**: 286-297.

LETOURNEAU, P.C., CONDIC, M.L., & SNOW, D.M. (1994). Interactions of developing neurons with the extracellular matrix. *Journal of Neuroscience* **14**: 915-928.

LIESI, P. & SILVER, J. (1988). Is astrocyte laminin involved in axon guidance in the mammalian CNS? *Developmental Biology* **130**: 774-785.

LOCHTER, A., VAUGHAN, L., KAPLONY, A., PROCHIANTZ, A., SCHACHNER, M., & FAISSNER, A. (1991). J1/tenascin in substrate-bound and soluble form displays contrary effects on neurite outgrowth. *Journal of Cell Biology* **113**: 1159-1171.

LOTTO, R.B. & PRICE, D.J. (1994). Evidence that molcules imfluencing growth and termination in the developing geniculocortical pathway are conserved between divergent mammalian species. *Dev Brain Res* **81**: 17-25.

LOTTO, R.B. & PRICE, D.J. (1995). The stimulation of thalamic neurite outgrowth by cortex-derived growth factors in vitro. The influence of cortical age and activity. *Euro J Neurosci* **7(2)**: 318-328.

LUMSDEN, A.G.S. & DAVIES, A.M. (1983). Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. *Nature* **306**: 786-788.

LUMSDEN, A.G.S. & DAVIES, A.M. (1986). Chemotropic effect of specific target epithelium in the developing mammalian nervous system. *Nature* **323**: 538-539.

LUMSDEN, A. & KEYNES, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature.* **337**: 424-428.

LUND, R.D. & BUNT, (1976). *J Comp Neurol* **165**: 247-264.

LUND, R.D. & MUSTARI, M.J. (1977). Development of the geniculocortical pathway in rats. *J Comp Neurol* **173**: 289-306.

LUO, Y., RAIBLE, D., & RAPER, J.A. (1993). Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75**: 217-227.

LUO, Y., SHEPHERD, I., LI, J., RENZI, M.J., CHANG, S., & RAPER, J.A. (1995). A Family of Molecules Related To Collapsin In the Embryonic Chick Nervous System. *Neuron* **14**: 1131-1140.

LUSKIN, M.B. & SHATZ, C.J. (1985a). Studies of the earliest generated cells of the cat's visual cortex: Cogeneration of subplate and marginal zones. *Journal of Neuroscience* **5**: 1062-1075.

LUSKIN, M.B. & SHATZ, C.J. (1985b). Neurogenesis of the cat's primary visual cortex. *Journal of Comparative Neurology* **242**: 611-631.

MACDONALD, R., XU, Q.L., BARTH, K.A., MIKKOLA, I., HOLDER, N., FJOSE, A., KRAUSS, S., & WILSON, S.W. (1994). Regulatory Gene-Expression Boundaries Demarcate Sites of Neuronal Differentiation In the Embryonic Zebrafish Forebrain. *Neuron* **13**: 1039-1053.

MACDONALD, R., BARTH, K.A., XU, Q.L., HOLDER, N., MIKKOLA, I., & WILSON, S.W. (1995). Midline Signaling Is Required For Pax Gene-Regulation and Patterning of the Eyes. *Development* **121**: 3267-3278.

MAFFEI, L., BERARDI, N., DOMENICI, L., PARISI, V., & PIZZORUSSO, T. (1992). Nerve growth factor (NGF) prevents the shift in ocular dominance distribution of visual cortical neurons in monocularly deprived rats. *Journal of Neuroscience* **12**: 4651-4662.

MAGOUN, H.W. & HANSON, M. (1942). The supraoptic decussation in the cat and monkey. *J Comp Neurol* **76**: 435-460.

MAISONPIERRE, P.C., BELLUSCIO, L., FRIEDMAN, B., ALDERSON, R.F., WIEGAND, S.J., FURTH, M.E., LINDSAY, R.M., & YANCOPOULOS, G.D. (1990). NT-3, BDNF, and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. *Neuron* **5**: 501-509.

MANSOURI, A., STOYKOVA, A., AND GRUSS, P. (1994). Pax genes in development. *Journal of Cell Science* **18**: 35-42.

MARCUS, R.C. & MASON, C.A. (1993). Early retinal axons growth in the mouse ventral diencephalon. *Soc. Neurosci. Abstr* **19**:1418.

MARCUS, R.C. & MASON, C.A. (1995). The First Retinal Axon Growth In the Mouse Optic Chiasm - Axon Patterning and the Cellular Environment. *Journal of Neuroscience* **15**: 6389-6402.

MARCUS, R.C., WANG, L.C., & MASON, C.A. (1996). Retinal Axon Divergence In the Optic Chiasm - Midline Cells Are Unaffected By the Albino Mutation. *Development* **122**: 859-868.

MARTI, E., BUMCROT, D.A., TAKADA, R., & MCMAHON, A.P. (1995). Requirement of 19k Form of Sonic Hedgehog For Induction of Distinct Ventral Cell-Types In CNS Explants. *Nature* **375**: 322-325.

MARTINEZ, S., WASSEF, M. & ALVARADO-MALLART, R-M. (1991). Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the hoemobox gene en. *Neuron* **6**: 971-981.

MASON, C.A. & GODEMENT, P. (1991). Growth cone form reflects interactions in visual pathways and cerebellar targets. In: *The nerve growth cone (Letourneau, P.C., Kater, S.B. & Macagno, E.R., eds)* 405-424 New York: Raven.

MASTICK, G.S., EASTER, S.S. (1996). Initial organization of neurons and tracts in the embryonic mouse forebrain and midbrain. *Dev Biol* **173**: 79-94.

MATSUNAMI, H., IWATA, H., OKUMARA, N., YOSHIDA, S., IMAIZUMI, K., LEE, Y., SHIRAISHI, S. & SHIOSAKA, S. (1992). Localization of basic fibroblastic growth factor-like immunoreactivity in the rat brain. *Brain Res* **587**: 49-65.

MATSUNAMI, H. AND TAKEICHI, M. (1995). Fetal brain subdivisions defined by R- and E-Cadherin expressions: evidence for the role of cadherin activity in region-specific, cell-cell adhesion. *Dev Biol* **172**: 466-478.

MATSUYAMA, A., IWATA, H., OKUMURA, N., YOSHIDA, S., IMAIZUMI, K., LEE, Y., SHIRAISHI, S. SHIOSAKA, S. (1992). Localization of basic fibroblast growth factor-like immunoreactivity in the rat brain. *Brain Res* B587**: 49-65.**

MATTSON, M.P. & KATER, S.B. (1988a). Isolated Hippocampal-Neurons In Cryopreserved Long-Term Cultures- Development of Neuroarchitecture and Sensitivity To NMDA. *International Journal of Developmental Neuroscience* **6**: 439|

MATTSON, M.P., DOU, P., & KATER, S.B. (1988b). Outgrowth-Regulating Actions of Glutamate In Isolated Hippocampal Pyramidal Neurons. *Journal of Neuroscience* **8**: 2087-2100.

MATTSON, M.P., LEE, R.E., ADAMS, M.E., GUTHRIE, P.B., & KATER, S.B. (1988c). Interactions Between Entorhinal Axons and Target Hippocampal-Neurons - A Role For Glutamate In the Development of Hippocampal Circuitry. *Neuron* **1**: 865-876.

MATTSON, M.P., GUTHRIE, P.B., & KATER, S.B. (1988d). Components of Neurite Outgrowth That Determine Neuronal Cytoarchitecture - Influence of Calcium and the Growth Substrate. *Journal of Neuroscience Research* **20**: 331-345.

MATTSON, M.P., TAYLORHUNTER, A., & KATER, S.B. (1988e). Neurite Outgrowth In Individual Neurons of A Neuronal Population Is Differentially Regulated By Calcium and Cyclic-Amp. *Journal of Neuroscience* **8**: 1704-1711.

MCADAMS, B.D. & MCLOON, S.C. (1995). Expression of chondroitin sulfate and keratan sulfate proteoglycans in the path of growing retinal axons in the developing chick. *Journal of Comparative Neurology* **352**: 594-606.

MCCAFFERY, P., NEVE, R.L., & DRAGER, U.C. (1990). A Dorsoventral Asymmetry In the Embryonic Retina Defined By Protein Conformation. *Proc Nat Acad Sci USA* **87**: 8570-8574.

MCCOBB, D.P., COHAN, C.S., CONNOR, J.A., & KATER, S.B. (1988a). Interactive Effects of Serotonin and Acetylcholine On Neurite Elongation. *Neuron* **1**: 377-385.

MCCOBB, D.P., HAYDON, P.G., & KATER, S.B. (1988b). Dopamine and Serotonin Inhibition of Neurite Elongation of Different Identified Neurons. *Journal of Neuroscience Research* **19**: 19-26.

MCCOBB, D.P., BEST, P.M., & BEAM, K.G. (1988c). Developmental-Changes In Ca-2+ Currents From Identified Chick Motoneurons. *Biophysical Journal* **53**: A 235

MCCONNELL, S.K. & KAZNOWSKI, C.E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**: 282-285.

MCINTIRE, S.L., GARRIGA, G., WHITE, J., JACOBSON, D., & HORVITZ, H.R. (1992).Genes necessary for directed axonal elongation or fasciculation in C. elegans. *Neuron* **8**: 307-322.

MCKANNA, J.A. (1993) Optic chiasm and indundibular decussation sites in the developing rat diencephalon are defined by glial raphes expressing p35 (lipocotin I, annexin I). *Dev Dynamics* **195**: 76-86

MCMAHON, A.P., JOYNER, A.L., BRADLEY, A., AND MCMAHON, J.A. (1992). The midbrain-hindbrain phenotype of Wnt-1-/Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69**: 581-595.

MESSERSMITH, E.K., LEONARDO, E.D., SHATZ, C.J., TESSIERLAVIGNE, M., GOODMAN, C.S., & KOLODKIN, A.L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* **14**: 949-959.

MILLER, B., SHEPPARD, A.M., PEARLMAN, A.C. (1992). Expression of two chondroitin sulfate proteoglycan core proteins in the subplate pathway of early cortical afferents. *Soc Neurosci Abstr* **18**: 778.

MILLER, B., CHOU, L., & FINLAY, B.L. (1993). The early development of thalamocortical and corticothalamic projections. *Journal of Comparative Neurology* **335**: 16-41.

MISSION, G-P., AUSTIN, C.P., TAKAHASHI, T., CEPKO, C.L., CAVINESS, V.S. (1991). The alignment of migrating neural cells in relation to neuropallial radial glial fiber system. *Cereb Cortex* **1**: 221-229.

MITROFANIS, J. & BAKER, G.E. (1993). Development of the thalamic reticular and perireticular nuclei in rats and their relationship to the course of growing corticofugal and corticopetal axons. *Journal of Comparative Neurology* **338**: 575-587.

MITROFANIS, J. & GUILLERY, R.W. (1993). New views of the thalamic reticular nucleus in the adult and the developing brain. *Trends In Neurosciences* **16**: 240-245.

MOLNAR, Z. & BLAKEMORE, C. (1990). Relationship of corticofugal and corticopetal projections in the prenatal establishment of projections from thalamic nuclei to specific cortical areas of the rat. *Journal of Physiology* **430**: 104P|

MOLNAR, Z. & BLAKEMORE, C. (1991). Lack of regional specificity for connections formed between thalamus and cortex in coculture. *Nature* **351**: 475-477.

MOLNAR, Z. & BLAKEMORE, C. (1995). How do thalamic axons find their way to the cortex?. *Trends In Neurosciences* **18**: 389-397.

MOODY, S.A., QUIGG, M.S., FRANKFURTER, A. (1987). Development of the peripheral trigeminal system in the chick revealed by an isotype-specific anti-beta-tubulin monoclonal antibody. *J Comp Neurol* **279**: 567-580.

MORISSETTE, N. & CARBONETTO, S. (1995). Laminin Alpha-2 Chain (M-Chain) Is Found Within the Pathway of Avian and Murine Retinal Projections. *Journal of Neuroscience* **15**: 8067-8082.

NAITO, J. (1986). Course of Retinogeniculate Projection Fibers In the Cat Optic-Nerve. *Journal of Comparative Neurology* **251**: 376-387.

NAITO, J. (1989). Retinogeniculate Projection Fibers In the Monkey Optic-Nerve - A Demonstration of the Fiber Pathways By Retrograde Axonal-Transport of Wga-Hrp. *Journal of Comparative Neurology* **284**: 174-186.

NAKAI, J. (1960). Studies on the mechanism determining the course of nerve fibers in tissue culture. II. The mechanism of fasciculation. *Z. Zellforsch.* **52**: 427-499.

NAKAJIMA, S. (1965). Selectivity in fasciculation of nerve fibers *in vitro. J Comp Neurol* **125**: 193-204.

NIIMI, K., JARADA, I., KUSAKA, Y., & KISHI, S. (1962). The ontogenetic development of the diencephalon of the mouse. *Tokushima J Exp Med* **8**: 203-238.

NORNES, J.O., DRESSLER, G.R., KNAPIK,E.W., DEUTSCH, U & GRUSS, P. (1990). Spatially and temporally restricted expression of Pax2 during murine neurogenesis. *Development* **109**: 797-809.

NORTHCUTT, R.G. AND BUTLER, A.B. (1993) The diencephalon and optic tectum of the longnose gar, Lepisosteus osseus (L): cytoarchitectonics and distribution of acetylcholinesterase. *Brain Behav Evol* **41**: 57-81.

NOVAK, N. & BOLZ, J. (1993). Formation of specific efferent connections in organotypic slice cultures from rat visual cortex cocultured with lateral geniculate nucleus and superior colliculus. *European Journal of Neuroscience* **5**: 15-24.

NUBLER-JUNG, K. (1979). *Wilhelm Roux Arch.* **186**: 211-233.

OOHIRA, A., MATSUI, F., WATANABE, E., KUSHIMA, Y., MAEDA, N. (1994). Developmentally regulation of a brain specific species of chondroitin sulfate proteoglycan neurocan identified with a monoclonal antibody IG2 in the rat cerebrum. *Neuroscience* **60**: 145-157.

PATTERSON, P.H. (1988). On the importance of being inhibited or saying no to growth cones. *Neuron* **1**: 263-267.

PINI, A. (1993). Chemorepulsion of axons in the developing mammalian central nervous system. *Science.* 261 . 95-98.

PINI, A. (1994). Axon guidance. Growth cones say no. *Curr Biol* **4 (2)**: 131-133.

PLAZA, S., DOZIER, C. AND SAULE, S. (1993) Quail PAX-6 (PAX-QNR) encodes a transcription factor able to bind and transactivate its own promoter. *Cell Growth Diff* **4**: 1041-1050.

POLLERBERG, G.E. & MACK, T.G.A. (1994). Cell adhesion molecule SC1/DMGRASP is expressed on growing axons of retina ganglion cells and is involved in mediating their extension on axons. *Developmental Biology* **165**: 670-687.

PORTEUS, M.H., BULFONE, A., CIARANELLO, R.D., AND RUBENSTEIN, J.L.R. (1991). Isolation and characterization of a novel cDNA clone encoding a homeo-domain that is developmentally regulated in the ventral forebrain. *Neuron* **7**: 221-229.

PORTEUS, M.H., BULFONE, A., LIU, J., PUELLES, L., LO, L., AND RUBENSTEIN, J.L.R. (1994). DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. *J Neuroscience* **14**: 6370-6383.

PRICE, M., LEMAISTRE, M., PISCHETOLA, M., DI LAURO, R., AND DUBOULE, D. (1991). A mouse gene related to distal-less shows a restricted expression in the developing forebrain. *Nature* **351**: 748-751.

PUELLES, L., AMAT, J.A., & DELATORRE, M.M. (1987). Segment-Related, Mosaic Neurogenetic Pattern In the Forebrain and Mesencephalon of Early Chick-Embryos . 1. Topography of AChE-Positive Neuroblasts Up To Stage Hh18. *Journal of Comparative Neurology* **266**: 247-268.

PUELLES, L. & RUBENSTEIN, J.L.R. (1993). Expression Patterns of Homeobox and Other Putative Regulatory Genes In the Embryonic Mouse Forebrain Suggest A Neuromeric Organization. *Trends In Neurosciences* **16**: 472-479.

PUSCHEL, A.W., ADAMS, R.H., & BETZ, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. *Neuron* **14**: 941-948.

RABBACHI, S.A., NEVE, R.L., DRAGER, U.C. (1991). A positional marker for the dorsal embryonic retina is homologous to the high-affinity laminin receptor. *Development* **109**: 521-531.

RAKIC, P. (1971). Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electron microscopic study in *Macacus rhesus*. *J Comp Neurol* **141**: 283-312.

RAKIC, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol **145**: 61-84.*

RAKIC, P. (1977). Prenatal development of the visual system in rhesus monkey. *Philos Trans R Soc Lond (Biol)* **278**: 245-260.

RAKIC, P. (1988) Specification of cerebral cortical areas. *Science* **241**: 170-176.

RAKIC, P. (1990). Principles of neural cell migration. *Experinentia* **46**: 882-891.

RAKIC, P. (1995). Radial versus tangential migration of neuronal clones in the developing cerebral cortex. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 11323-11327.

REESE, B.E. & BAKER, G.E. (1993) The re-establishment of the representation of the dorso-ventral axis in the chiasmatic region of the ferret. *Vis Neurosci* **10**: 957-968.

RENNIE, S. (1992). In vivo and in vitro development of the geniculocortical pathway in the mouse. *PhD Thesis. University of Edinburgh.*

RENNIE, S., LOTTO, R.B., & PRICE, D.J. (1994). Growth-promoting interactions between the murine neocortex and thalamus in organotypic co-cultures. *Neuroscience* **61**: 547-564.

ROBINSON, G.W., WRAY, S., AND MAHON, K.A. (1991). Spatially restricted expression of a member of a new family of murine distal-less homeobox genes in the developing forebrain. *New Biol* **3**: 1183-1194.

ROELINK, H., PORTER, J.A., CHIANG, C., TANABE, Y., CHANG, D.T., BEACHY, P.A., & JESSELL, T.M. (1995). Floor Plate and Motor-Neuron Induction By Different Concentrations of the Amino-Terminal Cleavage Product of Sonic Hedgehog Autoproteolysis. *Cell* **81**: 445-455.

ROMIJN, H.J., VANHUIZEN, F., & WOLTERS, P.S. (1984). Towards An Improved Serum-Free, Chemically Defined Medium For Long-Term Culturing of Cerebral-Cortex Tissue. *Neuroscience and Biobehavioral Reviews* **8**: 301-334.

RUBENSTEIN, J.L.R. & PUELLES, L. (1994). Homeobox Gene-Expression During Development of the Vertebrate Brain. *Current Topics In Developmental Biology* **29**: 1-63.

RUBENSTEIN, J.L.R., MARTINEZ, S., SHIMAMURA, K., & PUELLES, L. (1994). The Embryonic Vertebrate Forebrain - the Prosomeric Model. *Science* **266**: 578-580.

RUGH, R. (1991). The mouse: its reproduction and development. *Oxford University Press, Oxford.*

RUTISHAUSER, U. (1984). Developmental biology of a neural cell adhesion molecule. *Nature* **310**: 549-554.

SAGA, Y., YAGI, T., IKAWA, Y., SAKAKURA, T., & AIZAWA, S. (1992). Mice develop normally without tenascin. *Genes and Development* **6**: 1821-1831.

SALINAS, P.C. & NUSSE, R. (1992). Regional Expression of the Wnt-3 Gene In the Developing Mouse Forebrain In Relationship To Diencephalic Neuromeres. *Mechanisms of Development* **39**: 151-160.

SCHMAHL, W., KNOEDLSEDER, M., FAVOR, J., & DAVIDSON, D. (1993). Defects of Neuronal Migration *and* the Pathogenesis of Cortical Malformations Are Associated With Small Eye (Sey) In the Mouse, A Point Mutation At the Pax-6-Locus. *Acta Neuropathologica* **86**: 126-135.

SHATZ, C.J. & LUSKIN, M.B. (1986). The relationship between the geniculocortical afferents and their cortical target cells during development of the cat's primary visual cortex. *Journal of Neuroscience* **6**: 3655-3668.

SHEPPARD, A.M., HAMILTON, S.K., PEARLMAN, A.L. (1991). Changes in the distribution of extracellular matrix components accompany early morphogenetic events of mammalian cortical development. *J Neurosci* **11**: 3928-3942.

SHIMAMURA, K., HARTIGAN, D.J., MARTINEZ, S., PUELLES, L & RUBENSTEIN, J.L.R. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121 (12)**: 3923-3933.

SIDMAN, R.L., ANGEVINE, J.B., TABER PIERCE, E. (1971). Atlas of the mouse brain and spinal cord. *Cambridge MA: Harvard UP*

SILVER, J. & SIDMAN, R.L. (1980). A mechanism for the guidance and topographic patterning of retinal ganglion cell axons. *J Comp Neurol* **189**: 101-111.

SILVER, J. & RUTISHAUSER, U. (1984). Guidance of Optic Axons In vivo By A Preformed Adhesive Pathway On Neuroepithelial Endfeet. *Developmental Biology* **106**: 485-499.

SIMON, D.K. & O'LEARY, D.D.M. (1992a). Development of Topographic Order In the Mammalian Retinocollicular Projection. *Journal of Neuroscience* **12**: 1212-1232.

SIMON, D.K. & O'LEARY, D.D.M. (1992b). Influence of Position Along the Medial-Lateral Axis of the Superior Colliculus On the Topographic Targeting and Survival of Retinal Axons. *Developmental Brain Research* **69**: 167-172.

SIMON, D.K. & O'LEARY, D.D.M. (1992c). Responses of Retinal Axons *In vivo* and *In vitro* To Position-Encoding Molecules In the Embryonic Superior Colliculus. *Neuron* **9**: 977-989.

SINGER, M., NORLANDER, R.H., EGAR, M. (1979). Axonal guidance during embryogenesis and regeneration of the spinal cord of the newt: the Blueprint Hypothesis of neuronal pathway patterning. *J Comp Neurol* **185**: 1-22.

SNOW, D.M. & LETOURNEAU, P.C. (1992). Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *Journal of Neurobiology* **23**: 322-336.

SRETAVAN, D.W. & SHATZ, C.J. (1987). Axon Trajectories and Pattern of Terminal Arborization During the Prenatal Development of the Cats Retinogeniculate Pathway. *Journal of Comparative Neurology* **255**: 386-400.

SRETAVAN, D.W. & REICHARDT, L.F. (1993). Time-Lapse Video Analysis of Retinal Ganglion-Cell Axon Pathfinding At the Mammalian Optic Chiasm - Growth Cone Guidance Using Intrinsic Chiasm Cues. *Neuron* **10**: 761-777.

SRETAVAN, D.W., FENG, L., PURE, E., & REICHARDT, L.F. (1994). Embryonic neurons of the developing optic chiasm express L1 and CD44, cell-surface molecules with opposing effects on retinal axon growth. *Neuron* **12**: 957-975.

STOYKOVA, A. & GRUSS, P. (1994). Roles of Pax-Genes In Developing and Adult Brain As Suggested By Expression Patterns. *Journal of Neuroscience* **14**: 1395-1412.

TANG, J., LANDMESSER, L., & RUTISHAUSER, U. (1992). Polysialic acid influences specific pathfinding by avian motoneurons. *Neuron* **8**: 1031-1044.

TAYLOR, J.S.H. (1991). The Early Development of the Frog Retinotectal Projection. *Development [Suppl]* **2**: 95-104.

TAYLOR, J.S.H. & GUILLERY, R.W. (1994). Early Development of the Optic Chiasm In the Gray Short-Tailed Opossum, *Monodelphis-Domestica. Journal of Comparative Neurology* **350**: 109-121.

TAYLOR, J.S.H. & GUILLERY, R.W. (1995a). The Effect of A Very Early Monocular Enucleation Upon the Development of the Uncrossed Retinofugal Pathway In Ferrets. *Journal of Comparative Neurology* **357**: 331-340.

TAYLOR, J.S.H. & GUILLERY, R.W. (1995b). Does Early Monocular Enucleation In A Marsupial Affect the Surviving Uncrossed Retinofugal Pathway. *Journal of Anatomy* **186**: 335-342.

TERADA, H., NAGAI, T. KIMURA, H. KITAHAMA, K., OKADA, S. (1996). Distribution of nitric oxide synthase-immunoreactive neurons in fetal rat brains at embryonic day 15 and day 19. *J Chem Neuroanat* **10 (3-4)**: 273-278.

TESSIER-LAVIGNE, M., PLACZEK, M., LUMSDEN, A.G.S. DODD,J. & JESSELL, T.M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**: 775-778.

THANOS, S., BONHOEFFER, F., & RUTISHAUSER, U. (1984). Fiber-fiber interaction and tectal cues influence the development of the chicken retinotectal projection. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 1906-1910.

THOMAS, D., GROUX-MASCATEUI, B., RAES, M-B., CARUELLE, J.P., STEHELIN, D., BARRITULT, D. & BOILLY, B. (1991). Developmental changes of acidic fibroblast growth factor (aFGF) transcription and expression in mouse brain. *Dev Brain Res* **59**: 117-122.

TON, C.C.T., HIROVENEN, H., MIWA, H., WEIL, M.W., MONAGHAN, A.P., JORDAN, T., VAN HEYNINGEN, V., HASTIE, N.D., MEIJERS-HEIJBOER, H., DRECHSLER, M., ROYER-POKORA, B., COLLINS, F., SWAROOP, A., STRONG, L.C., AND SAUNDERS, G.F. (1991). Positional cloning and characterization of a paired box and homeobox containing gene from the aniridia region. *Cell* **67**: 1059-1074.

TORREALBA, F., GUILLERY, R.W., EYSEL, U., POLLEY, E.H., & MASON, C.A. (1982). Studies of Retinal Representations Within the Cats Optic Tract. *Journal of Comparative Neurology* **211**: 377-396.

TOYAMA, K., KOMATSU, Y., YAMAMOTO, N., KUOTANI, T. YAMANDA,K. (1991). In vitro approach to visual cortical development and plasticity. *Neurosci Methods* **12**: 57-71.

TREISMAN, F., HARRIS, E., AND DESPLAN, C. (1991). The paired box encodes a second DNA-binding domain in the Paired homeodomain protein. *Genes Dev* **5**, 594-604.

TREVARROW, B., MARKS, D.L., & KIMMEL, C.B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4**: 669-679.

TRISLER, G.D., SCHNEIDER, M.D., & NIRENBERG, M. (1981). A Topographic Gradient of Molecules In Retina Can Be Used To Identify Neuron Position. *Proc Nat Acad Sci USA* **78**: 2145-2149.

TSUCHIDA, T., ENSINI, M., MORTON, S.B., BALDASSARE, M., EDLUND, T., JESSELL, T.M., & PFAFF, S.L. (1994). Topographic Organization of Embryonic Motor-Neurons Defined By Expression of Lim Homeobox Genes. *Cell* **79**: 957-970.

TUTTLE, R., SCHLAGGAR, B.L., BRAISTED, J.E., & O'LEARY, D.D.M. (1995). Maturation-dependent upregulation of growth-promoting molecules in developing cortical plate controls thalamic and cortical neurite growth. *Journal of Neuroscience* **15**: 3039-3052.

WALTER, J., HENKEFAHLE, S., & BONHOEFFER, F. (1987a). Avoidance of posterior tectal membranes by temporal retinal axons. *Development* **101**: 909-913.

WALTER, J., KERNVEITS, B., HUF, J., STOLZE, B., & BONHOEFFER, F. (1987b). Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. *Development* **101**: 685-696.

WALTHER, C. & GRUSS, P. (1991). Pax-6, A Murine Paired Box Gene, Is Expressed In the Developing CNS. *Development* **113**: 1435-1449.

WANG, L-C., GODEMENT, P., MASON, C.A. (1992). Cells of the optic chiasm midline inhibit uncrossed retinal fiber outgrowth in vitro. *Soc Neurosci Abstr* **18**:222.

WESSELLS, N.K., LETOURNEAU, P.C., NUTTALL, R.P., LUDUENA-ANDERSON, M., GEIDUSCHEK, J.M. (1980). Responses to cell contacts between growth cones, neurites and ganglionic non-neuronal cells. *J Neurocytol* **9**: 647-664.

WHITTEMORE, S.R., EBENDAL, T., LARKFORS, L., & ET AL. (1986). Developmental and regional expression of beta nerve growth factor messenger RNA and protein in the rat central nervous system. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 817-821.

WILLIAMS, R.W., HOGAN, D., & GARRAGHTY, P.E. (1994). Target Recognition and Visual Maps In the Thalamus of Achiasmatic Dogs. *Nature* **367**: 637-639.

WILLIAMS, E.J., WALSH, F.S., DOHERTY, P.(1994). Tyrosine kinase inhibitors can differentially inhibit integrin-dependent and CAM-stimulated neurite outgrowth. *J Cell Biol.* **124**: 1029-1037.

WILSON, S.W., ROSS, L.S., PARRETT, T., & EASTER, S.S. (1990). The Development of A Simple Scaffold of Axon Tracts In the Brain of the Embryonic Zebrafish, *Brachydanio rerio. Development* **108**: 121-145.

WILSON, S.W. & EASTER, S.S. (1991). A Pioneering Growth Cone In the Embryonic Zebrafish Brain. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 2293-2296.

WILSON, S.W. & EASTER, S.S. (1991). Stereotyped Pathway Selection By Growth Cones of Early Epiphysial Neurons In the Embryonic Zebrafish. *Development* **112**: 723-746.

WILSON, S.W., PLACZID, M. & FURLEY, A.J. (1993). Border disputes: do boundaries play a role in growth-cone guidance? *TINS* **16 (8)**: 316-323.

WINDEL, W.F. (1935). Neurofibrillar development of the cat embryo: extent of development in the telencephalon and diencephalon up to 15mm. *J Comp Neuro* **63**: 139-172.

WINDEL, W.F. & BAXTER, R.E. (1935). The first neurofibrillar development in albino rat embryos. *J Comp Neurol* **63**: 173-188.

WIZEMNANN, A., THANOS, S., BOXBERG, Y.V., BONHOEFFER, F. (1993). Differential reactions of crossing and non-crossing rat retinal axons on cell membrane preparations from the chiasm midline: an in vitro study. *Development* **117**: 725-735.

WOLFER, D.P., HENEHANBEATTY, A., STOECKLI, E.T., SONDEREGGER, P., & LIPP, H.P. (1994). Distribution of TAG-1/axonin-1 in fibre tracts and migratory streams of the developing mouse nervous system. *Journal of Comparative Neurology* **345**: 1-32.

WOOD, J.G., MARTIN, S., PRICE, D.J. (1992) Evidence that the earliest generated cells of the murine cerebral cortex form a transient population in the subplate and marginal zone. *Dev Brain Res* **66**: 137-140.

YAGINUMA, H. & OPPENHEIM, R.W. (1991). An experimental analysis of in vivo guidance cues used by axons of spinal interneurons in the chick embryo: Evidence for chemotropism and related guidance mechanisms. *Journal of Neuroscience* **11**: 2598-2613.

YAMADA, T., PLACZEK, M., TANAKA, H., DODD, J., & JESSELL, T.M. (1991). Control of Cell Pattern In the Developing Nervous-System -Polarizing Activity of the Floor Plate and Notochord. *Cell* **64**: 635-647.

YAMAMOTO, M., KUROTANI, T. & TOYAMA, K. (1989). Neuronal connections between the lateral geniculate nucleus and the visual cortex in vitro. *Science* **245**: 192-194.

YAMAMOTO, M., HASSINGER, L.& CRANDALL, J.E. (1990). Ultrastructural localization of stage-specific neurite-associated proteins in the developing rat cerebral and cerebellar cortices. *J Neurocytol* **19**: 619-627.

YAMAMOTO, M., YAMADA, K., KUROTANI, T. & TOYAMA, K. (1992). Laminar specificity of extrinsic cortical connections studied in co-culture preparations. *Neuron* **9**: 217-228.

ZIMMER, A. & ZIMMER, A. (1992). Induction of A RAR-Beta-2-gal Transgene By Retinoic Acid Reflects the Neuromeric Organization of the Central-Nervous-System. *Development* **116**: 977-983.