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The role of *Pax7* in the formation of the superior colliculus and the vertebrate visual system

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The Role of *Pax7* in the Formation of the Superior Colliculus and the Vertebrate Visual System

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USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

Abstract

This thesis has explored the role of *Pax7* in formation and maturation of the mouse superior colliculus, an important brain region associated with topographic visual input and the capacity to evoke appropriate visuomotor responses to environmental stimuli. The research performed was explicitly targeted at understanding the relationship between *Pax7* expression and the formation of the topography of the superior colliculus, including neuron specification and differentiation, boundary formation, polarisation and retinocollicular mapping.

By employing histochemical, immunohistochemical and immunofluorescent techniques, together with quantification utilising Optimas Digital Image Analysis, this project assessed *Pax7* expression in diverse mutant and wildtype mice throughout a variety of developmental timepoints. Quantification revealed dynamic *Pax7* expression patterns consisting of gradients of both cellular distribution and cellular protein levels, which act in summation to polarise the superior colliculus. Graded Pax7 cellular distribution and protein levels are optimal during retinal innervation and axon arborisation in wildtype mice. This contrasts with that of *Pax6* mutant mice which demonstrate reduced Pax7 protein levels as a result of optic nerve hypoplasia. These results demonstrate a responsive role for *Pax7* in retinocollicular mapping, and therefore visual system development.

Examination of *Pax7* mutant mice divulged a greater understanding of the role of *Pax7* during superior collicular development, and provided the first determination of central nervous system defects in *Pax7* mutant animals. Results indicate that *Pax7* is redundant for neuronal specification and differentiation, but is requisite, in a dosage-dependent manner, for maintenance of a subpopulation of dorsal superior collicular neurons and subsequently superior collicular polarity.

The paralogue *Pax3* is thought responsible for sustaining embryonic development in *Pax7* mutant mice, resulting in a less severe phenotype. Therefore, this project explored

temporospatial patterns of *Pax3* expression relative to *Pax7* expression during all embryonic stages examined. Findings detail a diverging expression pattern between paralogues that is spatially and temporally associated with neuronal differentiation and maturation within the superior colliculus. Moreover, *Pax3* expression is spatially perturbed within the central nervous system of *Pax7* mutant mice, exhibiting changes in the time at which *Pax3*-expressing cells exit from the germinal region.

The results of the research undertaken in this thesis have significantly contributed to the understanding of developmental mechanisms occurring within the superior colliculus, and the contribution of *Pax7* to these processes. Findings delineate that the functional repertoire of *Pax7* within the superior colliculus embraces neuronal maturation and maintenance, determination of polarity and participation in retinocollicular mapping.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

(i)   incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;

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Preface

Policy Content A PhD by publication shall conform to Rule 52 plus the following:

(i) completion of the requirements for Confirmation of Candidature (Rule 34);

(ii) a full explanatory introduction, general discussion and conclusion shall be included to link the separate papers and to place them in the context of an established body of knowledge;

(iii) if an adequate contextual literature review is not otherwise presented, it shall be included as a separate chapter;

(iv) if detailed data and descriptions of methods are not otherwise given, they shall be included as appendices;

(v) only papers published in refereed scholarly media during the period of enrolment may be included in a thesis submitted in the form of a series of published papers. However, papers which have been accepted for publication but have not yet appeared in refereed scholarly media may also be included as part of the thesis;

(vi) the number of papers submitted should be sufficient for the body of work to constitute a substantial and original contribution to knowledge;

(vii) any published paper of which the candidate is a joint author may only be included in the thesis provided the work done by the candidate is clearly identified. The candidate must provide to the University Research Students and Scholarships Committee, at the time of submission of the thesis, a written statement from each co-author attesting to the candidate's contribution to a joint publication included as part of the thesis; and

(viii)   the sources from which the candidate's information is derived, the extent to which the work of others has been used and/or for which the assistance of individuals, associations or institutions has been obtained, shall be acknowledged generally in a preface or introduction, and specifically in notes, references and appendices.

Within this thesis, Chapters 4, 5 and 6 were published as co-authored Papers 1,2, and 3 respectively, and were jointly written by the candidate (Jennifer Thompson) and supervisor (Dr Melanie Ziman).

Paper 1 (Chapter 4), a review article published in the journal, Development, Growth and Differentiation, includes an initial preview of laboratory work performed by the candidate on *Pax6* mutant and wildtype mice. Paper 2 (Chapter 5) contains a research paper based on laboratory work conducted by the candidate on *Pax6* mutant and wildtype mice. The animals were generously supplied by Associate Professor Frank Lovicu, who performed the initial handling of the animals including mating, anaesthesia, sacrifice, genotyping, and tissue preservation. In support of this collaboration, Associate Professor Lovicu appears as a co-author on the abovementioned Papers 1 and 2 (Chapters 4 and 5). A paragraph in the methods section of Chapter 5 describing genotyping experiments was written by Associate Professor Lovicu. As a co-author, Associate Professor Lovicu critically reviewed and approved the manuscript.

Paper 3 (Chapter 6) is a research paper generated from experimental results obtained by the candidate on *Pax7* mutant and wildtype mice. These animals were generously donated by Professor Ahmed Mansouri, Max Planck Institute of Biophysical Chemistry, Germany.

Professor Mansouri generated and maintains the *Pax7* mutant mouse line. Animal matings, anaesthesia, sacrifice, genotyping and tissue preservation were performed by Dr Andreas Zembrzycki at the aforementioned institute. As collaborators, Professor Mansouri and Dr Zembrzycki appear as co-authors on Paper 3 (Chapter 6). A portion of the methods section of Paper 3 was reviewed by Dr Zembrzycki, and the manuscript was critically reviewed and approved by both Professor Mansouri and Dr Zembrzycki.

Both research Papers 2 and 3 (Chapters 5 and 6) were jointly conceived and written by Jennifer Thompson and Dr Melanie Ziman, whilst the laboratory experimentation, not including contributions mentioned above, was performed soley by the candidate.

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Index of Abbreviations

ANOVA .... Analysis of Variance

CNS .... Central nervous system

LGN .... Lateral geniculate nucleus

MHB .... Midbrain-hindbrain boundary

PNS .... Peripheral nervous system

RGC .... Retinal ganglion cell/s

SGFS .... *Stratum griseum et fibrosum superficiale*

Definition of Terms

PAX GENES - *PAX* refers to the gene encoding a human PAX transcription factor, whereas *Pax* refers to the gene encoding a vertebrate Pax transcription factor.

SUPERIOR COLLICULUS/TECTUM – The tectum in higher vertebrates, located in the dorsal mesencephalon, encompasses the superior and inferior colliculi. The superior colliculus in mammals is homologous to the tectum in lower vertebrates. As this project is undertaken in mice, the term "superior colliculus" will be used unless referring to early stages of development prior to differentiation of the colliculi, or when the information provided directly relates to experimentation in lower vertebrates, in which case the term "tectum" will be used.

ANIMAL MODEL TERMINOLOGY – Where referring to specific genotypes, the generally accepted nomenclature will be used (eg $Pax7^{+/-}$ refers to *Pax7* heterozygous knockout mice in which one allele is knocked out). When referring to multiple genotypes of a mouse model the term "mutant" will be used (eg *Pax7* mutant mice refers to mice in which either one or both alleles of *Pax7* have been knocked out).

CELLULAR EXPRESSION TERMINOLOGY – Cells positive for a particular protein are designated "protein$^+$" (eg Pax7$^+$ cells are positive for Pax7 protein).

SUPERIOR COLLICULAR TOPOGRAPHY – This term encompasses the functional components of superior collicular development that were assessed in this thesis, specifically superior collicular cytoarchitecture, boundary formation, polarity and retinocollicular mapping. For the purpose of brevity, the collective term "superior collicular topography" will be used unless discussing only one or a number of these components.

CHAPTER 1

INTRODUCTION

"Men love to wonder, and that is the seed of science."

Ralph Waldo Emerson, 1803-1882.

CHAPTER 1: Introduction

1.1 Overview

1.1.1 *Pax* genes

Pax genes are a family of genes that encode highly conserved transcriptional regulators that are crucial for vertebrate development (Balczarek *et al.* 1997); correct gene dosage is also critical (Schedl *et al.* 1996). *Pax6, Pax7* and *Pax3*, members of this pivotal gene family, pattern the developing embryo along its dorsoventral and anteroposterior neuraxes (Goulding *et al.* 1991; Ericson *et al.* 1996). Early extensive expression is believed to confer regional identity, whereas later restricted expression in specific subsets of cells regulates processes such as cell differentiation, proliferation, adhesion, migration, sorting, and axon guidance (Kawakami *et al.* 1997; Stoykova *et al.* 1997; Pratt *et al.* 2000; Stoykova *et al.* 2000; Jones *et al.* 2002; Pratt *et al.* 2002).

Within the developing neural tube, *Pax7* and *Pax3* are expressed in, and subsequently dorsalise, the neural tube along the entire anteroposterior axis. In contast, *Pax6* acts to ventralise the neural tube anteroposteriorly (Goulding *et al.* 1991; Jostes *et al.* 1991; Ericson *et al.* 1996; Mansouri and Gruss 1998). Thereafter, expression becomes restricted to subsets of cells, with these genes playing important roles in the formation of specific brain regions and structures of the vertebrate visual system (Kawakami *et al.* 1997; Nomura *et al.* 1998; Matsunaga *et al.* 2001). *Pax6* functions in forebrain (prosencephalon) specification, formation of the forebrain boundary with the midbrain (mesencephalon), and differentiation of all neuronal cells of the eye (Schwarz *et al.* 1999; Pratt *et al.* 2000; Marquardt *et al.* 2001). Alternatively, *Pax7* and *Pax3* establish midbrain identity and specification of the cells of the superior colliculus (the midbrain area in higher vertebrates to which the optic nerve projects) (Kawakami *et al.* 1997; Nomura *et al.* 1998; Matsunaga *et al.* 2001). *Pax7* and *Pax3* are thought functionally redundant, adding complexity to revealing individual paralogue functions (Mansouri *et al.* 1996a).

This project has examined the role of *Pax7* in establishing superior collicular topography, specifically cytoarchitecture, boundary formation, polarity and retinocollicular mapping. This was determined by comparison of the spatial expression of *Pax7* in wildtype mice relative to that in *Pax6* and *Pax7* mutant mice.

Moreover, a comparative analysis of *Pax7* and *Pax3* expression profiles within the embryonic brain explored temporospatial variations between paralogues and revealed a functional dichotomy related to neuronal differentiation and maturation.

The role of *Pax7* in specification of superior collicular neurons was delineated and here, for the first time, the role of *Pax7* in maturation and maintenance of a subpopulation of neurons was identified.

1.1.2 The superior colliculus/tectum and the lateral geniculate nucleus

In lower vertebrates the tectum is the primary visual centre. In higher vertebrates, the tectum comprises the corpora quadrigemina, constituting the paired superior and inferior colliculi. Most retinal ganglion cells (RGCs) of higher vertebrates project to the laternal geniculate nucleus (LGN) of the diencephalon, which acts as a relay to the visual cortex, the primary visual centre (Hollander and Sanides 1976; Illing and Wassle 1981; Jeffery 1989). A lesser proportion of RGCs project to the superior colliculus. Although the superior colliculus is not the primary visual centre in higher vertebrates, it plays an important role in the visual system by acting to integrate a variety of stimuli (visual, auditory, tactile) with oculomotor functions to facilitate orientation of the eyes and head to environmental stimuli (visuomotor system). The superior colliculus also participates in the reflexive control of eye position, stabilizing images in the retina during movement of the eyes and/or head (Martin 2003, p.162).

The dorsal layers of the superior colliculus receive input from RGCs and interpret visual perception with respect to the environment (Godement *et al.* 1984; Edwards *et al.* 1986b). The ventral layers of the superior colliculus function in eye and neck muscle control, and

processing of somatic sensory and auditory information (Stein 1978; Stein 1984; Martin 2003, pp. 169-170). This input from stimuli is topographically mapped, with the spatial arrangement of the environment preserved and integration of sensory data performed within the superior colliculus to form a spatial map of the environment (Jay and Sparks 1984; Rees 1996). Correct retinocollicular mapping is therefore crucial for a coordinated visuomotor reaction in response to visual stimuli.

1.2 Significance of the study

Induction of brain repair and/or successful nerve regeneration will require fundamental knowledge of the normal biology of neural tissue. In particular, knowledge of genes involved in neuronal cell specification and differentiation and guidance of axon growth cones from their place of origin to the correct target tissue for accurate connectivity will be important. Identification of the hierarchy of gene expression involved in these processes will provide insight into the processes of neural and visual system formation, superior collicular patterning and retinocollicular map formation, as well as differentiation of specific neural subtypes in the important brain region, the superior colliculus.

Previous studies in our laboratory and that of others have detailed the expression of *Pax7* in the superior colliculus and chick tectum, and identified it as important for their formation (Kawakami *et al.* 1997; Nomura *et al.* 1998; Matsunaga *et al.* 2001; Thomas *et al.* 2004; Thomas *et al.* 2006). However, the multiphasic nature of this gene throughout development of mouse superior collicular topography has never been assessed. Moreover, a thorough assessment of the temporospatial relationship between *Pax7*, *Pax6* and *Pax3* at multiple stages of superior collicular development has not been defined. Therefore, in this project *Pax7* expression profiles were examined in the mouse superior colliculus. Moreover, its correlation with *En-1*, *ephrin-A2* and other markers of cellular status were examined during development and maturation, with a view to clarifying the role of *Pax7* in superior collicular development. The abovementioned genes together with *Pax3* were also analysed in the superior colliculus of embryonic and postnatal *Pax6* and *Pax7* mutant mice. These experiments revealed, for the first time, the functional significance of *Pax7* in superior collicular neuronal maturation and maintenance.

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1.3 Overall aim of the study

The purpose of this research was to elucidate the role of *Pax7* in defining the functional characteristics of the superior colliculus, including determination of superior collicular cytoarchitecture, boundary formation, polarity, and retinocollicular mapping. This was achieved utilising wildtype mice, as well as *Pax6* and *Pax7* mutant mice in which the visual system and map formation are/may be perturbed.

The mouse visual system, well studied to date, offers an excellent model for assessing gene expression and regional polarity relative to neural mapping *in vivo*. The genes investigated in this study are involved in axonal mapping of the superior colliculus as well as neural developmental pathways outside the visual system. Therefore, identification of their association with each other in establishment of superior collicular polarity and mapping in the visual system may provide important information with respect to their mutual relationship within the central nervous system (CNS) in general.

Investigation of *Pax7* gene function during neuronal specification and differentiation within the superior colliculus was determined in order to provide important neurobiological information which may be harnessed to augment cell replacement modalities within the mesencephalon and/or other brain regions.

CHAPTER 2

REVIEW OF THE LITERATURE

"It is the mark of an educated mind to be able to entertain a thought without accepting it."

Aristotle, 384 BC - 322 BC.

CHAPTER 2: Review Of The Literature

2.1 *Pax* genes

Overview

The highly conserved *Pax* genes encode transcriptional regulators of vertebrate embryonic development and have arisen from a single ancestral gene as a result of gene duplications throughout evolution.   There are nine known members in vertebrates and seven in *Drosophila*, with the nine vertebrate *Pax* genes grouped into four categories based on sequence homology (Balczarek *et al.* 1997) (Figure 2.1).
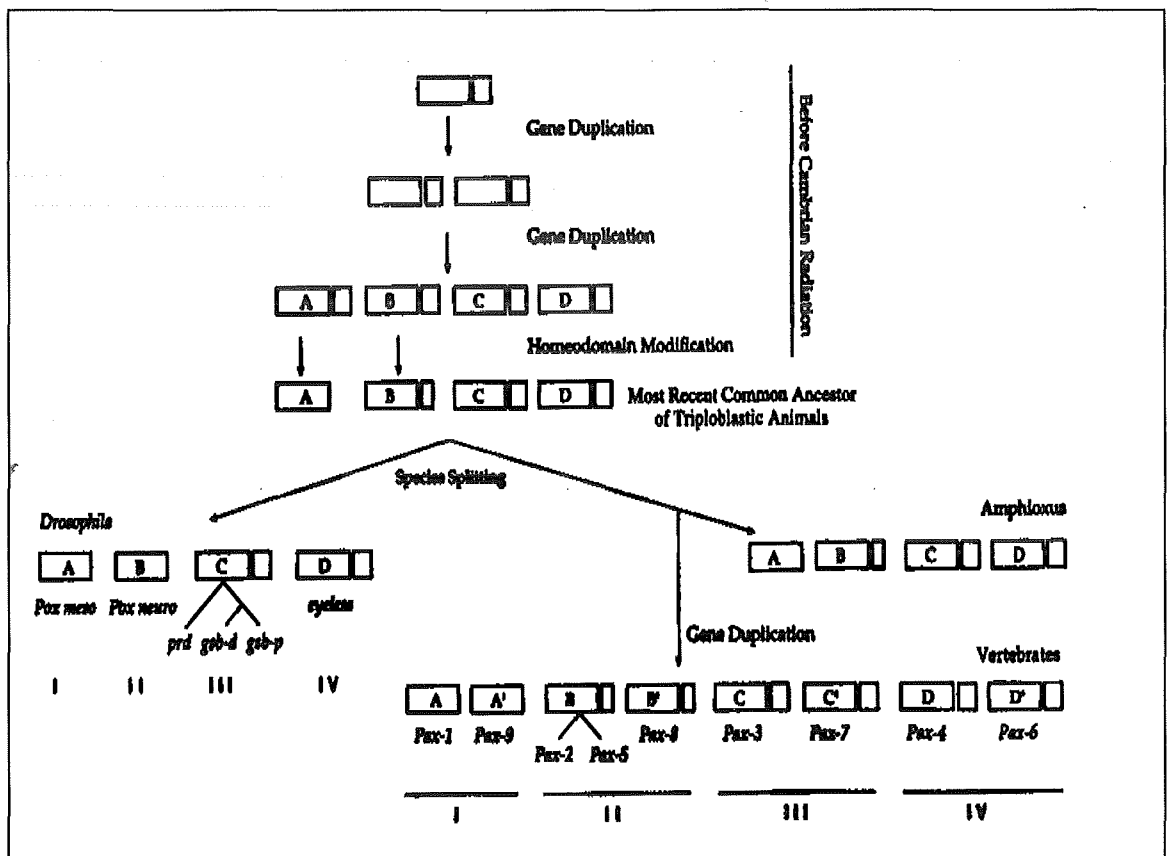
Figure 2.1. *A diagrammatic representation of the evolution of* Pax *genes based on gene duplication and sequence homology (Balczarek et al. 1997).*

Most *Pax* genes are expressed in the developing vertebrate CNS, indicating a clear role for the *Pax* family in vertebrate neurogenesis (Chalepakis *et al.* 1993; Stoykova and Gruss 1994; Peters *et al.* 1998).

2.2 Formation of the vertebrate neuraxis

2.2.1 Neurulation

Overview

Subsequent to gastrulation is the process of neurulation - formation of the neural plate, neural tube, and central and peripheral nervous systems (PNS) (Twyman 2001, pp. 172, 271). The neural plate derives from the dorsal ectoderm (Figure 2.2a) in response to ventralizing signals mediated by Sonic hedgehog (Shh) secreted from the notochord and floor plate (Echelard *et al.* 1993; Roelink *et al.* 1995; Chiang *et al.* 1996; Ericson *et al.* 1996). The lateral portions of the neural plate differentiate with a dorsal perspective due to signals, mediated by the Bone Morphogenetic Protein (BMP) family (Basler *et al.* 1993; Liem *et al.* 1995; Lee and Jessell 1999). This polarization occurs as the neural plate folds and the lateral portions fuse, forming the dorsal midline point and subsequently the neural tube (Figure 2.2b-d). The roof plate forms from the lateral neural plate cells (prosecephalon) (Lee and Jessell 1999) or from cells migrating from the isthmic node, positioned at the isthmic organizer within the dorsocaudal mesencephalon (mesencephalon and rhombencephalon) (Alexandre and Wassef 2003). The roof plate consists of specialized dorsal midline glial cells important in patterning the dorsal neural tube. Neural crest cells arise from the lateral margin of the neural plate and migrate extensively, forming neurons and glial cells of the PNS, as well as melanocytes and other non-neural cell types (Lee and Jessell 1999; Basch *et al.* 2006). Thus, the notochord, roof plate and floor plate are important in patterning the developing embryo along anteroposterior and dorsoventral axes (Lee and Jessell 1999).

Within the neural tube, the neuroepithelial sheet, from which the neural tube derives, becomes the ventricular zone - an area of mitotic activity that generates stem cells used to populate the entire CNS. Postmitotic cells migrate from the ventricular zone outwards, forming the mantle layer, from which grey matter is derived. In this manner, the initial patterning of the neural tube (along anteroposterior, dorsoventral and mediolateral axes) is translated into brain morphogenesis and circuit formation via cell migration, cell sorting and aggregation, axon elongation, fasciculation, target recognition and synapse formation.
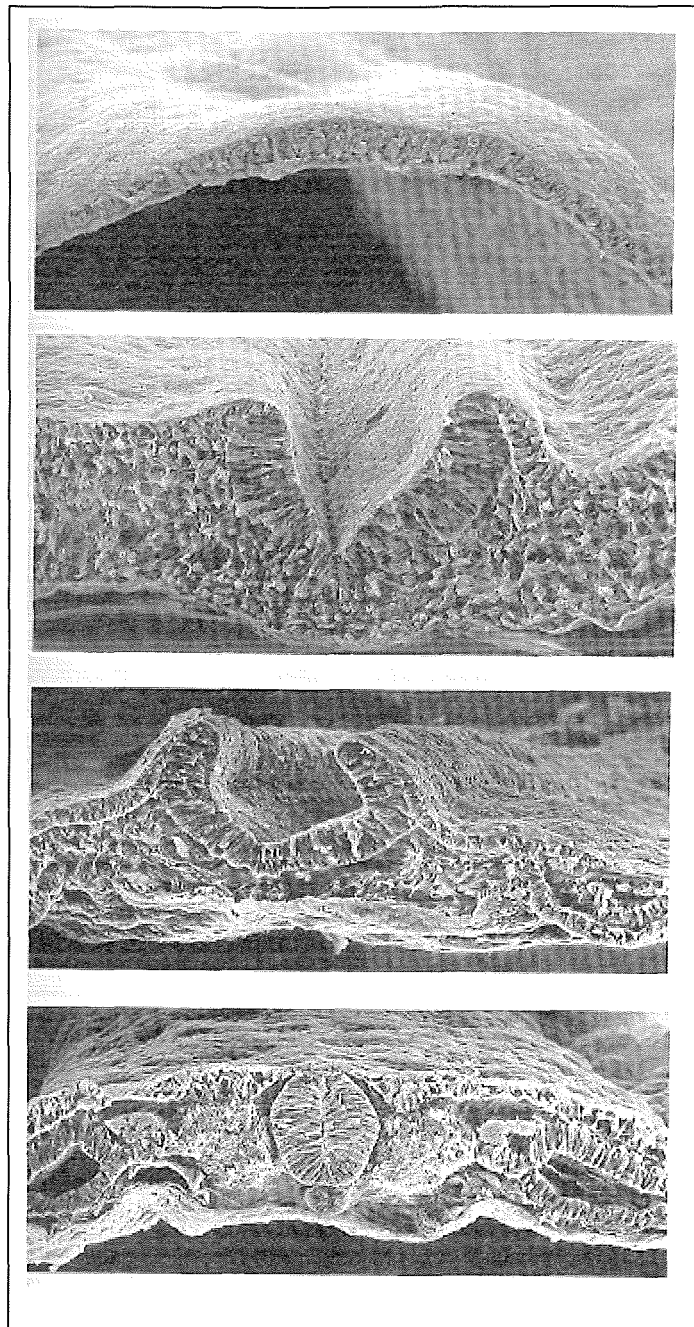
Figure 2.2. **Neurulation - formation of the neural plate, neural tube, central and peripheral nervous systems (chick).** *a) Neural plate; b) folding and elevation of the neural plate; c) convergence of the neural folds; d) neural folds close, forming the neural tube. The neural tube gives rise to the central nervous system. Neural crest cells from the lateral neural plate migrate after closure of the neural tube and form the peripheral nervous system. (Gilbert 2000, p. 381).*

This translation is facilitated by the expression of cell adhesion molecules, and axonal guidance cues of both an attractive and repulsive nature, which function to guide both

migrating neurons and elongating axons. Aggregations of cells, in response to differential cell signals and cell adhesion molecules, eventually form the brain nuclei, from which specific regions of the brain arise (reviewed in Lumsden and Krumlauf 1996; Tanabe and Jessell 1996; Redies and Puelles 2001).

It has been suggested that the increased size and complexity of brains of higher species occurs in response to repeated rounds of patterning necessitated by signal dilution from organizing centres patterning larger distances. Initial expanded growth of a brain region may therefore result in a differentiation trigger causing further expansion, explaining the six-layered neocortex of mammals in comparison to the two-layered allocortex of birds and reptiles (Fernandez *et al.* 1998; Redies and Puelles 2001).

The role of *Pax6* in polarization of the neural tube

The transcription factor Pax6 is one of the earliest gene products expressed in the developing embryo (Walther and Gruss 1991). Initially, *Pax6* is expressed in the neural plate (but not in the floor plate) whereas just after closure of the neural tube it is expressed in the lower ventral region except in the most ventral cells, acting to ventrally polarize the neural tube (Goulding *et al.* 1993) (Figure 2.3).
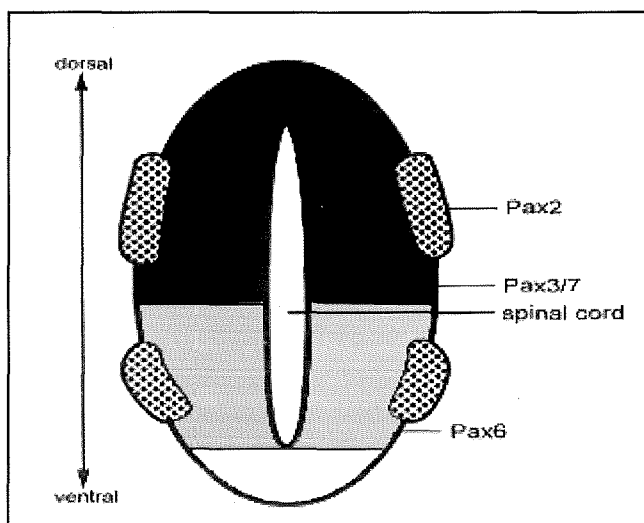
Figure 2.3. *A diagram depicting the polarised dorsoventral expression of* Pax6 *and* Pax7 *in the developing neural tube.* Pax6 *expression is restricted to the mid and ventral neural tube (except the most ventral cells) and* Pax7 *expression is restricted to the dorsal neural tube (adapted from Ziman et al. 2001a).*

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The role of *Pax7* in polarization of the neural tube

Pax7 is expressed prior to neurulation, initially in the presumptive neural crest domain in gastrulating chick embryos (Basch *et al.* 2006) and later, during neurulation, in the midline of the newly forming neural plate before becoming restricted to the lateral neural plate, which is destined to become the dorsal portion of the neural tube (Ericson *et al.* 1996). Initially, *Pax7* expression occurs in the anterior portion of the neural plate, and is not expressed in the more posterior regions until closure of the neural tube. Thereafter expression occurs along the entire anteroposterior axis, but is distinctly limited to the dorsal region (Figure 2.3). *Pax7* expression remains in the dorsal ventricular zone until it becomes the ependyma, at which stage the tissue is no longer mitotically active and becomes *Pax7*-negative (Jostes *et al.* 1991).

2.2.2 Patterning along the anteroposterior axis of the brain

The anterior end of the neural tube eventually segments into three distinct primary vesicles that result in the eventual formation of the fore- (prosencephalon), mid- (mesencephalon) and hindbrain (rhombencephalon) (reviewed in Lumsden and Krumlauf 1996). These primary vesicles are divided functionally (generally) with respect to the senses of smell, vision and hearing (Feduccia and McCrady 1991, p. 421) (Figure 2.4).
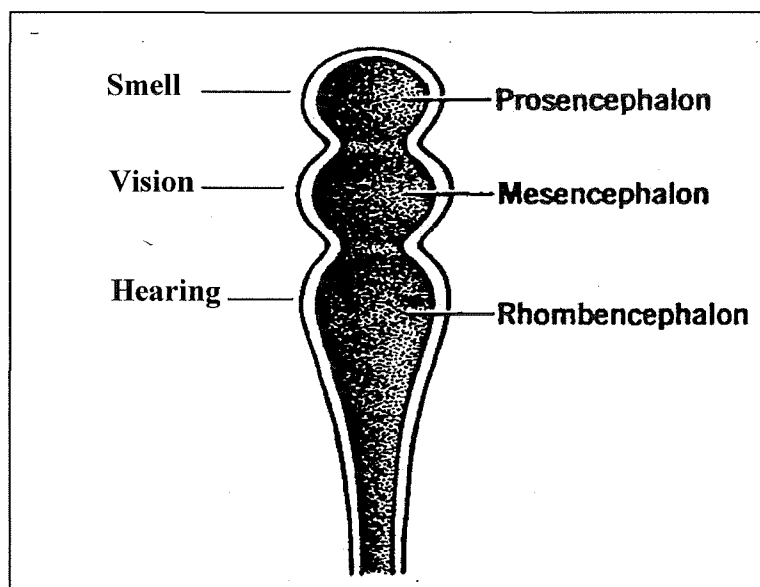
Figure 2.4. A diagram depicting brain segmentation along the anteroposterior axis of the developing neural tube (Feduccia and McCrady 1991, p. 421).

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The first vesicle, the prosencephalon, further differentiates into the telencephalon and diencephalon. The second vesicle forms the mesencephalon. The third vesicle, the rhombencephalon, ultimately gives rise to the metencephalon and the myelencephalon (Feduccia and McCrady 1991, pp. 421-422), with the posterior portion of the neural tube forming the spinal cord (Twyman 2001, p. 277) (Figure 2.5).
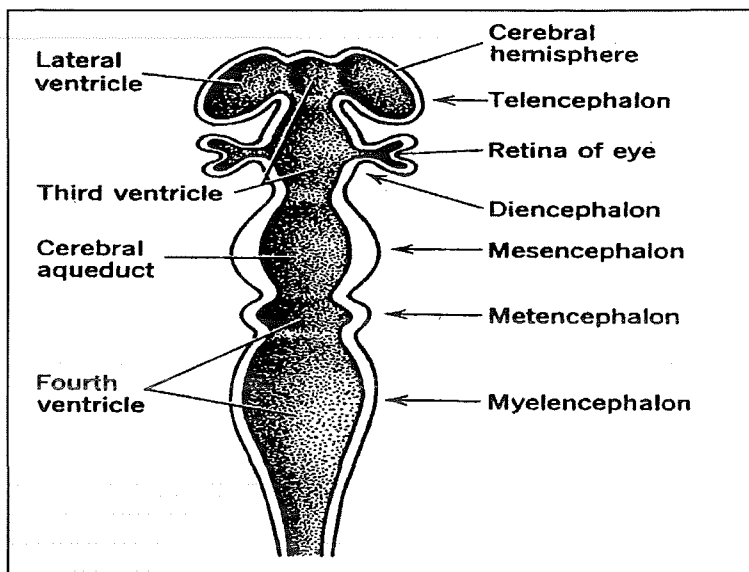
Figure 2.5. A diagram depicting further brain segmentation, resulting in differentiation of the prosencephalon and rhombencephalon into distinct regions (Feduccia and McCrady 1991, p. 422).

The prosencephalon and rhombencephalon are subdivided into neuromeres (prosomeres and rhombomeres, respectively) (Puelles and Rubenstein 1993), with neuromere segments demonstrating specific temporal and spatial patterns of gene expression (Marshall et al. 1992). Regionalisation of the prosencephalon into prosomeres 1 - 6 is initiated by cellular differentiation and migration, generating the region-specific tissues of the forebrain, such as the eye. Each is further subdivided into several longitudinal compartments with prosomere 1 (p1) being the most caudal (Puelles and Rubenstein 1993). Regionalization regulates the subpopulation of neurons, and the navigation of their axons (Mastick et al. 1997).

With the exception of limited exchange of signalling molecules or cells between units, most neurons remain in the domain of their birthplace, giving rise to specific regions with distinct boundaries (Redies and Puelles 2001). Boundary regions have previously been

defined as "interfaces between cells with different molecular properties that reflect distinct programs of gene expression" (Ingham and Martinez-Arias 1992).

Patterning of the anteroposterior axis is achieved through a series of continuous refinements orchestrated by the different inductive properties of organizing centres, from which region-specific gene products are secreted. Each region affords specific identity to its subpopulation of neuronal cells each expressing genes encoding different combinations of region-specific transcription factors (refs: Lumsden and Krumlauf 1996; Nakagawa et al. 1996). Region-specific transcription factor expression is likely to be promoted by the discrete, spatial expression/secretion of appropriate regulatory factors (Lang et al. 2003).

Genes encoding transcription factors known to be involved in prosencephalic specification include *Pax6* (Mastick et al. 1997; Matsunaga et al. 2000) as well as members of the homeobox and winged-helix genes such as *Emx1*, *Emx2*, *Dlx-1*, *Lim1/2*, *Mbx*, *Nbx*, *Otx2*, *BF-1*, *BF-2*, and the Brachyury homolog *Tbr-1* (Bang and Goulding 1996; Fernandez et al. 1998; Perea-Gomez et al. 1999; Shawlot et al. 1999; Kawahara et al. 2002). Region-specific expression of transcription factors such as *Pax6* in the prosencephalon, and *Engrailed/Pax2* in the mesencephalon, together with the secreted factor Fgf8, control the positioning and maintenance of the diencephalic-mesencephalic boundary (Araki and Nakamura 1999; Matsunaga et al. 2000; Scholpp and Brand 2003; Scholpp et al. 2003).

The mesencephalic vesicle is defined by and dependent upon the nested expression of *Pax2* between the rostral (diencephalon) and caudal (rhombencephalon) domains of *Pax6* expression (Schwarz et al. 1999). Mesencephalic specification is also determined by the overlapping expression of the homeobox gene *Otx2* with *En-1* and *Pax2* (Araki and Nakamura 1999; Broccoli et al. 1999; Nakamura 2001a). The interface between the expression domains of the mutually repressive *Otx2* and *Gbx2* positions the isthmic organizer (Wassarman et al. 1997; Broccoli et al. 1999). This important regulatory centre controls patterning of the mesencephalon due to the graded secretion of the isthmic-related proteins Fgf8 and Wnt-1, leading to upregulation of *Pax2*, *Pax5*, *Pax8* and *Engrailed* in the cells surrounding the isthmus in the region of the midbrain-hindbrain boundary (McMahon et al. 1992; Urbanek et al. 1994; Crossley et al. 1996; Joyner 1996). Fgf8, together with *Eng2/Eng3*, is required for maintaining mesencephalic identity in the developing neural

plate (Scholpp *et al.* 2003). Consistent with this paradigm, *Aussicht* mutant zebrafish, which show upregulated *Pax2* and *Fgf8* expression, exhibit perturbation to anteroposterior and mesencephalic patterning (Heisenberg *et al.* 1999).

Homeobox genes, including members of the *Hox* family *(Hoxa-1, Hoxa-2, Hoxb-2, Krox-20, kreisler, Phox2b)* and the *HOM* family, together with retinoic acid, are important in regulating specificity and segmentation of the rhombencephalon (refs: Lumsden and Krumlauf 1996; Pattyn *et al.* 2000).

It is this precise anteroposterior segmentation and refinement together with the position-specific expression of regulating transcription factors and developmental control genes that is responsible for regional specialization, and consequently the blueprint of the CNS.

2.2.3 Patterning along the dorsoventral axis of the brain

Competing dorsalizing and ventralizing signals, secreted from the overlying ectoderm and roof plate (dorsalizing) (Lee and Jessell 1999; Lee *et al.* 2000) and notochord and floor plate (ventralizing) (Tanabe and Jessell 1996; Ericson *et al.* 1997), result in the differential expression of transcription factors that in turn specify the fate of the neuronal subtypes along the dorsoventral axis. In the dorsal neural tube, commissural neurons and association neurons arise, whereas motor neurons and ventral interneurons arise in the ventral neural tube (Basler *et al.* 1993; Goulding *et al.* 1993; Liem *et al.* 1995; Nomura and Fujisawa 2000). This polarised specification of neuronal subtypes within the neural tube imparts regional characteristics which in turn induce specialization of cell nuclei, translating into regional brain morphogenesis (Redies and Puelles 2001).

The dorsoventral axis of the brain subdivides into the basal plate, alar/basal boundary and alar plate (Altman and Bayer 1984) (Figure 2.6).
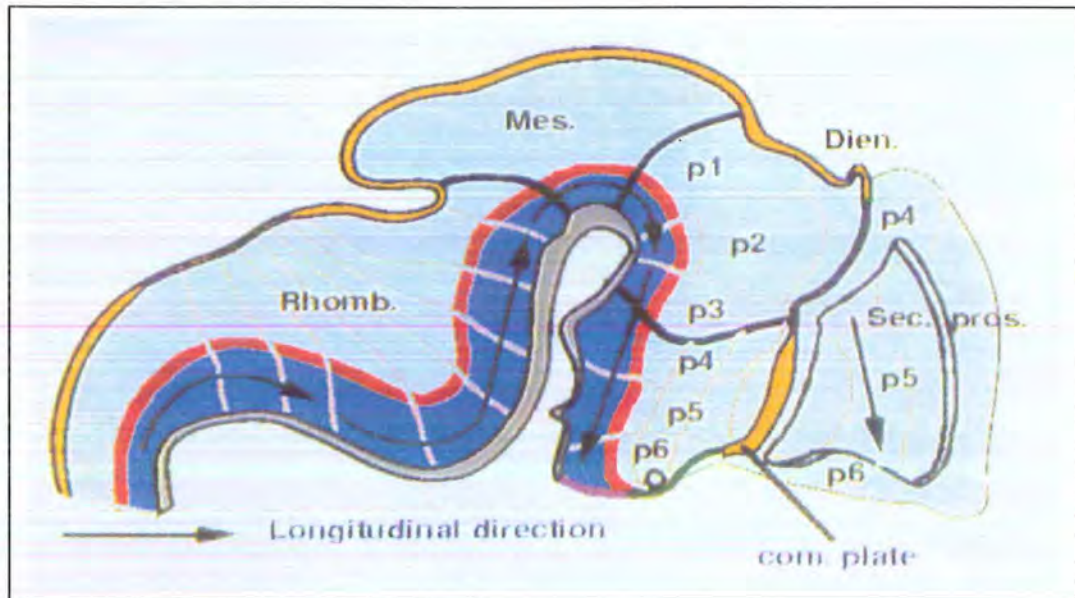
Figure 2.6. A diagram depicting the dorsoventral patterning of the fore-, mid- and hindbrain, identifying the floor plate (grey), basal plate (dark blue), alar/basal boundary (red), alar plate (light blue) and roof plate (yellow) (Puelles and Rubenstein 1993).

There are a panoply of genes that cooperate in patterning the dorsoventral axis of the brain. Although it is understood that it is the joint action of these genes that determine regional specification and polarity, this discussion will focus on the role of *Pax6* and *Pax7* in dorsoventral axis patterning of the brain and specification of visual system brain regions.

2.3 Formation of the vertebrate visual system

2.3.1 The brain regions involved in the visual system

Specialisation of the prosencephalon

Overview

As previously mentioned, the prosencephalon divides along its rostrocaudal axis into the telencephalon (rostral) and diencephalon (caudal). The telencephalon gives rise to the cerebral cortices (which contain the primary visual centre), the hippocampus, basal ganglia

and olfactory bulb. The diencephalon gives rise to the thalami (epi/hypo/ventral/dorsal) incorporating the LGN, and the pretectum and eyes (Puelles and Rubenstein 1993; Fernandez *et al.* 1998).

The role of *Pax6* in specialisation of the prosencephalon

Initially, *Pax6* is expressed throughout the prosencephalon (Kawakami *et al.* 1997) before it becomes restricted to the alar plate of the prosencephalon, immediately below the roof plate (Puelles and Rubenstein 1993). The crucial role of *Pax6* in regionalisation of the developing prosencephalon is demonstrated in *Pax6* mutant mice. *Sey (Pax6$^{-/-}$)* mice show extensive defects in prosencephalic patterning, including loss of eye structures, subsets of cells in the cortex and alteration of the diencephalic-mesencephalic boundary (Hill *et al.* 1991; Mastick *et al.* 1997; Stoykova *et al.* 2000). *Pax6$^{lacZ/lacZ}$* mice also exhibit alterations to thalamocortical and corticofugal axon pathfinding (Jones *et al.* 2002; Pratt *et al.* 2002). Moreover, *Pax6* is required for the normal development of the dorsal thalamus (Pratt *et al.* 2000). In the telencephalon *Pax6* modulates dorsoventral patterning of brain structures via inhibition and activation of region-specific genes (Stoykova *et al.* 2000). *Pax6*, therefore, plays a crucial role in the correct patterning and formation of the prosencephalon.

Specialisation of the mesencephalon

Foreword: A synopsis of some of the following data is encompassed in a review paper which constitutes the body of Chapter 4. However, this Chapter (2) addresses these processes in greater detail and has therefore been included for clarity.

Overview

The mesencephalon can be subdivided functionally and histologically into two separate domains – the inferior and superior colliculi/tectum (dorsal) and the tegmentum (ventral). The superior colliculus/tectum originates from the mesencephalic alar plate and the tegmentum from the mesencephalic basal plate (Nomura and Fujisawa 2000). Dorsoventral

differentiation of the mesencephalon is due to region-specific expression of transcription factors and the interplay between many gene products across both dorsoventral and anteroposterior axes, as discussed below.

Dorsoventral patterning of the mesencephalon

The mesencephalic floor plate and the notochord participate in establishing ventral mesencephalic polarity. The ventral limit of the superior colliculus, at the interface between the superior colliculus and tegmentum, is defined by secretion of ventralizing signals such as Shh from the mesencephalic floor plate and notochord. Shh activates the expression of ventral-specific genes such as *Pax6* (Agarwala *et al.* 2001) (Figure 2.7) and also assists in patterning the ventral mesencephalon into discrete arcs of neuronal identity across the mediolateral axis (Agarwala and Ragsdale 2002).
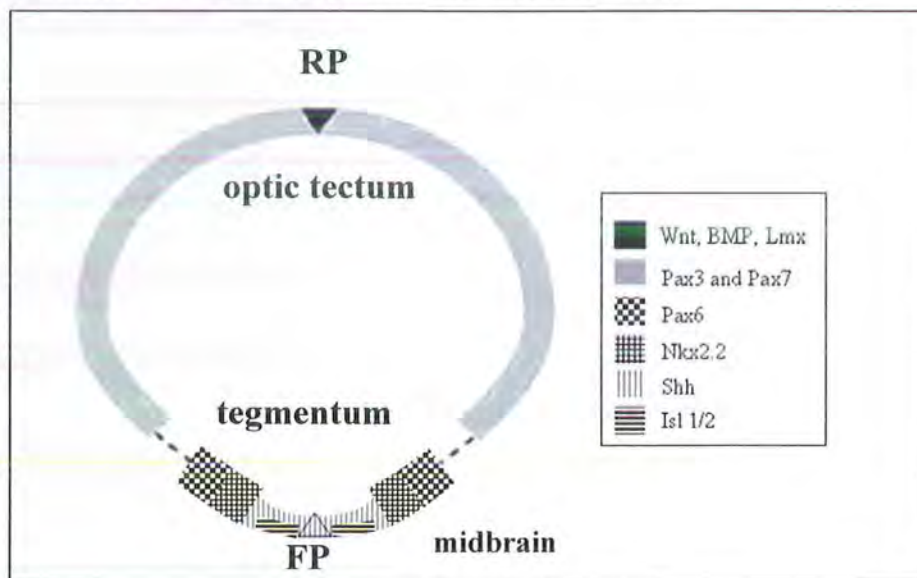
Figure 2.7. A diagram depicting expression domains of genes involved in dorsoventral patterning of the mesencephalon. Abbreviations: RP, roof plate; FP, floor plate (adapted from Alexandre and Wassef 2005).

In a recent study, quail mesencephalic ventral floor plate, expressing *Shh*, was transplanted into the dorsal mesencephalon of chick embryos (*Shh*-negative), resulting in induction of an ectopic tegmentum in the dorsal mesencephalon (and alteration to retinotopic mapping). Furthermore, the importance of Shh secretion from the notochord was demonstrated using Shh-secreting quail fibroblasts transplanted into the dorsal mesencephalon where an ectopic

tegmentum was formed (Nomura and Fujisawa 2000). These results highlight the roles of the mesencephalic floor plate and the notochord (including Shh secretion) in dorsoventrally polarising the mesencephalon (refer to section 2.3.1 [pp.22-23] of this thesis for further details).

BMP genes are expressed dorsally, initially in the neural folds and subsequently (after neural tube closure) by the roof plate and surrounding tissues (Basler *et al.* 1993; Liem *et al.* 1995; Lee *et al.* 1998), and act to dorsally polarize the mesencephalon by activating the expression of *Pax3* and *Pax7*, amongst other genes (Liem *et al.* 1995; Monsoro-Burq *et al.* 1996). Interestingly, the mesencephalic roof plate, a key determinant of dorsal polarity, is generated by migration of cells from a node within the isthmic organizer during anteroposterior development (Alexandre and Wassef 2003) and is positionally constrained by Shh (Fogel *et al.* 2008).

The initial dorsoventral polarity dictated by *Shh* and *BMP* genes imparts positional specification upon mesencephalic cells, determining the morphologically distinct, laminated tectum/superior colliculus from the discrete nuclei of the tegmentum. This positional specification eventually becomes inherent within the cell, and thus is independent of signaling centres after a specific developmental stage (stage 16 in chick), as elucidated recently by elegant, temporal transplantation experiments (Li *et al.* 2005).

Importantly, *Pax3* and *Pax7* are thought to specify dorsal mesencephalic identity, as distinct from that of the ventral tegmentum, culminating in formation of the superior colliculus (Nomura *et al.* 1998; Matsunaga *et al.* 2001). In the developing chick brain, *Pax7* expression initially occurs along the roof plate and alar plate, eventually becoming restricted to the roof plate of the telencephalon, the epithalamus, and pretectum anteriorly, and to the alar plate of the mesencephalon and cerebellum posteriorly (Figure 2.8).

Ultimately, *Pax7* expression becomes restricted to the dorsal midbrain, specifically the tectum and epiphysis (Kawakami *et al.* 1997). Within the developing mouse tectum (E13), *Pax7* and *Pax3* share extensive overlapping expression domains (Stoykova and Gruss 1994).
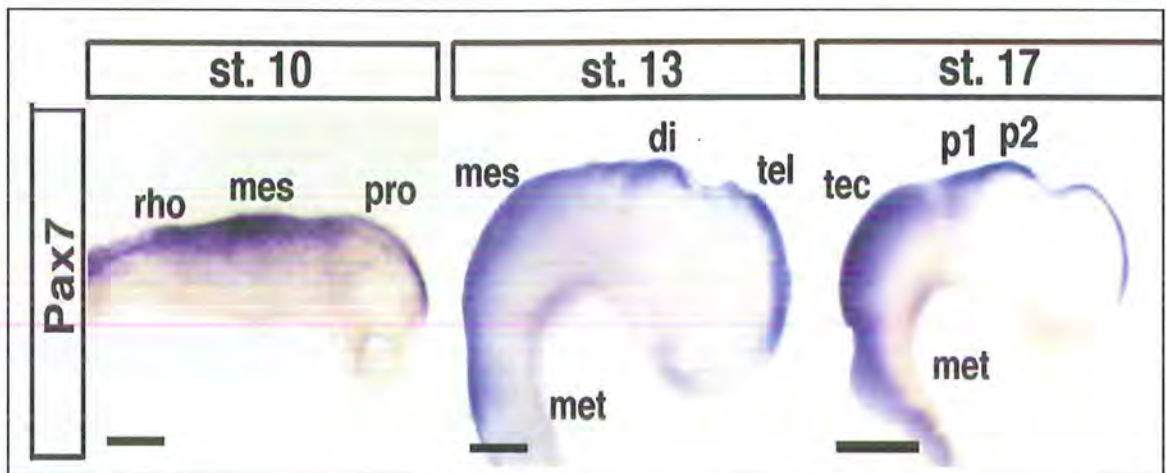
Figure 2.8. Expression pattern of Pax7 at various stages in the developing chick brain. Detected by whole mount in situ hybridization. Stage 10 (10 somite stage); Stage 13 (18 somite stage) and Stage 17 (E2.5). Abbreviations: di, diencephalon; mes, mesencephalon; met, metencephalon; pro, prosencephalon; p1, prosomere 1; p2, prosomere 2; rho, rhombencephalon; tec, tectum; tel, telencephalon. Scale bars 200µm Stages 10/13; 500µm stage 17 (Matsunaga et al. 2001).

Anteroposterior patterning of the mesencephalon

The mesencephalon demonstrates anteroposterior polarity due to the graded distribution of secreted molecules from the organizing centre, the isthmus (situated caudally), in particular Fgf8 and Wnt-1, which activate transcription factors in surrounding cells, including *En, Pax2, Pax5, Pax8,* and *ephrin-A2* (McMahon and Bradley 1990; Thomas and Capecchi 1990; Thomas *et al.* 1991; Krauss *et al.* 1992; McMahon *et al.* 1992; Urbanek *et al.* 1994; Drescher *et al.* 1995; Crossley *et al.* 1996; Favor *et al.* 1996; Lee *et al.* 1997; Schwarz *et al.* 1997; Urbanek *et al.* 1997; Lun and Brand 1998; Funahashi *et al.* 1999; Okafuji *et al.* 1999; Nomura and Fujisawa 2000) (Figure 2.9).

This cascade of activation induces differentiation, migration and/or survival capacity in adjacent tissue (reviewed in Hidalgo-Sanchez *et al.* 2005) and is initiated via complex combinatorial genetic expression, briefly discussed below. Furthermore, by producing the cells that eventually form the roof plate, the isthmic organizer also influences dorsoventral patterning (Alexandre and Wassef 2003).

**Figure 2.9.** A diagram depicting overlapping expression domains of genes involved in specification of regional patterning in the chick anteroposterior neuraxis (adapted from Nakamura and Watanabe 2005).

Correct positioning of the isthmic organizer is, therefore, crucial for correct establishment of anteroposterior polarity. The mutually repressive relationship between _Gbx2_ (expressed in the rhombencephalon) and _Otx2_ (expressed in the prosencephalon and mesencephalon) occurs from early neural plate stages, with the interface between their expression domains acting to correctly position the isthmic organizer, as evidenced by induction of _Fgf8_ and _Wnt-1_ expression at this site (Simeone _et al._ 1992; Millet _et al._ 1996; Wassarman _et al._ 1997; Broccoli _et al._ 1999; Hidalgo-Sanchez _et al._ 1999; Irving and Mason 1999; Katahira _et al._ 2000).   Fgf4 (a notochord-derived vertical signal involved in anteroposterior patterning of the mesencephalon) has also been shown to induce the isthmus-related genes _Fgf8, Wnt-1, En-1, En-2_ and _Pax2_ (Shamim _et al._ 1999).   _Lmx1b_, expressed in the mesencephalon, is induced by _Otx2_, and acts to repress _Fgf8_ except at the _Wnt-1_ domain, where it indirectly regulates _Fgf8_ via _Wnt-1_, acting to restrict the anterior expression of _Fgf8_ to the isthmic region (reviewed in Nakamura and Watanabe 2005).   _Pax2_, itself inhibited anteriorly by _Grg4_ in the mesencephalon, is essential for induction and correct

positioning of *Fgf8* (Ye *et al.* 2001). *Gbx2* and *Otx2* are not, however, required for *Fgf8* induction, as Fgf8 is detected in *Otx2/Gbx2* single and double mutant mice (Li and Joyner 2001; Martinez-Barbera *et al.* 2001). Multiple misexpression and transplantation experiments therefore highlight the key status of the *Otx2/Gbx2* interface as crucial for correct positioning, but not induction, of the isthmic organizer (Millet *et al.* 1996; Broccoli *et al.* 1999; Hidalgo-Sanchez *et al.* 1999; Irving and Mason 1999; Millet *et al.* 1999; Katahira *et al.* 2000).

Fgf8 misexpression in the chick anterior mesencephalon or posterior diencephalon results in production of an ectopic isthmus (Crossley *et al.* 1996; Martinez *et al.* 1999; Shamim *et al.* 1999) via a negative feedback loop resulting in repression of *Otx2* expression (Martinez *et al.* 1999). Absence of *Fgf8* in homozygous *acerebellar* zebrafish (Reifers *et al.* 1998) and in *Fgf8* mutant mice (Meyers *et al.* 1998) results in deletion of the cerebellum and mesencephalon, encompassing the isthmic region, with deletion of the mesencephalon due to extensive cell death (Chi *et al.* 2003). These experiments indicate a crucial role for *Fgf8* in induction and correct positioning of the isthmic organizer, and maintenance of mesencephalic identity, and as such determination of cell fate, polarity and cell survival (Echevarria *et al.* 2005).

Otx2 is also required for initiating *En-1* expression (Rhinn *et al.* 1998). *Engrailed, Pax2* and *Fgf8* repress *Pax6* activity, limiting its anterior expression domain to the prosencephalon, with upregulation of other mesencephalic-related genes and formation of the mesencephalon (Araki and Nakamura 1999; Okafuji *et al.* 1999; Scholpp *et al.* 2003), culminating in formation of the diencephalic-mesencephalic boundary (Araki and Nakamura 1999; Matsunaga *et al.* 2000; Scholpp *et al.* 2003) (refer to section 2.3.1 [pp. 24-26] in this thesis for further details).

En-1 has an anterior limit of expression at the rostral boundary of the mesencephalon, whereas the posterior limit of *Otx2* expression forms the caudal boundary of the mesencephalon, and their overlapping expression, as shown in Figure 2.9, together with *Pax2* and *Fgf8*, may be pivotal in defining the mesencephalon along the anteroposterior axis (Araki and Nakamura 1999; Broccoli *et al.* 1999; Nakamura 2001a; Scholpp *et al.* 2003). In support of this, misexpression of either *En1/2, Pax2/5* or *Fgf8* forms a feedback

loop with subsequent ectopic tectal formation, identifying these genes as substantial determinants of mesencephalic identity (reviewed in Nakamura and Watanabe 2005).

The role of *Pr 7* and *En* in mesencephalic specification and formation of the superior colliculus

Previous misexpression experiments in chick embryos have clearly defined *Pax7* as a pivotal gene in specifving the tectum from the mesencephalon (Matsunaga *et al.* 2001). Following initial expression in the alar plate (Matsunaga *et al.* 2001), *Pax7* is strongly expressed in differentiating neuronal cells prior to laminae formation. Strong expression of *Pax7* can still be detected in a subset of cells which begin migrating radially toward the pia of the tectum after formation of laminae commences, finally concentrating in a layer known as the *stratum griseum et fibrosum superficiale* (SGFS), the target site for retinal axons (LaVail and Cowan 1971; Kawakami *et al.* 1997). Recent studies have demonstrated variations in subpopulations of chick tectal cells during development, with $Pax7^+$ cells fated to more dorsal laminar fates (Fedtsova *et al.* 2008). $Pax7^+$ cells remain in the adult mouse superior colliculus (Stoykova and Gruss 1994) and chick tectum (Shin *et al.* 2003; Thomas *et al.* 2004) where they are expressed in decreasing caudorostral and dorsoventral gradients (Thomas *et al.* 2004), implicating *Pax7* in determination of mesencephalic polarity and therefore map formation.

Initially, tectum formation correlates with the coordinated expression of *Pax7* and suppression of *Pax6* (Nomura *et al.* 1998). Shh secreted from the mesencephalic floor plate has been shown to repress *Pax7* expression (Nomura and Fujisawa 2000; Watanabe and Nakamura 2000), thereby acting to limit *Pax7* expression to the dorsal mesencephalon, the tectum (Nomura *et al.* 1998). However, whilst Shh activates *Pax6* expression ventrally it does not initiate the dorsal restriction of *Pax7*, although it is required for maintaining this restriction, as demonstrated in *Shh* mutant mice (Fogel *et al.* 2008).

When *Pax3* or *Pax7* are misexpressed in either the tegmentum or the diencephalon of chick embryos, the repression of *Pax6* and induction of other tectum-related genes such as *Fgf8* and *En-2* lead to formation of ectopic tectal tissue. This confirms a significant role for *Pax3* and *Pax7* in defining the tectum from the mesencephalic alar plate where they act in a

feedback loop together with other tectal-related genes, such as *En* and *Fgf8* (Matsunaga *et al.* 2001). Conversely, when quail mesencephalic floor plate was transplanted to the chick tectum, suppression of *Pax7* caused the tectum to adopt a tegmental identity (Nomura and Fujisawa 2000). Similarly, mice lacking Shh exhibit Pax7$^+$ neurons, typical of the tectum, in the tegmentum (Fedtsova and Turner 2001; Fogel *et al.* 2008). Collectively, these experiments indicate that in the mesencephalon the expression of *Pax7* is required for tectum formation, and tegmentum is formed in its absence (Nomura *et al.* 1998).

Expression of *En* in the mesencephalon is controlled by Fgf8, Wnt-1 (Crossley *et al.* 1996; Martinez *et al.* 1999) and *Pax2* (Song *et al.* 1996). Like *Pax7*, *En* is initially expressed along the entire anteroposterior neuraxis and in the early mesencephalon (Davis *et al.* 1991). Disruption of expression has been shown to cause severe defects in mesencephalic development (Wurst *et al.* 1994). *Pax7* expression, however, becomes restricted to the superior colliculus, whereas *En* is expressed in both the superior colliculus and tegmentum. *En* may function during normal mesencephalic development to impose mesencephalic fate on precursor cells, or to inactivate an inhibitor of this fate subsequently allowing differentiation, growth and survival of mesencephalic cells (Araki and Nakamura 1999) (refer section 2.4.2 [pp.40-41] of this thesis for further details). Thus, previous research provides evidence of the vital roles of both *Pax7* and *En* in superior collicular formation and maintenance, however their relationship remains obscure.

In order to study tectum formation, Nomura *et al.* (1998) investigated chick/quail chimeras in which the isthmic region was ablated. *En-2* was not expressed in the ablated tectum, consistent with the role of the isthmic organizer in regulating *En* expression in the mesencephalon (McMahon *et al.* 1992; Crossley *et al.* 1996; Martinez *et al.* 1999). The dorsal mesencephalon continued to express *Pax7* as normal, and a small tectum was formed, which gradually regressed and eventually disappeared. These results indicate firstly that induction of *Pax7* expression (initially expressed in the neural plate) (Ericson *et al.* 1996) and subsequent tectum formation is independent of the isthmus and *En*, and secondly that either the isthmus or *En* is required for the **maintenance** of *Pax7* expression and the tectum. Similar results were obtained in zebrafish *noi* (*no isthmus*) mutants, which lack a functional Pax2 protein (and thus lack engrailed, wnt-1 and zash1A); in these

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zebrafish, a tectum initially forms but later degenerates (Brand *et al.* 1996). In addition, misexpression of *En-2* in the chick diencephalon resulted in a rostral shift of the diencephalic-mesencephalic boundary into the caudal diencephalon, and induced ectopic expression of *Pax7* and *ephrin-A2*, with concomitant repression of *Pax6* (Araki and Nakamura 1999).

Taken together, these results suggest that *Pax7* and *En* may participate in a feedback loop with isthmic-related genes (such as *Fgf8* and *Wnt-1*) and transcription factors (such as Pax2, Otx2 and Gbx2) to determine the formation and differentiation of the dorsal mesencephalon, the superior colliculus, and as such may together define the topographical properties which in turn control map formation (refer section 2.4.2 [pp.39-42] of this thesis for further details on the relationship between Pax7, En and map formation).

Complementary expression of *Pax6* and *Pax7* defines brain regions and participates in boundary formation

As addressed previously, the diencephalic-mesencephalic boundary is thought formed by the repressive interaction between *Pax6* (diencephalon) and *En-1/Pax2* (mesencephalon) (Araki and Nakamura 1999; Matsunaga *et al.* 2000). Further experimentation has indicated, however, that these are not the only genes that participate in diencephalic-mesencephalic boundary formation. In *noi* (*no isthmus*) mutant zebrafish, in which *pax2.1* is non-functional leading to ablation of *Pax5*, *Pax8* and *engrailed* expression, initial formation of the diencephalic-mesencephalic boundary occurs, as indicated by markers for this area, but it is altered at later stages (Brand *et al.* 1996; Pfeffer *et al.* 1998; Scholpp and Brand 2003). This indicates that it is possible for the diencephalic-mesencephalic boundary to form in the absence of *Pax2* and *En*. Furthermore, the combined loss of *Fgf8* and *Eng-2/Eng-3* has been shown to perturb formation of the diencephalic-mesencephalic boundary in zebrafish (Scholpp and Brand 2003; Scholpp *et al.* 2003), demonstrating that this boundary is not formed by repressive interaction between *Pax6* and *En-1/Pax2* expression alone, and that other genes participate in its formation and/or maintenance.

In this thesis the role of *Pax6* and *Pax7* in defining the placement of rostral and ventral superior collicular boundaries was explored. The rationale for this investigation came from observations that complementary expression domains of *Pax6* and *Pax7* define neighbouring brain regions (Stoykova and Gruss 1994; Kawakami *et al.* 1997; Nomura *et al.* 1998; Matsunaga *et al.* 2000).

Therefore, the diencephalic-mesencephalic boundary may also be defined by the border of expression of *Pax6* (diencephalon) together with *Pax7* (superior colliculus/tectum) (Kawakami *et al.* 1997; Matsunaga *et al.* 2000). In support of this hypothesis, misexpression of *Pax6* in the embryonic chick mesencephalon results in a caudal shift of the diencephalic-mesencephalic boundary (Figure 2.10), repression of *Pax7*, and fate change of the rostral tectal swelling (mesencephalon) to the pretectum (diencephalon) (Matsunaga *et al.* 2000). Conversely, when *Pax7* is misexpressed in the diencephalon, *Pax6* is repressed and tectal genes are upregulated, causing fate change of the dorsal diencephalon to the tectum (Araki and Nakamura 1999; Matsunaga *et al.* 2001).
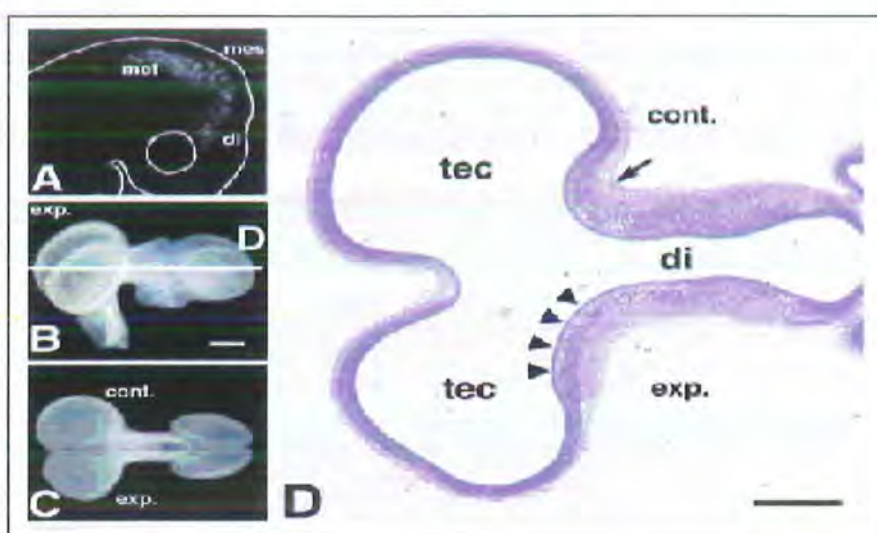
Figure 2.10. **Caudal shift of the diencephalic-mesencephalic boundary due to Pax6 misexpression in the embryonic chick mesencephalon.** *A) Pax6 misexpression after electroporation. Transfection of Pax6 in the neural tube occurred from the diencephalon to the metencephalon on the experimental side. B) Lateral view (showing plane of cut for figure D) C) Dorsal view D) Arrowheads indicate caudal shift of the diencephalic-mesencephalic boundary on the experimental side in contrast to the control side. Scale bar 250μm. (Matsunaga et al. 2000).*

Pax6⁻ᐟ mice also exhibit fate change of the most caudal portion of the diencephalon, the pretectum, to that of the mesencephalon, resulting in defective diencephalic-mesencephalic boundary formation (Mastick *et al.* 1997). The results of the abovementioned studies suggest that reciprocal *Pax6* and *Pax7* expression may assist in defining the diencephalic-mesencephalic boundary and the superior collicular/tegmental boundary.

2.3.2 Formation of vertebrate eyes from the diencephalon

Overview

Initial eye specification occurs as a band of *Pax6* expression within the midline of the anterior neural plate (Li *et al.* 1997). *Shh* initiates *Pax2* expression within the mid region of the *Pax6* band, acting to downregulate *Pax6* medially, bisecting the band into two bilaterally symmetrical prospective eye fields. This establishes proximodistal axis specification, with *Pax2* proximally specifying the optic stalks and *Pax6* distally specifying retinal tissue (Macdonald *et al.* 1995; Chiang *et al.* 1996).

Morphologically, the sprouting of optic vesicles from the sides of the diencephalon at E8.5 signifies the beginning of eye formation (Figure 2.11a). The vesicles grow laterally, with distal portions enlarging, in contrast to the connection to the diencephalon, which constricts to form the optic stalk (Figure 2.11b). The growing distal surface of the optic vesicle flattens as it approaches the non-neural ectoderm. Subsequent thickening of this ectodermal tissue results in the formation of the lens placode (Figure 2.11c), which in turn invaginates in concert with the optic vesicle at E9.5 (Figure 2.11d), and together they form the lens vesicle and optic cup, respectively. The lens placode pinches off forming the lens vesicle, distinct from the remaining ectoderm (Figure 2.11e). The separated ectoderm fuses, covers the lens and becomes the cornea (Moore 1988, p.403; Browder *et al.* 1991, pp. 655-688; Feduccia and McCrady 1991, p.466; Chow and Lang 2001).
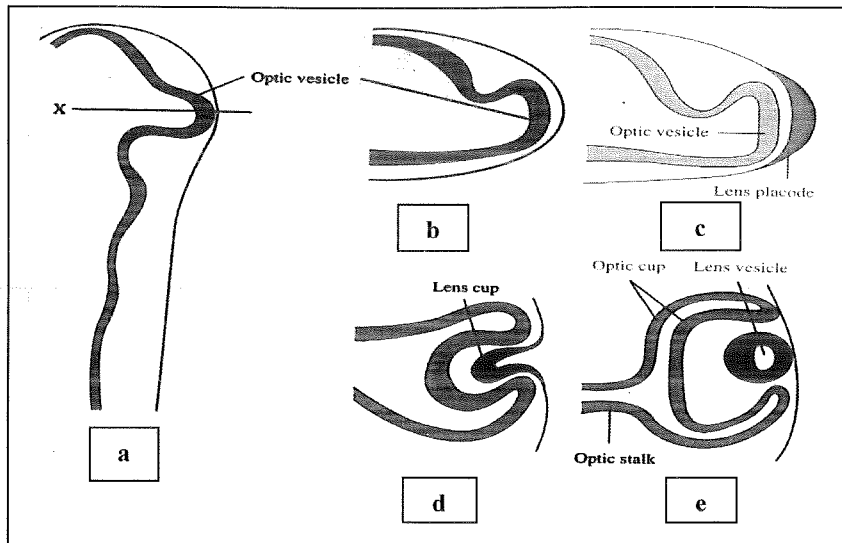
Figure 2.11. *A diagrammatic representation of the embryonic development of the eye.*
a) Sprouting of the optic vesicle; b) lateral growth of the optic vesicle; c) formation of the lens placode; d) invagination of the optic vesicle and lens placode; e) formation of the optic cup and lens vesicle (Browder et al. 1991, p. 686).

The two layers of the optic cup further differentiate into the retinal pigmented epithelium and the neural retina (Moore 1988, p. 403) (Figure 2.12).
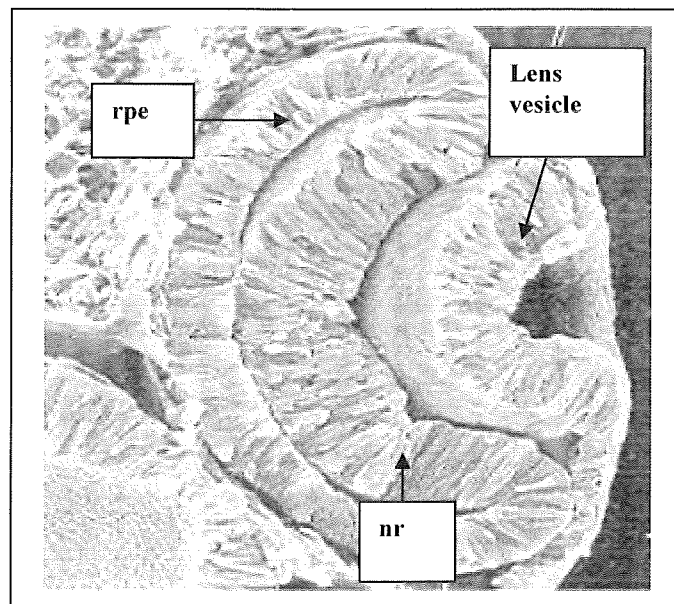
Figure 2.12. *The two layers of the optic cup – the retinal pigmented epithelium (rpe) and the neural retina (nr)(Gilbert 2000, p. 400).*

The neural retina (the sensory retinal layer) differentiates into functional photoreceptor rods and cones, bipolar neurons and ganglionic neurons (Moore 1988, p. 466; Feduccia and McCrady 1991, p. 403) (Figure 2.13) with ganglionic cells being the first neurons formed in the retina (Young 1985; Harman and Beazley 1989). In the mouse, RGC neuronogenesis commences in the central retina at E11.5 and continues through to E18, whilst spreading peripherally (Drager 1985).
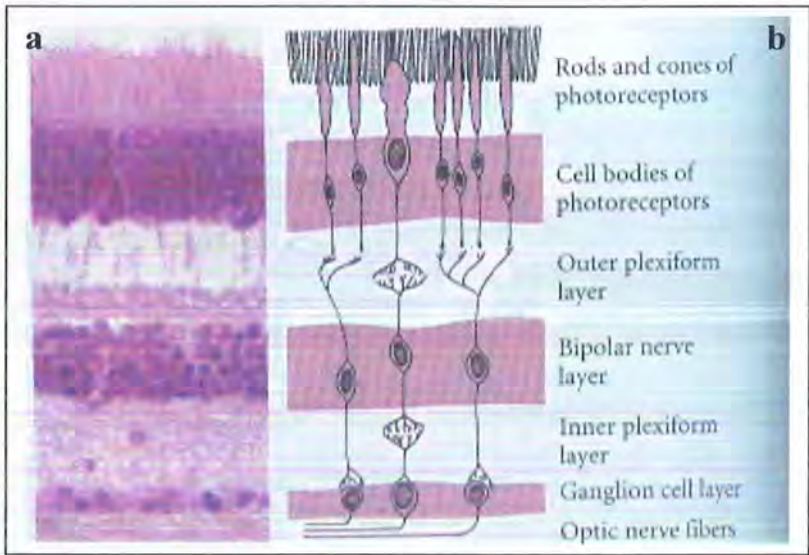
Figure 2.13. *The layers of the human retina. a) Three separate layers of neurons (photoreceptors, bipolar nerves and ganglion cells) in the adult retina, indicating the synapses between neural layers; b) a diagrammatic representation of the major retinal neuronal pathway (Gilbert 2000, p. 402).*

Ganglion cells in the superficial layer of the mouse neural retina project their axons to the optic disc at the centre of the retina from E13 (Figure 2.14). The axons exit the retina, enter the optic nerve head and proceed down the optic stalk to the diencephalon forming the optic nerve (Birgbauer *et al.* 2000). The axons project through the optic chiasm and optic tract (E12-E16) and traverse the side of the diencephalon, through neurons of the collicular plate and pass underneath the pia to the superior colliculus, which they invade around the time of birth (between E15-P3) (Godement *et al.* 1984; Edwards *et al.* 1986b; Sachs *et al.* 1986). Contralateral-projecting RGC axons precede their ipsilateral counterparts in invading their target tissue (Godement *et al.* 1984; Sachs *et al.* 1986). Mapping of the RGC axons to the dorsal LGN (retinogeniculate projection) occurs within a similar timeframe (Godement *et al.* 1984).
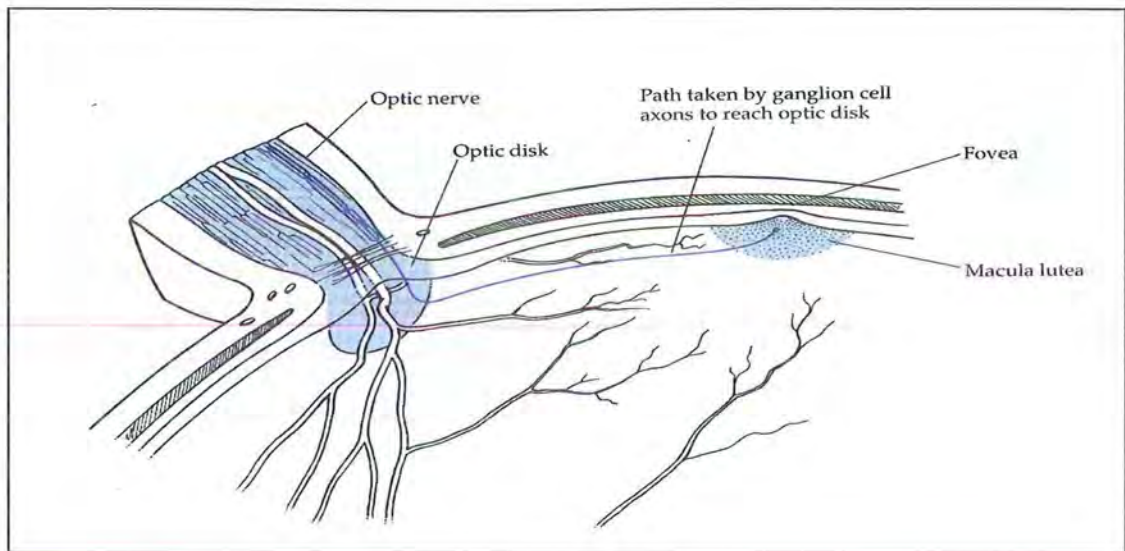
Figure 2.14. A diagram depicting the projection of retinal ganglion cell axons from the neural retina along the optic nerve (Martin 2003, p. 164).

During the first postnatal week, mapping of RGC axons within the superior colliculus dynamically alters, leading to transformation of the lamina pattern and arborization, followed by map refinement (Godement *et al.* 1984; Edwards *et al.* 1986b). The axonal projection of the ganglionic neurons of the retina to the superior colliculus is known as the retinocollicular projection (retinotectal projection in lower vertebrates).

Throughout this process, the acquisition of polarity within the developing optic vesicle and optic cup occurs initially across the proximodistal axis (vesicle), followed by the dorsoventral axis (cup), orchestrated by the functionally and spatially distinct expression of *Bmp4* and *Shh*. *Bmp4* directs distal (vesicle) and dorsal (cup) polarising activity complementary to the proximal (vesicle) and ventral (cup) activity of *Shh* (Sasagawa *et al.* 2002). Across the proximodistal axis, *Pax2* is expressed proximally (Sasagawa *et al.* 2002) and is involved in optic stalk formation (Otteson *et al.* 1998) whereas *Pax6* is expressed distally (Sasagawa *et al.* 2002) and is involved in eye/retinal development (Hill *et al.* 1991; Marquardt *et al.* 2001). At the *Pax2/Pax6* interface, the retinal pigmented epithelium is specified by combined *Pax2* and *Pax6* activity (Baumer *et al.* 2003). The proximal versus distal boundary of the optic vesicle is demarcated by reciprocal transcriptional repression of *Pax2* and *Pax6* (Schwarz *et al.* 2000) and when this demarcation is perturbed, as occurs in *Krd* (*Pax2* haploinsufficient) mice (Otteson *et al.* 1998), or resulting from *Pax2* or *Pax6*

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misexpression experiments (Schwarz *et al.* 2000), defects in the retina and optic disc/stalk occur.

The polarity of the optic cup is determined by the graded expression of genes, such as *Pax6*, *Tbx5*, *Vax1/2* and the *Eph* receptor family and dictates correct projection of RGC axons to and within the target tissue (Koshiba-Takeuchi *et al.* 2000; Baumer *et al.* 2002; Ziman *et al.* 2003). Whilst it is acknowledged that there are a myriad of genes involved in normal eye development, this discussion will now focus on *Pax6*, the master gene in eye formation across phylogeny.

The role of *Pax6* in vertebrate eye formation

As a master control gene in eye development, *Pax6* controls processes such as eye formation and specification of eye cells. As RGC axons are projecting to the superior colliculus, graded *Pax6* expression culminates in graded expression of guidance cues in the retina. Any perturbation to gradients or levels of *Pax6* expression results in incorrect eye development and topographic mapping (Thieler *et al.* 1978; Hogan *et al.* 1986; Hill *et al.* 1991).

Ectopic expression of *Pax6* in *Drosophila* embryos results in formation of ectopic, compound eyes on legs, wings and antennae (Halder *et al.* 1995), and misexpression in *Xenopus* embryos results in eyes and partial eye structures in various ectopic locations (Chow *et al.* 1999). In contrast, absence of *Pax6* results in failure of eye formation (anophthalmia). In *Sey (Pax6$^{-/-}$)* mutant mice, embryos do not form lens or nasal placodes, resulting in the absence of eyes and nasal cavities (Thieler *et al.* 1978; Hogan *et al.* 1986; Hill *et al.* 1991) (refer section 2.6.1 [p. 42-45] of this thesis for further information).

Importantly, the correct dosage of *Pax6* is required for correct vertebrate eye development. Whereas haploinsufficiency or homozygosity results in abnormal eye development (microphthalmia with haploinsufficiency/anophthalmia with homozygosity), overexpression of this master control gene results in microphthalmia, with dysplasia of the retina, cornea and iris (Schedl *et al.* 1996). In humans mutations in *PAX6* (generally due to truncation of the protein) or haploinsufficiency result in aniridia, with patients displaying

30
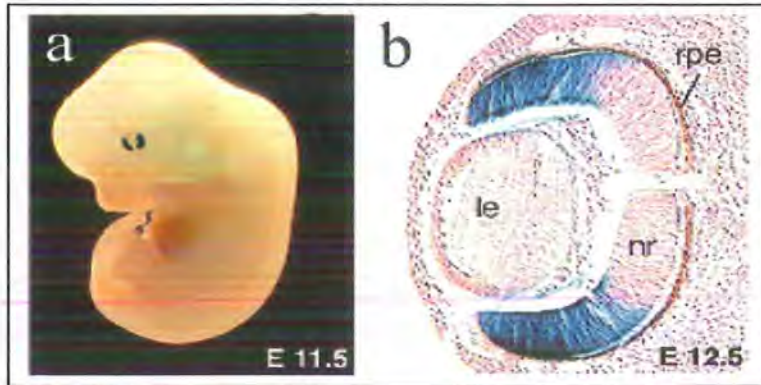
Figure 2.15. Sites of **Pax6** *expression in the embryonic mouse retina as indicated by the* **tau-lacZ** *reporter gene, under the control of the* **α-enhancer** *region of the* **Pax6** *promoter, which reveals specific tau-β-gal on* **Pax6⁺** *cells. A) Note expression in the nasal and temporal retina of E11.5 embryo. B) Expression in the distal nasal and temporal neural retina of E12.5 embryo (counterstained with neutral red)* (Baumer et al. 2002). *Abbreviations: le lens; nr neural retina; rpe retinal pigmented epithelium.*

Further evidence supporting a role for *Pax6* in specification of retinal axes and, subsequently, topographic mapping of RGCs to the superior colliculus has been determined in previous studies. Using a transgenic α-CRE; *Pax6$^{flox/flox}$* mouse model, in which *Pax6* is virtually eliminated in cells of the distal nasal and temporal retina (Marquardt *et al.* 2001), Baumer *et al* (2002) demonstrated altered expression of *BF-1, BF-2,* and *Tbx5,* which have been shown to participate in topographic mapping of RGCs (Yuasa *et al.* 1996; Koshiba-Takeuchi *et al.* 2000). Furthermore, expanded expression of ventral markers (*Vax1/Vax2*) indicate altered specification of the nasotemporal and dorsoventral retinal axes (Baumer *et al.* 2002).

In the embryonic chick retina, *Pax6* is expressed in a dorsal^{low} to ventral^{high} gradient. Recently, *in vivo* expression patterns of *Pax6* in the retina were shown to correlate with the expression pattern of the *EphB2* receptor (Ziman *et al.* 2003), a known axon guidance molecule involved in retinotopic mapping across the dorsoventral axis (Braisted *et al.* 1997). Furthermore, the ability of *Pax6* to regulate *EphB2* was demonstrated *in vitro* showing that *Pax6* induces cell differentiation along a neurogenic lineage and it upregulates *EphB2* in *Pax6*-transfected P19 cells (Ziman *et al.* 2003). The results of the

abovementioned studies indicate a role for *Pax6* in autoregulation, specification of retinal axes and retinotopic mapping.

Taken together, *Pax6* governs retinal development by defining positional information in the developing optic cup and retina, thus determining cell fate and topography of axonal projections to specific areas of the superior colliculus (Marquardt *et al.* 2001; Baumer *et al.* 2002; Ziman *et al.* 2003). *Pax6* is therefore believed to have several roles in eye formation – early specification of the eye primordium and later differentiation of specific subsets of cells in the retina (Kawakami *et al.* 1997; Marquardt *et al.* 2001; Baumer *et al.* 2002), together with determination of retinal polarity and topographic map formation (Marquardt *et al.* 2001; Baumer *et al.* 2002; Ziman *et al.* 2003).

2.4 Axon trajectory and molecular guidance cues

2.4.1 Retinocollicular mapping

Axons of RGCs project from the retina through the optic pathways to the superior colliculus and (in higher vertebrates) through the LGN to the visual cortex (Hollander and Sanides 1976; Illing and Wassle 1981; Jeffery 1989).

Within the superior colliculus, the RGC axon synapses with specific neurons in a topographic manner, maintaining the spatial relationship between retinal neurons and target cells of the superior colliculus to conserve correct mapping of information (Sperry 1963; reviewed in Holt and Harris 1993; Cheng *et al.* 1995). Polarity reversal occurs during the retinocollicular projection; dorsal retinal axons project to the ventral superior colliculus and ventral retinal axons project to the dorsal superior colliculus. Nasal retinal axons project to the posterior superior colliculus and temporal retinal axons project to the anterior superior colliculus. This recognition of target region is mediated by gradients of molecular guidance cues within the superior colliculus (Cheng *et al.* 1995; Drescher *et al.* 1995; Nakamoto *et al.* 1996). The termination of axons in their chosen territory is related to a balance

between attractive and repulsive properties provided by cell surface and secreted molecular guidance cues (Drescher *et al.* 1995; Goodhill and Urbach 1999).

2.4.2 Molecular guidance cues

Molecular guidance cues vary in their mechanism of action, being either long-range, diffusible molecules or short-range, contact-mediated molecules or structures. Many receptor-ligand systems have been identified to date, and they show remarkable conservation between species (reviewed in Tessier-Lavigne and Goodman 1996). This thesis will focus on the Ephrin family of mapping determinants.

The *Eph/ephrin* gene family

The *Eph/ephrin* gene family encode cell surface receptors and ligands involved in early embryogenesis, functioning in cell sorting, adhesion, migration and axonal guidance, and are crucial for correct axon trajectory. The Eph receptor and ephrin ligand family chaperone the growing tip of the axon, the growth cone, towards the target region (Pasquale *et al.* 1992; Henkemeyer *et al.* 1994; Pasquale *et al.* 1994; Drescher *et al.* 1995; Winslow *et al.* 1995; Henkemeyer *et al.* 1996; Nakamoto *et al.* 1996).

The Eph/ephrin family of receptors/ligands have been implicated in retinocollicular mapping (Bishop *et al.* 2000; Nakamoto 2000) and formation of topographic maps in other brain regions (e.g. hippocampus and septum). In particular, *ephrin-A2* and *ephrin-A5* are ligands expressed in the superior colliculus in an anterior^{low} to posterior^{high} gradient, where they are responsible for correct retinocollicular mapping of RGC axons (Drescher *et al.* 1995; Logan *et al.* 1996). The mapped projection of the retinal axons must be highly ordered for correct visual perception. They are also thought to convey specificity to connections of spinal motor and ganglionic neurons in the PNS and to regulate migration of cells during neurogenesis (Holder *et al.* 1998).

The Eph/ephrin family have similar roles in many species, although the individual interactions and molecules may vary (reviewed in Coulthard *et al.* 2002). The different roles of these receptors and ligands in different subsets of cells may depend on the specific neuronal population together with the developmental stage of the embryo (Gao *et al.* 2000). The early developmental expression of *ephrins*, at the gastrula stage, suggests a role for these ligands in the regulation of events such as neurogenesis, precursor migration and neuronal differentiation in specific cell populations (Nieto *et al.* 1992; Xu *et al.* 1994; Gao *et al.* 2000). *Ephrin* receptor expression in *Drosophila* prior to morphological segmentation suggests a role in determining segment-specific qualities, such as compartmentation by segment-specific gene expression (Nieto *et al.* 1992). Dominant-negative experiments highlight the vital role of the *Eph/ephrin* family, specifically *ephrin-A2, ephrin-A5* and *EphA3*, in formation of the neural tube, somites and notochord during zebrafish embryogenesis (Oates *et al.* 1999). Other studies have demonstrated their role in specifying somite polarity and boundary formation (Durbin *et al.* 1998; Durbin *et al.* 2000).

Eph/ephrins engage in interactions resulting in either attraction or repulsion. Up-regulation of integrin-mediated adhesion to extracellular matrix proteins provides an attraction mechanism for ephrin^{+} cells (Bohme *et al.* 1996; Jones *et al.* 1998; Davy *et al.* 1999; Davy and Robbins 2000; Gu and Park 2001). *EphB2/EphB3* double mutant mice exhibit cleft palates, and *ephrin-A5*[-] mice have cranial defects due to failure of the neural folds to adhere in the dorsal midline (Frisen *et al.* 1999). Examples of repulsive mechanisms are restriction of cell mixing between domains resulting in regional specification of tissues such as the rhombomeres of the hindbrain (Xu *et al.* 1999), or directing advancing RGC axons to terminate at appropriate sites in the superior colliculus (Drescher *et al.* 1995).

Eph and *ephrin* expression also occurs in adult tissues, where they are thought to regulate plasticity in the nervous system, perhaps via synaptic remodelling. They may also guide the migration and connection of new neurons generated from stem cells in the adult brain (Frisen *et al.* 1999).

The *Eph/ephrin* family and their mechanism of action

Ephrin ligands are membrane-anchored proteins that bind to Eph receptors (Flanagan and Vanderhaegen 1998). The two categories of *Eph/ephrins* are classified according to the manner in which ligands are attached to membranes. Ephrin-A ligands are anchored to the membrane via a glycosylphosphatidylinositol moiety, whereas ephrin-B ligands are anchored via a transmembrane segment (reviewed in Pasquale 2000). Mostly, ephrin-A ligands bind to EphA receptors, and ephrin-B ligands bind to EphB receptors, however promiscuity between the different classes does occur (Flanagan and Vanderhaegen 1998), and different ligands appear to activate different receptors with varying affinity (Holder *et al.* 1998; Frisen *et al.* 1999). Their function *in vivo* may therefore depend on their spatiotemporal co-expression and on the varying degrees of interaction between different receptors and ligands (Holder *et al.* 1998).

Currently fourteen Eph receptors and nine ephrin ligands have been identified (Lemke 1997; Menzel *et al.* 2001). Ephrin ligands are unusual in that they participate in signal transduction which results in a bidirectional mode of signalling, allowing forward and reverse signalling to both the ligand- and receptor-bearing cells as a means of contact-dependent communication (Holland *et al.* 1996; Flanagan and Vanderhaegen 1998; Davy *et al.* 1999; Mellitzer *et al.* 1999; Davy and Robbins 2000; Cowan and Henkemeyer 2001; Huai and Drescher 2001). This enables ephrin- and Eph-bearing cells from neighbouring areas, each with distinct genetic identities, to reciprocally influence each other. This interaction is generally of a repulsive nature, with ephrin- and Eph-expressing cells repelled from each other to occupy distinct spatial domains, thus restricting cell mixing (reviewed in Pasquale 2000). Bidirectional signalling has been shown to regulate the cellular movements that are at the basis of developmental patterning (reviewed in Coulthard *et al.* 2002).

After ligand-receptor binding, intercellular signals result in cytoskeletal changes, alterations in cell adhesion and collapse of the growth cone (Davenport *et al.* 1998; Hattori *et al.* 2000). Signalling between receptor/ligand may also functionally modulate integrin adhesion molecules causing cytoskeletal changes (Davy *et al.* 1999; Davy and Robbins 2000; Gu and Park 2001). Binding usually occurs with high affinity, with the resulting

connection cleaved by a metalloprotease enzyme, eg Kuzbanian, severing the external portion of the ephrin ligand from the cell membrane (Hattori *et al.* 2000).

The *Eph/ephrin* family in boundary formation in the developing brain

The expression of *Eph* and *ephrins* appears to coincide with the expression of *Hox* genes in the developing embryo. Evidence that *Hox* genes, such as *En*, *Hoxa1*, *Hoxa2*, and *Hoxb1* are involved in the regulation of this signalling family during anteroposterior embryonic patterning is substantial (Itasaki *et al.* 1991; Itasaki and Nakamura 1992; Logan *et al.* 1996; Frisen *et al.* 1999). At the midbrain-hindbrain boundary, *in situ* hybridisation experiments in the mouse have shown that *ephrin-A2* is highly expressed, with complementarily high expression of the appropriate receptors near the diencephalic-mesencephalic boundary and in the rhombencephalic region, highlighting their possible role in boundary formation via repulsive mechanisms (Cheng *et al.* 1995). Furthermore, Xu *et al* (1996) demonstrated expansion of the zebrafish eye field into the diencephalon and loss of diencephalic structures when *ephrin* (rtk-1) expression was disrupted in forebrain patterning. Perturbation of this receptor resulted in ectopic expression of *Pax6* in the ventral rather than dorsal diencephalon, revealing the role of *Eph/ephrins* in boundary formation (Xu *et al.* 1996). Thus, it is apparent that *Eph/ephrins* influence embryonic patterning by cell-cell interactions as well as axonal guidance and are important in mesencephalic patterning and its boundary formation with the diencephalon and rhombencephalon. In the context of this thesis, it is possible that *Pax7*-regulation of *ephrin-A2* (Thomas *et al.* 2004) and *Pax6*-regulation of Eph receptors (Ziman *et al.* 2003) may provide a mechanism for *Pax6/Pax7* participation in boundary formation between the diencephalon/mesencephalon, and the superior colliculus/tegmentum.

The *Eph/ephrin* family and retinocollicular mapping

Eph receptors and ephrin ligands confer positional information to neurons and their growing axons within the retina and superior colliculus/tectum (Cheng *et al.* 1995; Drescher *et al.* 1995; Feldheim *et al.* 2000; Yamada *et al.* 2001). The complementary gradients of EphB/ephrin-B, and EphA/ephrin-A molecules in the retina and superior

colliculus dictate topographic mapping along dorsoventral and nasotemporal axes, respectively (Cheng *et al.* 1995; Braisted *et al.* 1997).

Retinal polarity

EphA receptors are expressed in a temporal^{high} to nasal^{low} retinal gradient, and EphB receptors have been detected in a ventral^{high} to dorsal^{low} retinal gradient. Ephrin ligands have opposing expression in the retina, with ephrin-A ligands in nasal^{high} to temporal^{low} gradients and ephrin-B ligands in dorsal^{high} to ventral^{low} gradients (Holder *et al.* 1998). Co-expression of ligands and receptors in the eye is thought to increase the sensitivity of the receptors for their appropriate ligands (Becker *et al.* 2000), possibly by triggering interaction between neighbouring RGC axons (Birgbauer *et al.* 2000) or reducing the sensitivity of the neuron to the ligand (Hornberger *et al.* 1999).

The ventral^{high} to dorsal^{low} gradient of EphB2 in the retina has been correlated to the similar gradient of *Pax6 in vivo*, and *in vitro* transfection of *Pax6* into undifferentiated P19 mouse embryonal cells has been shown to upregulate EphB2 (Ziman *et al.* 2003), suggesting a role for *Pax6* in regulating EphB2 expression.

Superior collicular polarity

As RGC axons innervate the superior colliculus and synapse in the SGFS in a topographic manner preserving relative positions of neighbouring retinal cells with target cells of the superior colliculus (Holt and Harris 1993), it is evident that Eph/ephrins play a key role in this topographic mapping. In the chick, both *ephrins-A2* and *–A5* are expressed in anterior^{low} to posterior^{high} gradients across the tectum, with *ephrin-A5* spatially restricted to a more posterior portion of the tectum (Logan *et al.* 1996). Ephrin-A2 repels temporal, but not nasal, RGCs and ephrin-A5 repels both temporal and nasal RGCs (Drescher *et al.* 1995). In *ephrin-A2/ephrin-A5* double null mutant mice, both nasal and temporal axons project aberrantly, resulting in converged termination zones at incorrect topographical locations (Feldheim *et al.* 2000). Yamada *et al.* (2001) used an ephrin-A2 monoclonal

antibody to support the role of *ephrin-A2* in establishment of the chick retinotectal projection. The repulsive effect of posterior tectal membranes was abolished, yet temporal and nasal projections were normal overall (with occasional diffuse termination zones and overshooting of axons) suggesting *ephrin-A5* conserves mapping via redundant mechanisms (Yamada *et al.* 2001).

The role of *Pax7* and *En* in regulation of *ephrin* expression

Growing evidence suggests that *Pax7* directly activates *ephrin-A2* expression. A recent study has indicated highly similar spatial and temporal graded expression patterns (caudal^{high} to rostral^{low}/dorsal^{high} to ventral^{low}) and co-localisation of *Pax7* and *ephrin-A2* in the developing chick tectum (E2 – E12). Moreover, *Pax7* upregulates the expression of *ephrin-A2* when transfected into embryonic cells *in vitro,* and this upregulation is abolished when *Pax7* expression is inhibited by antisense oligonucleotides (Thomas *et al.* 2004).

In avian brains, *En-1* and *En-2* pattern mesencephalic tissue, where they are expressed in a graded distribution prior to the graded expression of axonal guidance cues, *ephrin-A2* and *ephrin-A5* and are postulated to regulate their graded expression (Itasaki *et al.* 1991; Itasaki and Nakamura 1992; Logan *et al.* 1996). However, the precise mechanism of the relationship between *En* and ephrin ligands has not been determined to date, and recent findings indicate that a direct, contact-dependent relationship may not exist. An indirect relationship between *En* and *ephrin-A2* is suggested by the finding that ephrin-A2 is not detected in other brain regions where *En-1* is expressed after retroviral misexpression, and it is not expressed *in vitro* in chick embryo fibroblasts retrovirally transfected with *En-1* (Logan *et al.* 1996), indicating the requirement of a tectal-related factor as an intermediary. For instance, after electroporation of *En-2* in the chick diencephalon, an ectopic tectum formed and isthmic-related genes including *Pax7* and *ephrin-A2* were expressed in the ectopic tectum, however their expression occurred 18 hours after electroporation. By contrast, *Pax6* was upregulated 3 hours after electroporation of *En-2*, suggesting a more direct relationship between *En* and *Pax6* (Araki and Nakamura 1999).

Recent studies have also elaborated the ability of En to be secreted from the cell and to be internalized by RGC axons, resulting in changes to axonal mapping (Prochiantz and Joliot 2003), thus directly affecting mapping independent of ephrin-A2. A relationship, therefore, with ephrin-A2 may not relate to a direct interaction but may be associated with its participation in the tectal feedback loop, acting to upregulate another direct mediator of ephrin-A2 expression. Furthermore, when mesencephalic floor plate or Shh-secreting cells were transplanted in the chick tectum, the retinotectal map was perturbed, yet *En* expression was not altered. In this instance, it is possible that alteration of the retinotectal map was due to Shh-mediated repression of *Pax7* expression (Nomura and Fujisawa 2000; Watanabe and Nakamura 2000), or to factors secreted from the mesencephalic floor plate, resulting in alteration of *ephrin* expression.

Coupled with the fact that in isthmus-ablated chicks a tectum was formed (albeit transiently) and *Pax7* was expressed in the absence of *En* expression (Nomura *et al.* 1998), it is likely that any relationship between *En* and *ephrin-A2* is indirect. Moreover, *En* is gradiently expressed in the mesencephalon in both the superior colliculus and the tegmentum, implicating other factors such as *Pax7* in maintaining *ephrin-A2* expression in the superior colliculus and the differential identity of the superior colliculus distinct from the tegmentum. Evidence has emerged regarding an *En*-regulated inhibitor (refer section 2.3.1 [p.23] of this thesis for further details) that may assist in mesencephalic polarization. This putative inhibitor has been identified as *Hes5*, and is believed to participate in establishment of rostrocaudal polarity within the developing chick tectum due to a rostral^{high} to caudal^{low} gradient. Misexpression studies reveal that *Hes5* is repressed by *En-2*, and upregulated when *En-2* is perturbed by siRNA; however, it is unknown whether this is a direct or indirect relationship. Furthermore, misexpression of *Hes5* in the mesencephalon repressed *ephrin-A2*, therefore in normal development high levels of *En-2* in the caudal mesencephalon permit expression of *ephrin-A2* via repression of *Hes5*. Conversely, low levels of *En-2* in the rostral mesencephalon diminish the expression of *ephrin-A2* due to high levels of *Hes5* (Kimura *et al.* 2004).

These findings identify *En-2* as a transcriptional repressor and an indirect regulator of *ephrin-A2*. However, this leaves open the question of what gene/s is/are responsible for activating and/or maintaining the expression of *ephrin-A2* in the superior colliculus. As

mentioned above, *En* expression is not limited to the dorsal mesencephalon. Furthermore, examination of the *Hes5* expression domain (Figure 2.16) reveals expression in the ventral mesencephalon which appears considerably more intense than that of the dorsal mesencephalon.
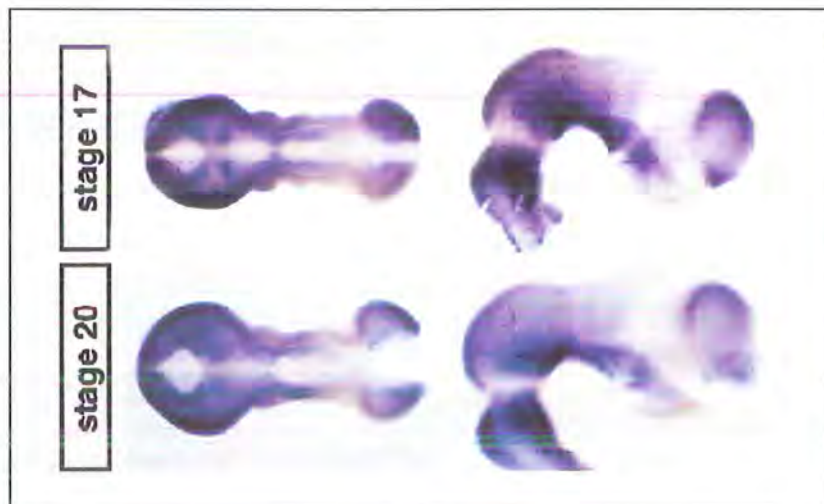
Figure 2.16. Whole-mount in situ hybridization for Hes5 expression in the developing chick brain, identifying variable expression in the dorsal and ventral mesencephalon (Kimura et al. 2004).

As *En* is expressed in a rostrocaudal gradient within the mesencephalon, it appears that other factor/s is/are responsible for variable expression of *Hes5* across the dorsoventral axis; *Hes5* expression is intense ventrally and most likely precludes *ephrin-A2* expression from the tegmentum. Conversely, *Hes5* is downregulated dorsally, resulting in a weaker graded expression pattern, allowing graded expression of *ephrin-A2*. Kimura *et al* concede that another molecule regulated by *En-2* may exist, as misexpression of *Hes5* in the mesencephalon did not affect expression of other tectum-related genes (*Pax5, Pax5, Wnt-1, Fgf8*) (Kimura *et al.* 2004). It is apparent that the genetic mechanisms underlying *ephrin-A2* regulation in the mesencephalon cannot be attributed to *Engrailed/Hes5* alone, and mechanisms governing direct, positive regulation are yet to be determined. In addition, the recent identification of specific domains within *Engrailed* that relate to nuclear export, secretion and internalization (Prochiantz and Joliot 2003) coupled with the finding that secreted Engrailed can be internalized by axonal growth cones, directly eliciting altered transcription with resultant axon turning (Brunet *et al.* 2005), adds greater complexity to the issue of retinocollicular mapping.

Taken together, it is possible that *En* acts to maintain the expression of *Pax7* in the superior colliculus, which in turn positively directs the expression of *ephrin-A2*, and in this manner the mutual, overlapping expression domains of *En* and *Pax7* dictate superior collicular polarity and retinocollicular mapping.

2.5 Summary

In summation, *Pax6, Pax7, Pax3, En* and the *Eph/ephrins* are involved in patterning the mesencephalon of the developing embryo, controlling regionalisation, specification, cytoarchitecture, polarity, boundary and map formation. In the visual system, *Pax6* and *Pax7* act to define the eye and the superior colliculus respectively, and upregulate the *Eph/ephrin* family of receptors and ligands, possibly together with *En*, culminating in correct axon guidance and retinocollicular mapping. It is clear that *Pax7* is significant in regulating formation of the superior colliculus, and its specific expression in the SGFS, to which the optic nerve projects, suggests a role in topographic mapping. It is feasible to surmise that coordinated expression of *Pax6, Pax7, En* and *ephrin*-A2 is required for boundary formation, specification of accurate topographic mapping and correct visual perception. However, prior to this thesis a spatial, temporal and functional correlation between these genes remained to be identified. A key focus of this study was to determine whether *Pax7, En* and *ephrin-A2* were co-expressed during development of normal retinocollicular mapping, and whether this relationship continued when the spatial and temporal levels of *Pax7* expression were perturbed, thereby illuminating the function of *Pax7* in determining superior collicular topography and in specification of dorsal neurons in the superior colliculus.

2.6 Animal models

2.6.1 The *Pax6 (Sey)* mutant mouse model

In order to investigate the role of *Pax7* in formation of the superior colliculus and establishment of superior collicular topography, a number of mouse models were used. *Sey* (*small eye*) mice (*Pax6$^{-/-}$* C57BL/6J x DBA/2J) have naturally occurring *Pax6* mutations

(Hill *et al.* 1991). As mentioned previously, correct levels of *Pax6* are critical for correct eye development and formation of the diencephalic-mesencephalic boundary, where interaction of *Pax6* and superior collicular-specific genes is thought required for correct superior collicular development (Schedl *et al.* 1996; Kawakami *et al.* 1997; Mastick *et al.* 1997; Matsunaga *et al.* 2000). *Sey* mice are therefore likely to exhibit altered superior collicular topography, and provide an ideal model in which to investigate the relationship between *Pax6* and *Pax7/En-1/ephrin-A2,* and their role in defining boundary and map formation.

There are two forms of *Sey* mutant mice; the *Sey^{Dickie} and Sey^{Neu}* variants (Roberts 1967; Hogan *et al.* 1986; Hill *et al.* 1991). Both forms arise from a point mutation (G - T transversion), however the mutations occur in different regions of the *Pax6* gene. The *Sey^{Dickie}* mutation occurs in the gene sequence encoding codon 194 of the protein, resulting in translation termination prior to the homeodomain. The point mutation in *Sey^{Neu}* occurs within an intron downstream of the homeobox, which introduces a novel splice site and abnormal intronic protein coding sequence and an intronic stop codon (Hill *et al.* 1991) (Figure 2.17). These mutations both result in a truncated, non-functional protein and accordingly produce similar phenotypes (Hill *et al.* 1991; Mastick *et al.* 1997).
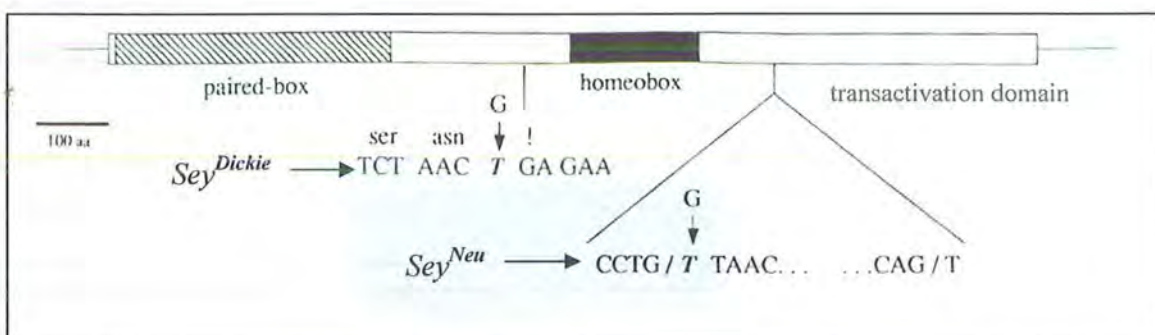
***Figure 2.17.** **Schematic diagram of the mouse** Pax6 **gene identifying the location and** nature of the* Sey^{Dickie} and Sey^{Neu} *mutations (adapted from Hill et al. 1991).*

Homozygous embryos of both *Sey* mutants exhibit enlarged optic vesicles but fail to develop eyes (Hogan *et al.* 1986) (Figure 2.18c). As indicated in Figure 2.19, homozygous mutant mice display defects in prosomere formation and altered morphology of the cerebral vesicle, the mesencephalic-p1 boundary, the dorsal midline and the hemispheric sulcus (Mastick *et al.* 1997). Analysis of markers at the altered mesencephalic-p1 boundary (*Pax6,*

Gsh-1, Lim-1, Dbx) indicates a shift in the identity of the p1 region to the mesencephalon (Mastick *et al.* 1997). Due to these and other severe brain defects, the mice die shortly after birth (Hogan *et al.* 1986).

Figure 2.18. Phenotypic variation in* Sey *mice compared to wildtype mice at E15. a) wildtype mouse b)* Sey *heterozygous mouse c)* Sey *homozygous mouse. Note the decreased eye size of the heterozygote (arrow) and the absence of eyes together with a shortened, imperforate snout in the homozygote (adapted from Hill et al. 1991).

By comparison, heterozygous individuals have small eyes (hence the name) (Figure 2.18b) and less severe CNS defects. The reduced eye size in heterozygous embryos causes a significant reduction in the number of RGC axons that form the optic nerve. This has been illustrated by the use of a transgenic mouse model (α-*tau-lacZ* X *Pax6*$^{Sey/+}$) in which axonal processes of Pax6$^+$ cells are labelled (Baumer *et al.* 2002). Consequently, heterozygous mice display hypoplastic optic nerves (Deiner and Sretavan 1999).

As the dosage of this regulatory gene is critical for correct brain regionalization and eye development, and for correct projection of the optic nerve to visual centres (Schedl *et al.* 1996; Kawakami *et al.* 1997; Mastick *et al.* 1997; Deiner and Sretavan 1999), heterozygous and homozygous *Sey* mice are a suitable model in which to study the role of *Pax7* on retinocollicular map formation.
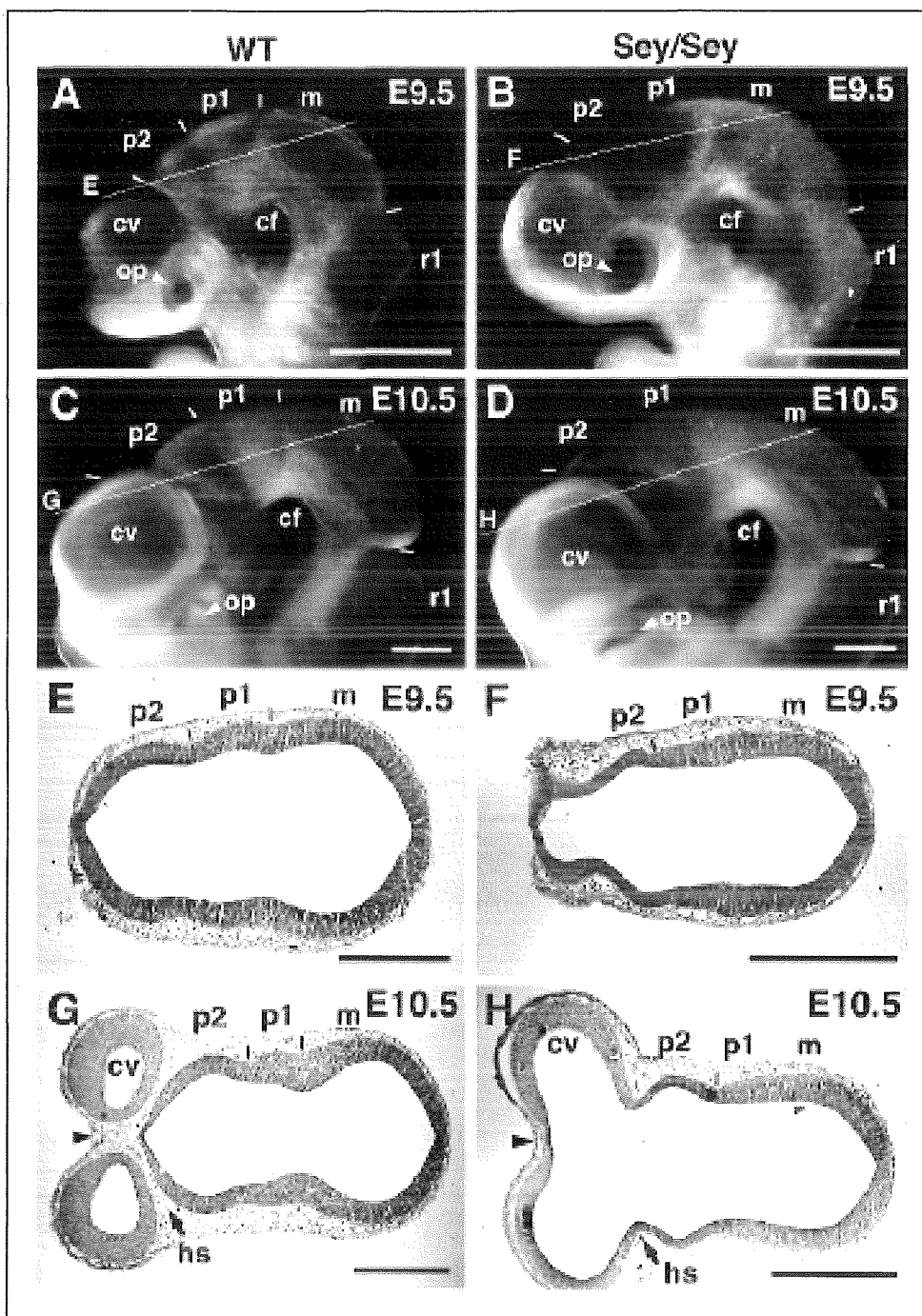
Figure 2.19. ***Altered morphology of the cerebral vesicle, mesencephalic-p1 boundary*** ***and hemispheric sulcus in* Sey (Pax6^{-/-})** *mice. Note loss of constriction of the mesencephalic-p1 boundary with alteration of the hemispheric sulcus (arrow), and incomplete separation of the cerebral vesicles (arrowhead) (Mastick et al. 1997). Figures A, B, C and D indicate the plane of cut for figures E, F, G and H. Scale bars 500μm. Abbreviations: cf, cephalic flexure; cv, cerebral vesicle; hs, hemispheric sulcus; m, mesencephalon; op, optic vesicle; p, prosomere (1/2); r1, rhombomere 1; WT, wildtype.*

2.6.2 The *Pax7* mutant mouse model

Pax7 heterozygous and homozygous mutant mice (*Pax7$^{+/-}$, Pax7$^{/-}$* X C57BL/6) were utilized as a model to study the role of *Pax7* in specification and differentiation of superior collicular topography. The *Pax7* mutant mouse model has been generated by homologous recombination of a *neomycin-lacZ* reporter construct into the *Pax7* locus in mouse embryonic stem cells. The vector containing a *neomycin* gene inserted into the first exon of the paired box of the *Pax7* gene, results in inactivation of the *Pax7* gene: absence of *Pax7* mRNA was confirmed by RT-PCR. The *β-galactosidase* gene *(lacZ)* also present in the vector is inserted in-frame with the *Pax7* sequences and acts as a reporter gene, indicating sites of normal *Pax7* expression (Mansouri *et al.* 1996b).

The predominant feature of *Pax7$^{/-}$* mice is malformation of cephalic neural crest cell derivatives, resulting in reduced maxillae (Figure 2.20) and serous gland tubules; *Pax7$^{/-}$* mice die shortly after weaning, usually within three weeks of birth (Mansouri *et al.* 1996b).
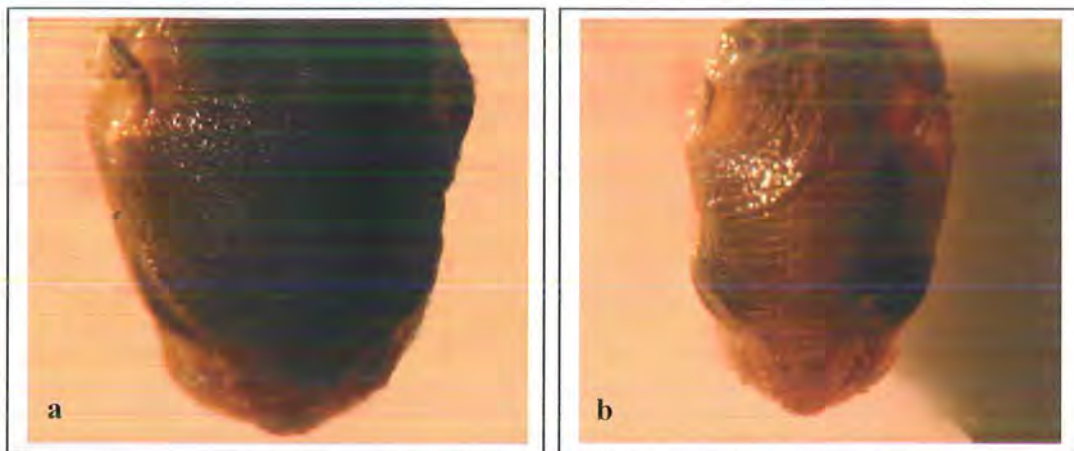
Figure 2.20. *A comparison of the phenotype of the* Pax7$^{/-}$ *mouse and the wildtype mouse at P5. a) wildtype mouse b) Pax7$^{/-}$ mouse. Note the reduced size of the mutant homozygote and the reduced maxilla.*

Analysis of skeletal muscle in *Pax7$^{/-}$* animals reveals severe depletion of skeletal muscle satellite cells and a marked decrease in muscle mass increasing in severity with age, with individuals failing to thrive (Seale *et al.* 2001). *Pax7$^{/-}$* animals also exhibit dilatations in the small intestine and appendix (Mansouri *et al.* 1996b). Alteration to the specification of

superior collicular topography has not previously been reported in *Pax7* mice. Thus the study of *Pax7* mutant mice to provide key insights into the role of *Pax7* in defining the functional aspects of the superior colliculus is novel.

Given the role of *Pax7* in specifying mesencephalic identity (refer section 2.3.1 [pp.22-24] of this thesis for further information), together with subpopulations of cells (Fedtsova *et al.* 2008) and persistent adult expression (Shin *et al.* 2003; Thomas *et al.* 2004; Thomas *et al.* 2006) in the chick tectum, it is reasonable to expect perturbation to superior collicular development in *Pax7* mutant mice. However, the lack of CNS deficits in *Pax7* mutant mice is considered to be more of an indication of the redundant nature of its paralogue, *Pax3*, than a testament to its developmental role. *Pax3* is expressed in the developing chick mesencephalon, but not in the adult mesencephalon (Matsunaga *et al.* 2001). A recent paper demonstrated upregulated levels of Pax7 within the unaffected neural tube of *Pax3* hypomorphic mice in which Pax3 protein levels were reduced to ~20% of wildtype levels (Zhou *et al.* 2008). Similarly, *Pax7* is ectopically expressed in *Pax3* mutant (Splotch) mice in the dorsal neural tube where only *Pax3* would normally be expressed in wildtype mice (Borycki *et al.* 1999). It is therefore possible that *Pax3* partially rescues some functions of its paralogue in specifying superior collicular identity in *Pax7* mutant mice.

CHAPTER 3

THEORETICAL FRAMEWORK

"We are made for solving problems and achieving goals."
"...man cannot discover new oceans until he has the courage to lose sight of the shore."

Sir Des Renford, MBE, 1927-1999.

CHAPTER 3: Theoretical framework

3.1 Rationale for the study

In contrast to *Pax6* and its role in eye development, a functional role for *Pax7* within the superior colliculus, other than initial specification of superior collicular identity (Nomura *et al.* 1998; Matsunaga *et al.* 2001), has not been explored in detail.

Firstly, the role of *Pax7* in polarization of the superior colliculus was determined. This was achieved by identification and characterization of graded *Pax7* expression throughout development, as well as assessment of temporal variations in the gradient to indicate functional differences. Protein levels of Pax7 (addressed further on the following page) and cellular distribution were assessed at key developmental stages in wildtype mice relative to *Pax6* and *Pax7*^{+/-} mutant mice to assess perturbation to expression. Secondly, *Pax7* is thought to regulate expression of *ephrin-A2*, a key guidance molecule during retinotopic map formation (Itasaki *et al.* 1991; Nomura and Fujisawa 2000). Demonstration of similarities in *Pax7* and *ephrin-A2* gradients spatially and temporally, and co-localisation in superior collicular neurons in wildtype mice would confirm this regulation (Papers 2 and 3/Chapters 5 and 6).

Differences in expression of *ephrin-A2*, either spatially or quantitatively in *Pax6* or *Pax7* mutant mice relative to wildtype mice were also assessed to confirm alterations to superior collicular polarity and map formation in these mutant mice (Papers 2 and 3/Chapters 5 and 6). *Pax7*^{+/-} and *Pax7*^{-/-} mutant mice also would be expected to exhibit a shift in the graded expression of *ephrin-A2* and altered map formation. Taken together, the results of these experiments would confirm a role for *Pax7* in retinocollicular mapping.

To further substantiate a role for *Pax7* in retinocollicular mapping, Pax7 protein levels were assessed in wildtype mice and compared to those in *Pax6* mutant mice, which have reduced retinal input (Deiner and Sretavan 1999). Any changes in *Pax7* expression profiles during

RGC innervation of *Pax6* mutant mice would indicate that Pax7$^+$ cells in the superior colliculus are sensitive to Pax6$^+$ RGC axons (Paper 2/Chapter 5).

This study also sought to test whether relative levels of *Pax6* and *Pax7* participate in superior collicular boundary formation. Matsunaga *et al.* (2000) have shown that opposing gradients of *Pax6* and *En-1/Pax2* define the diencephalic-mesencephalic boundary by mutual repression. However, when they misexpressed *Pax7* in the diencephalon, the diencephalon adopted a mesencephalic fate (Matsunaga *et al.* 2001). Similarly, misexpression of *Pax6* in the chick mesencephalon caused fate change of the mesencephalon (tectum) to the diencephalon. While these experiments indicate that *Pax6* and *Pax7* expression levels are crucial for maintaining the differential identity between the diencephalon and the mesencephalon (Nomura *et al.* 1998), the role of *Pax7* in boundary formation has not been explored to date. Considering Mastick *et al.* (1997) detected loss of the diencephalic-mesencephalic boundary in homozygous *Pax6* mutant embryonic mice, it is reasonable to expect that reduced levels of either *Pax6* or *Pax7* expression would affect the formation of these boundaries. This thesis explored alteration to the diencephalic-mesencephalic boundary in *Pax6* heterozygote mutant mice as well as *Pax7* mutant mice, relative to wildtype. Additionally, boundary formation between the superior colliculus and the tegmentum (ventral mesencephalon), where *Pax6* is expressed across the mediolateral axis and abuts *Pax7* expression in the dorsal mesencephalon (Stoykova and Gruss 1994; Nomura *et al.* 1998; Agarwala *et al.* 2001; Matsunaga *et al.* 2001) was investigated. Changes in boundary formation were assessed by measuring the *Pax7* expression domain in *Pax6* mutant mice and by assessing the *Pax6* expression domain in *Pax7* mutant mice, relative to wildtype. Furthermore, midbrain-hindbrain boundary formation was assessed in wildtype and *Pax7* mutant mice using the marker *En-1* to assess altered caudal superior collicular boundary formation subsequent to an altered *Pax7* profile, and results demonstrated no perturbation at the developmental stages examined (Paper 3/Chapter 6).

As *Pax7* is expressed very early in embryogenesis, and has been implicated in imparting regional identity to specified cells (Stoykova and Gruss 1994), it is likely that the gene plays a role in specifying subpopulations of cells within the superior colliculus.

To further illuminate the role of *Pax7* in neural development, a comparative analysis of the expression profiles of *Pax7* and its paralogue *Pax3* during development and maturation of the brain was performed. Brain regions external to the superior colliculus were also assessed for wildtype *Pax3* expression domains relative to *Pax7*, and for perturbation to the *Pax3* expression profile in *Pax7* mutant mice. Results provide details of a temporospatial relationship between *Pax3* and *Pax7* relative to markers of neuronal differentiation, and comparisons between wildtype and *Pax7* mutant mice are shown. Research performed in this thesis, therefore, provides an assessment of the role of *Pax7* in superior collicular formation and function.

Hypotheses and specific aims of the study

Hypothesis 1

Pax7 plays a role in development of superior collicular polarity and the visual system of the mouse (Papers 1 and 2)

Aim 1.1: **To explore a role for *Pax7* in determination of mouse superior collicular polarity and visual system development**

The *Pax7* expression profile in wildtype mice was detected immunohistochemically at key stages of visual system development. Expression of the retinocollicular polarity marker *ephrin-A2* was analysed relative to *Pax7* expression. Confocal microscopy was used to detect co-localisation of Pax7 and ephrin-A2 in wildtype mice at P5-7. Cellular distribution and protein levels were quantified using Optimas Digital Image Analysis, and graded expression was assessed by statistical methods.

Aim 1.2: **To assess the *Pax7* expression profile in the superior colliculus of *Pax6* mutant mice at a variety of developmental timepoints, and relate changes to altered superior collicular polarity**

To assess the impact of retinal innervation on Pax7$^+$ cells we compared Pax7 protein levels in wildtype to those in *Pax6* mutant mice where RGC input is reduced (Deiner and Sretavan 1999). Also, the *Pax7* cellular distribution was assessed for spatial variations in *Pax6* mutant mice relative to wildtype mice. Assessment was conducted at key developmental timepoints and significant variances were detected by statistical analysis.

The spatial distribution and cellular protein levels of ephrin-A2$^+$ cells were assessed in the superior colliculus of *Pax6* mutant mice at P5-7 and results were compared with the *Pax7* profile and to wildtype mice. Changes in polarity and cellular response to retinal input were statistically analysed.

Aim 1.3:   To **assess diencephalic-mesencephalic boundary formation in** *Pax6*
              mutant mice relative to wildtype

Spatial distribution of Pax7$^+$ cells in wildtype and *Pax6* mutant mice were analysed to assess the positioning of the diencephalic-mesencephalic boundary in *Pax6* mutant mice, using *Pax7* as a marker of the dorsal mesencephalon.

Hypothesis 2

Pax7 plays a key role in development of mouse superior collicular topography and neuronal cell specification (Paper 3)

Aim 2.1: To analyse the *Pax7* expression profile during specification and maturation of the mouse superior colliculus in wildtype and *Pax7$^{+/-}$* mice

The temporospatial expression profile of Pax7$^+$ cells was determined immunohistochemically in wildtype and *Pax7$^{+/-}$* mice from mid-embryonic to early adult stages when cells are are undergoing neuronogenesis and maturation to become differentiated cells. The effects of haploinsufficiency on brain cytoarchitecture were quantified and statistically analysed.

Aim 2.2: To investigate a role for *Pax7* in neuronal proliferation, specification, and differentiation within the mouse superior colliculus

Histochemical, immunohistochemical and immunofluorescence were used to assess the relationship between *Pax7* expression and neuronal proliferation, differentiation, apoptosis and retinocollicular polarity in wildtype and *Pax7* mutant mice utilizing a variety of markers at appropriate timepoints. Results for *Pax7* mutant mice were compared to those of wildtype.

Aim 2.3: To assess superior collicular boundary formation in *Pax7* mutant mice, relative to wildtype

Immunohistochemistry was used to assess the role of *Pax7* on boundary formation of the diencephalon/mesencephalon, superior colliculus/tegmentum (*Pax6*) and mesencephalon/metencephalon (midbrain-hindbrain) (*En-1*) boundaries in wildtype and *Pax7* mutant mice.

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Hypothesis 3

The roles of *Pax7* and *Pax3* diverge during development of the brain (Paper 3)

Aim 3.1: **To assess *Pax7* and *Pax3* expression profiles in the brain of wildtype and *Pax7* mutant mice throughout development**

The expression profiles of *Pax7* and *Pax3* were analysed immunohistochemically during embryonic development in wildtype and *Pax7* mutant mice. *Pax3* expression, detected immunohistochemically, was explored in all brain regions during embryonic development.

Aim 3.2: **To relate *Pax7* and *Pax3* to neuronal proliferation, specification and differentiation in the mouse superior colliculus**

Markers of neuronal proliferation and differentiation were detected immunofluorescently in the superior colliculus of wildtype and *Pax7* mutant mice and results were compared spatially with those for *Pax7* and *Pax3* expression.

CHAPTER 4

PAPER 1

The Role of *Pax7* in Determining the Cytoarchitecture of the Superior Colliculus

"One needs to be slow to form convictions, but once formed they must be defended against the heaviest odds."

Mahatma Ghandi, 1869-1948.

CHAPTER 4: PAPER 1

The Role of *Pax7* in Determining the Cytoarchitecture of the Superior Colliculus

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Running Title: Pax7 and superior colliculus formation

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4.1 Abstract

Pax genes are a family of transcriptional regulators vital for embryonic development. One member of the family, *Pax7*, functions early in neural development to establish dorsal polarity of the neural tube, and continuous refinement of its expression affords regional identity to brain nuclei, in particular the superior colliculus. *Pax7* expression within the superior colliculus is eventually restricted to the *stratum griseum et fibrosum superficiale* (*SGFS*), the retinorecipient layer to which the optic nerve projects. The key role of *Pax7* in specification of the superior colliculus has been highlighted by misexpression studies which result in ectopic formation of superior collicular tissue with characteristic laminae innervated by RGC axons. Here we review the role of *Pax7* in formation of the superior colliculus and discuss the possibility that *Pax7* may also assist in refinement of correct topographic mapping.

Key Words: brain mapping, mesencephalon, optic tectum, *Pax7*, superior colliculus.

4.2 Introduction

Pax genes are a family of developmental transcriptional regulators that direct embryonic patterning. Their highly conserved nature reflects their critical role in embryogenesis (Balczarek *et al.* 1997), particularly during formation of the CNS (Stoykova and Gruss 1994).

One member of the *Pax* gene family, *Pax7*, is involved in CNS and skeletal muscle development (Kawakami *et al.* 1997; Seale *et al.* 2001). *Pax7* expresses a number of alternate transcripts which are thought responsible for directing embryonic cells along a neurogenic or myogenic lineage (Ziman and Kay 1998), with a single transcript able to initiate neural cell differentiation *in vitro* (Ziman *et al.* 2001a). *Pax7* acts early in CNS development to establish dorsal polarity of the neural tube (Jostes *et al.* 1991), before being restricted to the roof plate of the telencephalon, the epithalamus and pretectum anteriorly, the alar plate of the mesencephalon medially and the cerebellum posteriorly. Continuous refinement of expression eventually results in *Pax7* restriction to the epiphysis and the dorsal mesencephalon, specifically the superior colliculus, a major visual brain center in vertebrates (homologous to the tectum of lower vertebrates) (Kawakami *et al.* 1997). As the laminae of the superior colliculus/tectum develop, *Pax7* is expressed in migrating neural cells that concentrate in the *stratum griseum et fibrosum superficiale* (*SGFS*), the dorsal retinorecipient layer to which the optic nerve projects (LaVail and Cowan 1971; Kawakami *et al.* 1997). It is the crucial role that *Pax7* plays in determining the formation and identity of the superior colliculus that is the focus of this review.

4.3 Formation of the mesencephalon

Complex interactions between secreted signalling proteins, transcription factors and cell surface molecules expressed in graded levels determine the polarity, cytoarchitecture and topographical mapping of the mesencephalon (Figure 4.1). *Pax7*, amongst other region-specific transcription factors, regulates partitioning of the mesencephalon into the superior colliculus/tectum and tegmentum in the dorsoventral plane and separates the mesencephalon from the diencephalon and metencephalon in the anteroposterior plane (Nomura *et al.* 1998; Matsunaga *et al.* 2001). Our own (Thomas *et al.* 2004) and other

recent experiments (Araki and Nakamura 1999; Matsunaga *et al.* 2001) indicate that *Pax7* is a crucial determinant of superior colliculus/tectum identity, and may be included in the feedback loop of superior collicular/tectal genes, *En-1, En-2, Pax2, Pax5, Fgf8 and Wnt-1*, that act in concert to regulate formation of this midbrain region.
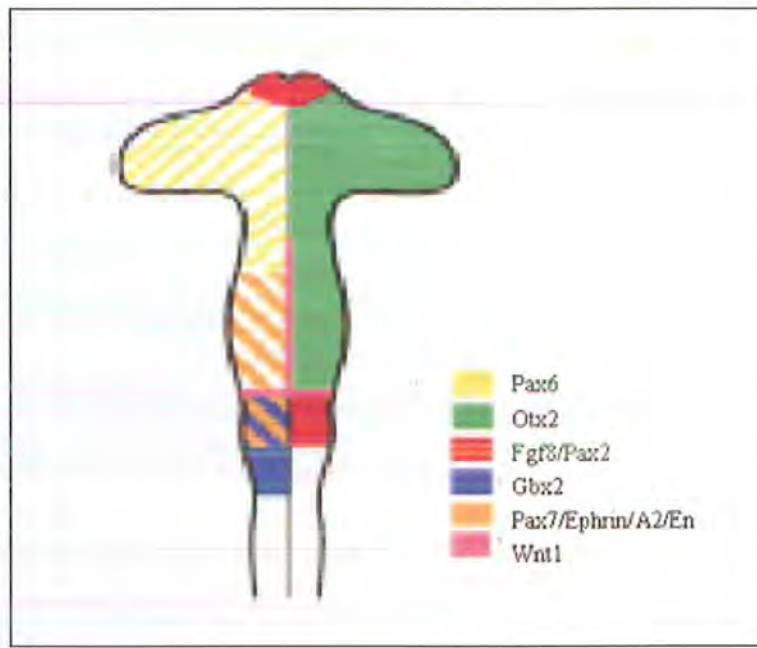
Figure 4.1. Expression patterns of mesencephalic-determining genes along the developing neuraxis (adapted from Nakamura 2001b).

4.3.1 Formation of the dorsoventral axis of the mesencephalon

Sonic Hedgehog (Shh), a strong ventralizing signal, is secreted from the mesencephalic floor plate and notochord. It suppresses *Pax7*, limiting its expression to the dorsal region, and prevents tectum formation in the ventral mesencephalon (Nomura *et al.* 1998; Watanabe and Nakamura 2000). Experiments confirm that ectopic expression of *Shh* in the mesencephalon or transplantation of Shh-secreting cells into chick dorsal mesencephalon inhibits development of the roof plate concomitant with suppression of *Pax7* and other region-specific genes, *En-2, Pax2, Pax5, Fgf8 and Wnt-1*, and induces ectopic tegmentum formation (Nomura and Fujisawa 2000; Watanabe and Nakamura 2000; Agarwala *et al.* 2001).

By contrast, members of the Bone Morphogenetic Protein (BMP) family (BMP2, BMP4 and BMP7) secreted from the dorsal epidermal ectoderm and roof plate initialize expression of dorsal transcription factors *Pax7* and *Pax3* (Basler *et al.* 1993; Liem *et al.* 1995; Lee and Jessell 1999). Specifically, it is thought that the dorsoventral polarity of the mesencephalon is achieved by Shh-mediated suppression of *Pax7* expression ventrally (Watanabe and Nakamura 2000; Nakamura 2001b) and activation of its expression dorsally by BMP-4 (reviewed in Lee and Jessell 1999).

4.3.2 Formation of the anteroposterior axis of the mesencephalon

From our own and other recent findings it is apparent that the regulated expression of *Pax7* in the dorsal mesencephalon, juxtaposed to *Pax6* expression within the diencephalon, affords regional identity to these tissues prior to their further differentiation into visual components, the superior colliculus/tectum in the mesencephalon and the eye in the diencephalon (Nomura *et al.* 1998; Matsunaga *et al.* 2000; Thomas *et al.* 2004; this paper).

The diencephalic-mesencephalic boundary at the anterior of the mesencephalon is thought to be formed by the repressive interaction between *Pax6* (diencephalon) and tectal markers *En-1/Pax2* (mesencephalon) (Araki and Nakamura 1999; Matsunaga *et al.* 2000). Loss of *Pax6* in *Sey (Pax6⁻/⁻)* mice results in fate change of the pretectum (the most caudal portion of the diencephalon) to mesencephalic tissue with defective diencephalic-mesencephalic boundary formation (Mastick *et al.* 1997).

However, these may not be the only genes required for correct boundary formation since a diencephalic-mesencephalic boundary is formed in the absence of expression of *engrailed* homologs; *noi* (*no isthmus*) mutant zebrafish have a mutation in the *Pax2.1* gene which leads to ablation of *engrailed* expression, yet the diencephalic-mesencephalic boundary initially forms and exhibits normal morphology as indicated by marker gene expression (Brand *et al.* 1996).

Since this boundary occurs between expression domains of *Pax6* and *Pax7*, repressive interactions between these two genes may play a role as suggested by transplantation and

misexpression studies. *Pax7* cDNA misexpressed in the embryonic chick diencephalon results in upregulation of endogenous *Pax7* and *En1* with concomitant repression of *Pax6* and fate change of the dorsal diencephalon to the tectum (Matsunaga *et al.* 2001). Conversely, misexpression of *Pax6* in the embryonic chick mesencephalon results in repression of tectum-related genes, including *Pax7* and *En1*, and a caudal shift of the diencephalic-mesencephalic boundary (Matsunaga *et al.* 2000).

Mutual repression of *Pax6* and *Pax7*, reported to occur in a cell autonomous manner (Matsunaga *et al.* 2000, 2001), may also act to limit the ventral as well as the anterior boundary of the superior colliculus/tectum. Our recent experiments, presented in this paper, confirm rostral and ventral expansion of *Pax7* expression in the superior colliculus of *Sey (Pax6$^{+/-}$)* mice (Figure 4.2). Taken together these experiments indicate that *Pax7* is one of several genes crucial for superior collicular boundary formation (Kawakami *et al.* 1997; Matsunaga *et al.* 2001).
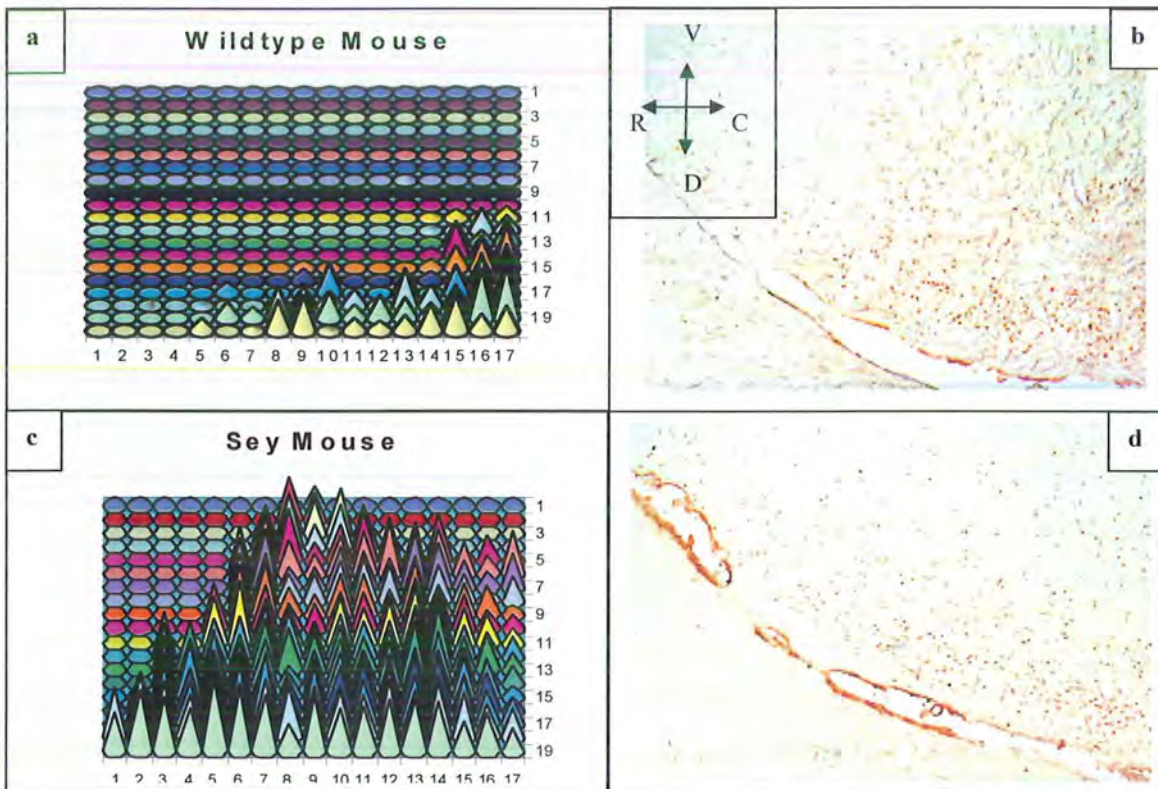
Figure 4.2. ***Immunohistochemistry demonstrating*** **Pax7** ***expression within the mouse superior colliculus of*** *(b)* ***wildtype and*** *(d)* **Sey (Pax6$^{+/-}$)** ***mice at P21 (sagittal section). Brain sections were incubated with Pax7 antibody (1/20, monoclonal, DSHB) followed by peroxidase labeled biotin-streptavidin and visualized with diaminobenzidine (DAB).***

Primary antibodies were withheld from control sections, which were immunonegative (result not shown). Note the anteroposterior and ventrodorsal (low to high) graded expression in the normal mouse, and the loss of gradient with concomitant expanded expression along both of these axes in the Sey mouse. (Magnification 100x/Scale bar 200 μm). Histograms showing intensity of Pax7 expression in the mouse superior colliculus of (a) wildtype and (c) Sey (Pax6$^{+/-}$) mice (sagittal section). Each cone represents one frame of analysis and its position within the tissue section. The peak of the cone denotes the intensity (optical density) of Pax7 signaling (refer to section 5.3.3 [p74] and appendix [pp153-156] for methodology). Note the loss of gradient in Sey mice, as well as the increased intensity. Although not yet quantified, the increase in signaling intensity appears to be due to increased numbers of Pax7-expressing cells, and an increase in the amount of Pax7 within each cell. Abbreviations: R, rostral; C, caudal; V, ventral; D, dorsal.

4.3.3 Midbrain-hindbrain boundary

Polarity of the anteroposterior axis of the mesencephalon correctly established due to secretion of factors from the isthmus, the organizing center situated at the boundary of the mesencephalon and rhombencephalon (midbrain/hindbrain) (Lumsden and Krumlauf 1996). Positioning of this isthmic organizer is established by complementary expression domains of *Otx2* (forebrain and midbrain) and *Gbx2* (hindbrain and spinal cord) (Figure 4.1) and alteration to either of these genes results in repositioning of the isthmus (Wassarman *et al.* 1997; Broccoli *et al.* 1999).

Fgf8, secreted from the isthmus, is implicated in regulating expression of *Wnt-1* in the mesencephalon, which in turn up-regulates the expression of *En* genes (McMahon *et al.* 1992; Crossley *et al.* 1996; Broccoli *et al.* 1999; Martinez *et al.* 1999; Millet *et al.* 1999; Katahira *et al.* 2000; Sugiyama *et al.* 2000) and *En* in turn initiates up-regulation of superior collicular/tectal genes. Furthermore, *Fgf8* is crucial for positioning of the diencephalic-mesencephalic boundary (Scholpp *et al.* 2003). Together, these genes act to specify this region of the mesencephalon.

En-1 is one of the most important factors in correct specification of the superior colliculus/tectum; loss of *En-1* expression results in absence of most of the colliculi and cerebellum (Wurst *et al.* 1994). *En-1* is believed to repress a tectal inhibitor, which allows subsequent expression of tectum-related genes including *Pax7*. This relationship between *En* and *Pax7* is thought to be crucial for superior colliculus/tectum specification and

maintenance (Nomura *et al.* 1998; Araki and Nakamura 1999; Matsunaga *et al.* 2000), a theory supported by isthmus-ablated chick/quail chimeras (lacking En-2 expression), in

which a small tectum expressing *Pax7* gradually regressed and eventually disappeared (Nomura *et al.* 1998). Similar results are observed in zebrafish *noi* (no isthmus) mutants, which also lack expression of *engrailed* genes (Brand *et al.* 1996).

4.4 What role does *Pax7* play in formation of the superior colliculus/tectum?

The pivotal role of *Pax7* in the formation of the superior colliculus/tectum has been highlighted by ectopic experiments indicating the ability of *Pax7* to act as a master control gene that instigates formation of superior collicular/tectal tissue (Matsunaga *et al.* 2001). Initially, *Pax7* defines the alar plate of the mesencephalon, and later the superior colliculus/tectum in the dorsal mesencephalon (Matsunaga *et al.* 2001). Transplantation experiments between chick and quail indicate that tectum formation critically depends upon induction and maintenance of *Pax7* expression and suppression of *Pax6* expression (Nomura *et al.* 1998). Transplantation of the chick mesencephalic floor plate to the tectum results in suppression of *Pax7* and fate change to the tegmentum, while *Pax7* misexpression in the tegmentum results in ventral expansion of tectal territory (Nomura *et al.* 1998). Misexpression of *Pax7* in the chick diencephalon results in induction of tectum-related genes, *Fgf8*, *En-2*, *Pax3* and endogenous *Pax7*, with subsequent formation of an ectopic tectum (Matsunaga *et al.* 2001). The ability of *Pax7* to induce tectum-related genes in the diencephalon where they are not normally endogenously expressed, coupled with the early expression of *Pax7* in the mesencephalic alar plate, from which the superior colliculus forms, suggests that *Pax7* may work at the forefront of the genetic cascade that is crucial to superior collicular/tectal development.

4.5 The superior colliculus/tectum and retinotopic mapping

RGCs project their axons to the superior colliculus/tectum in a highly ordered manner, which maintains the spatial relationship between neurons. The graded expression of the

molecular guidance cues *ephrin-A2* and *ephrin-A5* within the superior colliculus/tectum refine this topographic mapping (Cheng *et al.* 1995; Drescher *et al.* 1995; Nakamoto *et al.* 1996). This has been illustrated by incorrect routing of retinal axons noted in *ephrin-A2/ephrin-A5* double null mutant mice (Feldheim *et al.* 2000).

4.6 A role for *Pax7* in defining superior collicular/tectal polarity and retinotopic map refinement

Graded expression of *Pax7* across both the dorsoventral (dorsal^{high} to ventral^{low}) and anteroposterior (caudal^{high} to rostral^{low}) axes has been unambiguously demonstrated in embryonic mouse superior colliculus (Figure 4.2) and chick tectum (Thomas *et al.* 2004) and *Pax7* expression gradients have been quantified using Optimas Digital Analysis (mouse) (Figure 4.2) and Leica Image Analysis (chick) Systems. Given that the expression of *Pax7* in the superior colliculus/tectum is restricted to the *SGFS* layer, it is feasible to question whether this gradient establishes the graded expression of molecular guidance cues that act to specify correct retinotopic mapping within this layer.

Evidence supporting a role for *Pax7* as a determinant of retinotopic map formation is clearly provided by *Pax7* misexpression studies in the chick diencephalon, in which an ectopic tectum was innervated by RGC axons (Matsunaga *et al.* 2001). Moreover, strong similarities in the spatial and temporal expression gradients of *Pax7* and *ephrin-A2* have been demonstrated in chick tecta, and *in vitro* and *in vivo* studies show *Pax7* regulation of *ephrin-A2* in a cell autonomous manner (Thomas *et al.* 2004). These experiments provide convincing support for the role of *Pax7* in regulation of *ephrin-A2* expression.

Previous research has suggested that *ephrin-A2* is regulated by *En* with a close correlation between the graded expression profiles of these genes in the superior colliculus/tectum (Itasaki *et al.* 1991; Itasaki and Nakamura 1992; Logan *et al.* 1996). However, careful consideration of experimental evidence suggests that this regulation may not be direct, and it is now felt that *En* suppression of a tectal repressor allows expression of tectal genes including *Pax7* and *ephrin-A2* (Araki and Nakamura 1999). Support for such an indirect mechanism of regulation comes from gene expression studies; *En* is expressed at high

levels around the isthmus and demonstrates a caudal[high] to rostral[low] gradient across the mesencephalon including both the superior colliculus/tectum and the tegmentum (Bally-Cuif *et al.* 1992; Bally-Cuif and Wassef 1994; Watanabe and Nakamura 2000). By contrast, *Pax7* and *ephrin-A2* exhibit similar graded expression across only the superior colliculus/tectum (Itasaki *et al.* 1991; Nomura and Fujisawa 2000).

Further evidence for an indirect role for *En* in regulating expression of *ephrin-A2* is demonstrated by *Acerebellar* mutant zebrafish (*fgf8* knockouts), which initially express all three zebrafish *engrailed* gene homologues in the midbrain; no isthmus is present yet tecta are formed. *Engrailed* expression is completely extinguished after 24 h yet *ephrin-A2* expression continues after *engrailed* expression is ablated and can be detected at 44 h, at the time of retinotectal projection to the tectum. However, ephrin-A2 expression is not graded as in the wildtype; it is evenly distributed at a level characteristic of the anterior tectum and the retinotectal map is lost (Reifers *et al.* 1998; Picker *et al.* 1999).

In chick embryos, misexpression of *En* in the diencephalon resulted in rapid repression of *Pax6* within three hours after electroporation, yet ectopic expression of *Pax7*, together with *ephrin-A2*, occurred after 18 hours (Araki and Nakamura 1999), supporting an indirect relationship between *En* and *Pax7* or *ephrin-A2*.

Our current research is aimed at exploring the relationship between *Pax7*, *En* and *ephrin-A2*. We predict that the graded expression of *Pax7* may specify positional information for *ephrin-A2*, and the graded expression of *En*, more intense closer to the isthmus, may regulate graded expression of both *Pax7* and *ephrin-A2* (caudal[high] to rostral[low]). The expression domains of *En*, *Pax7* and *ephrin-A2* may work in concert to afford regional polarity to the superior colliculus/tectum and facilitate refinement of retinotopic map formation.

4.7 Conclusion

It is evident that *Pax7* plays a pivotal role in neurogenesis, from the very early stages of CNS development through to maturation of the brain. Initial extended expression dictates a

neurogenic lineage, with subsequent restriction of expression acting to dorsalize the neural tube and specify brain regions, particularly the superior colliculus/tectum. The final expression of *Pax7* specifically within the *SGFS* layer of the superior colliculus/tectum, in a gradient similar to that reported for ephrin ligands, argues against a role limited to dorsalization only. We speculate that *Pax7* may also function in retinotopic map refinement via regulation of the molecular guidance cue, *ephrin-A2*, within the retinorecipient layer of the superior colliculus/tectum.

Clearly *Pax7* plays a major role in the formation of the vertebrate visual system, and research aimed at resolving and elucidating the many roles of this important regulatory gene will be crucial to ensuring future successful regeneration of the optic nerve.

4.8 Acknowledgments

CHAPTER 5

PAPER 2

Pax7 and Superior Collicular Polarity:
Insights from *Pax6 (Sey)* Mutant Mice

"Failing is not being knocked down; it is not getting up again."

Sir Des Renford, MBE, 1927-1999.

CHAPTER 5:

Pax7 and Superior Collicular Polarity: Insights from *Pax6* (*Sey*) Mutant Mice

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Running title: *Pax7*- mediated collicular polarity is altered in *Sey* mice

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5.1 Abstract

Pax genes are important modulators of CNS development. *Pax7* and *Pax6* polarise the neural tube and regionalise the brain. *Pax7* is pivotal in specifying the superior colliculus/tectum, an important centre for integration of visuomotor responses and a target for *Pax6* RGC axons during retinocollicular mapping.

Whilst initial *Pax7*-specification of the mesencephalon is well-established, a role in regulating polarity within the maturing mouse superior colliculus is yet to be defined, although already detailed for the chick tectum. We therefore quantified *Pax7* cellular distribution and expression levels at three functionally distinct stages of superior collicular development, and analysed *Pax7* expression in response to aberrant axonal input and altered forebrain-midbrain boundary placement in *Pax6* mutant mice. Comparative expression profiles of *ephrin-A2* and its co-localisation with *Pax7* were determined in wildtype and *Pax6* mutant mice.

Results indicate that graded *Pax7* expression in wildtype mice is perturbed in *Pax6* mutant mice; changes manifest as a shift in polarity, loss of graded expression and dramatically reduced protein levels during RGC synaptogenesis. *Ephrin-A2* expression is similarly altered. These results implicate *Pax7* as an important determinant of polarity within the mouse superior colliculus, and suggest a role in retinotopic mapping.

5.2 Introduction

Pax genes are important regulators of CNS development (Jostes *et al.* 1991; Chalepakis *et al.* 1993; Stoykova and Gruss 1994; Schwarz *et al.* 1999). Their activity is multiphasic, typically achieved by dynamic spatial and temporal expression patterns (Stoykova and Gruss 1994; Kawakami *et al.* 1997; Thomas *et al.* 2004; Thomas *et al.* 2006).

Pax6 and *Pax7* are expressed very early in the developing CNS (Walther and Gruss 1991; Ericson *et al.* 1996; Basch *et al.* 2006) where they polarise the anteroposterior and dorsoventral axes of the neural tube and specify brain regions (Goulding *et al.* 1993; Ericson *et al.* 1996; Kawakami *et al.* 1997; Schwarz *et al.* 1999; Gilbert 2003, p.401). Expression, misexpression and transplant studies have identified *Pax7* as pivotal for specification of the mesencephalic tectum, homologue of the mammalian superior colliculus (Kawakami *et al.* 1997; Nomura *et al.* 1998; Matsunaga *et al.* 2001). Notably, *Pax7* misexpression in the embryonic chick tegmentum or diencephalon induced ectopic tectum formation, repression of *Pax6* and RGC innervation of ectopic tissue (Matsunaga *et al.* 2001). These, together with previous results, indicate *Pax7* expression and *Pax6* repression are prerequisites for correct tectum formation (Nomura *et al.* 1998).

Additional studies in developing chicks implicate *Pax7* in regulation of tectal polarity; initial extensive expression in undifferentiated cells of the ventricular zone becomes restricted to neurons of the dorsal retino recipient laminae in decreasing caudorostral and dorsoventral gradients, with persistent graded expression in adults (Kawakami *et al.* 1997; Thomas *et al.* 2004; Thomas *et al.* 2006). Furthermore, *Pax7* regulates expression of the molecular guidance cue *ephrin-A2*; *in vivo*, Pax7 co-localises with ephrin-A2 and *in vitro*, *ephrin-A2* is upregulated in *Pax7* transfected cells (Thomas *et al.* 2004). Taken together, it is apparent that *Pax7* is vital for chick tectal polarity and topography; however, these functional roles have yet to be detailed for *Pax7* within the mouse superior colliculus.

By contrast, the role of *Pax6* in mouse eye and forebrain specification, in positioning of the forebrain-midbrain boundary, and in retinal cell specification is well established (Stoykova *et al.* 1996; Mastick *et al.* 1997; Stoykova *et al.* 1997; Schwarz *et al.* 1999; Matsunaga *et*

al. 2000; Marquardt et al. 2001; Philips et al. 2005). *Pax6* polarises the ocular axis, thereby regulating accurate projection to target tissues (Ziman et al. 2003). Reduced *Pax6* expression in *Pax6*-deficient mice results in microphthalmia (Hill et al. 1991) and a 20-30% reduction in the axonal projection within the optic nerve (Deiner and Sretavan 1999).

To clarify the role of *Pax7* in determination of polarity within the developing mouse superior colliculus, we quantified *Pax7* cell distribution and protein levels at three key, functionally distinct stages: during optic nerve invasion (embryonic day (E)18), during formation of initial synaptogenesis (postnatal days (P)5-7) and in the mature, innervated superior colliculus (P21). Similarly, we analysed *Pax7* expression in developing and adult *Pax6* mutant mice in which the forebrain-midbrain boundary (Mastick et al. 1997; Schwarz et al. 1999) and RGC number (Philips et al. 2005) and projection to the superior colliculus (Deiner and Sretavan 1999) are altered.

Results indicate that *Pax7* exhibits dynamic spatial and temporal expression patterns in the developing mouse superior colliculus, where it specifies neurons of the dorsal retinorecipient laminae, similar to that of the chick tectum (Thomas et al. 2004; Thomas et al. 2006). In developing *Pax6* mutant mice, *Pax7* expression patterns are dramatically altered; expression extends rostrally with loss of graded expression, and protein levels are dramatically reduced during initial synaptogenesis (P5-7). To relate observed alterations in *Pax7* expression to polarity, we quantified *ephrin-A2* expression at P5-7, and found its expression similarly shifted with extended rostral expression and perturbed protein levels rostrally. Taken together, the expression patterns in wildtype mice and the significant changes observed for *Pax7* and *ephrin-A2* expression in *Pax6* mutant mice, together with evidence of their cellular co-localisation, strongly support a role for *Pax7* in regulation of polarity and topography within the mouse superior colliculus.

5.3 Materials and methods

5.3.1 Mouse tissue

Mice were obtained from timed matings of $Pax6^{+/-}$ (Sey^{Neu})/C57BL/6 mice. Noon of the day of vaginal plug appearance was designated E0.5. Genotyping of Sey^{Neu} mice was carried out as described previously (Xu *et al.* 1997). In brief, a 336 bp PCR fragment was amplified using primers SeyA (5'-GCATAGGCAGGTTATTTGCC-3') and SeyB (5'-GGAATTCCTGAGGAACCAGAGAAGACAGGC-3'). As a novel *Hind*II site arises from the single-base pair change of Sey^{Neu} allele within the *Pax6* gene (Hill *et al.* 1991), *Hind*II digestion of the amplified PCR fragment results in 114 bp and 222 bp fragments. This digested DNA was analysed using agarose gel electrophoresis.

Three to four normal ($Pax6^{+/+}$) and three heterozygote ($Pax6^{+/-}$) littermates were utilised at each time point, E18, P5-7 and P21. Three $Pax6^{-/-}$ null embryos were also assessed at E18. Embryonic brains were removed and postfixed in 4% paraformaldehyde. Postnatal mice were intracardially perfused, brains removed and postfixed with 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose before sectioning in the sagittal plane at 20µm, and stored at -80°C until required.

Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, and animal experimental procedures conformed to National Health and Medical Research Council of Australia guidelines, with approval by the Animal Ethics Committees of Edith Cowan University and the University of Sydney.

5.3.2 Immunohistochemistry

Consecutive tissue sections were treated with 0.2% Triton-X100/PBS (10 min), 1.5% H_2O_2/PBS (2x10 min). and blocked with 10% fetal calf serum/PBS (30 min) prior to incubation overnight at 4°C with primary antibodies; Pax7 antibody (1:20, mouse, monoclonal, DSHB; Figure 5.2a) or ephrin-A2 antibody (1:500, rabbit, polyclonal, Santa

Cruz; Figure 5.2b). Sections were then incubated with biotinylated anti-mouse/anti-rabbit IgG (Dako) followed by streptavidin/HRP complex (Dako) (20 mins each at room temperature) and visualized with diaminobenzidine (Dako) before processing through an ethanol series/xylene, and mounting in DePex. Control slides without primary antibodies were immuno-negative.

5.3.3 Immunohistochemical quantification and analysis

Images were captured with a Leica DC300 camera attached to an Olympus BX41 microscope and analysed using Optimas 6.5 Digital Image Analysis software (Media Cybernetics). The microscope was equilibrated prior to analysis, and microscope and image software settings were standardised. Optical density and cell distribution measurements for Pax7 immunostaining were obtained on a frame-by-frame basis (at 400X magnification), encompassing the entire $Pax7^+$ portion of the superior colliculus, thereby representing a serial reconstruction of the region (Coggeshall and Lekan 1996). For embryonic sections, frame sizes were halved for better resolution of graded expression. Optical density and cell distribution measurements were similarly obtained for ephrin-A2, with analysis of 1 in every 3.5 frames across the rostrocaudal axis and 1 in every 4 frames across the dorsoventral axis (at 1000x magnification). Ephrin-A2 quantification was limited to protein present on the soma to exclude ligand expression on axons or dendritic processes of neighbouring neurons. Each individual cell measurement was plotted back into its topographic position within Excel and values were normalised against immuno-negative adjacent tissue. Results were subsequently analysed and graphed as either average cellular protein level or average cell number at each axis point. Standard errors are shown. Refer to Appendix for more details of methodology (pp153-156).

5.3.4 Double immunofluorescence/confocal analysis

Tissue sections were treated with 0.2% Triton-X100/PBS (10 min), blocked with 10% fetal calf serum/PBS (30 min) and incubated overnight at 4°C with primary antibody: Pax7 (1:10) and either ephrin-A2 (1:50) or βIII tubulin (TuJ1)(1:500, rabbit, polyclonal, Abcam).

To assess variation in spatial expression between wildtype and *Pax6* mutant mice across both axes at P5-7, the number of frames containing Pax7 or ephrin-A2 staining were compared by ANOVA. For the purpose of brevity, significant values not considered pertinent to our discussion have been reported on the appropriate table, but not discussed in the text. To comply with statistical assumptions of normal distribution, $Pax7^+$ cell distribution values were transformed using $x^{0.08}$, where x is the $Pax7^+$ cell count per axis point. All statistical analyses were conducted utilising SPSS Version 13.0 Statistical Software, with a 95% confidence interval.

5.4 RESULTS

The suitability of the Pax7 antibody for use in mouse tissue has previously been established using Western Blot analysis (Seale *et al.* 2004).

5.4.1 Quantification of Pax7 within the developing mouse superior colliculus

Distribution of $Pax7^+$ cells

Wildtype Mice: Rostrocaudal axis (Figure 5.1a-c; Table 5.1)

At E18, $Pax7^+$ cells exhibit a curvilinear distribution along the rostrocaudal axis of the mouse superior colliculus (Figure 5.1a), i.e. there is a significant difference between the number of cells in the rostral and mid regions (p=0.002) and mid and caudal regions (p=0.042), but not between the rostral and caudal regions (p>0.05). At P5-7, the number of cells remains high midway across the axis (R-M, p=0.016), however there are now many more $Pax7^+$ cells caudally, culminating in a significant $rostral^{low}$ to $caudal^{high}$ gradient (Figure 5.1b; p=0.014; Table 5.1) which persists in the adult (Figure 5.1c; p=0.010; Table 5.1).

Figure 5.1 (Following page): **Pax7 expression profiles detailing $Pax7^+$ cell distribution *(a-f) and cellular protein levels (g-l) in the developing superior colliculus of Pax6 mutant mice and wildtype littermates, identifying rostrocaudal and dorsoventral expression patterns at E18, P5-7 and P21. Values are mean cell numbers ± SEM (a-f) or mean optical density ± SEM (g-l) (n =3, except $Pax6^{+/+}$ E18, where n = 4).***

Pax7+ cell distribution in the mouse superior colliculus

Rostrocaudal Axis   R ◄►C

E18

a

P5-7

b

P21

c

Dorsoventral Axis   V ◄►D

d

e

f

Pax7 cellular protein levels in the mouse superior colliculus

Rostrocaudal Axis   R ◄►C

E18

g

P5-7

h

P21

i

Dorsoventral Axis   V ◄►D

j

k

l
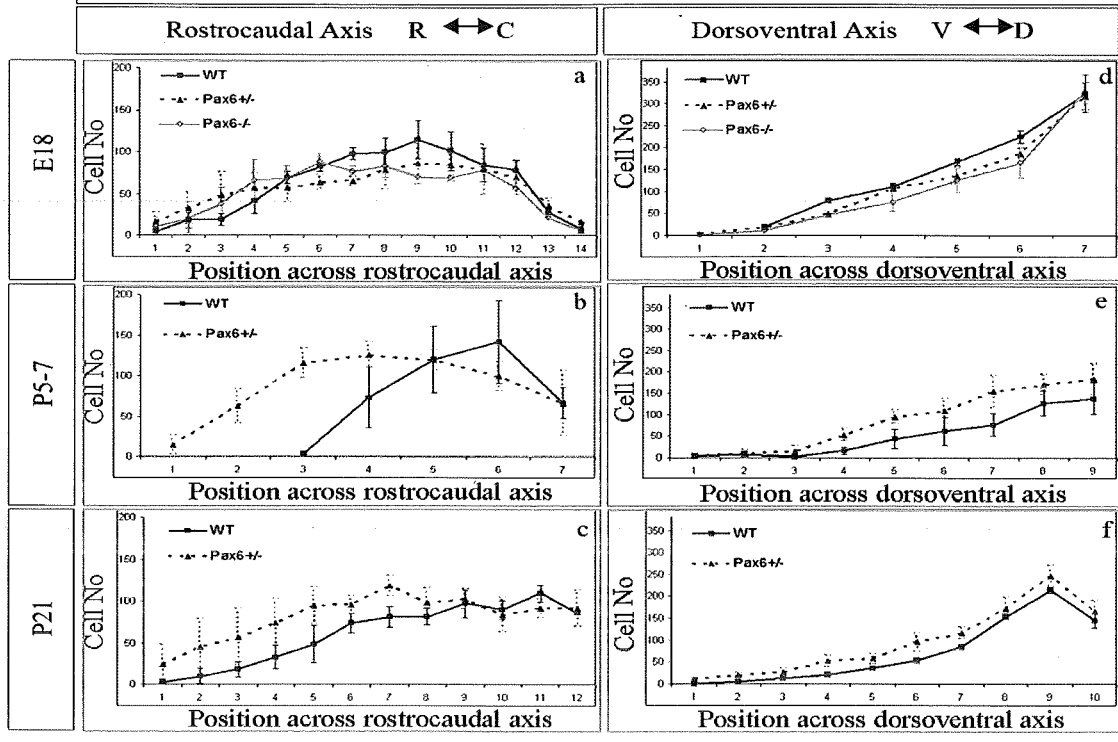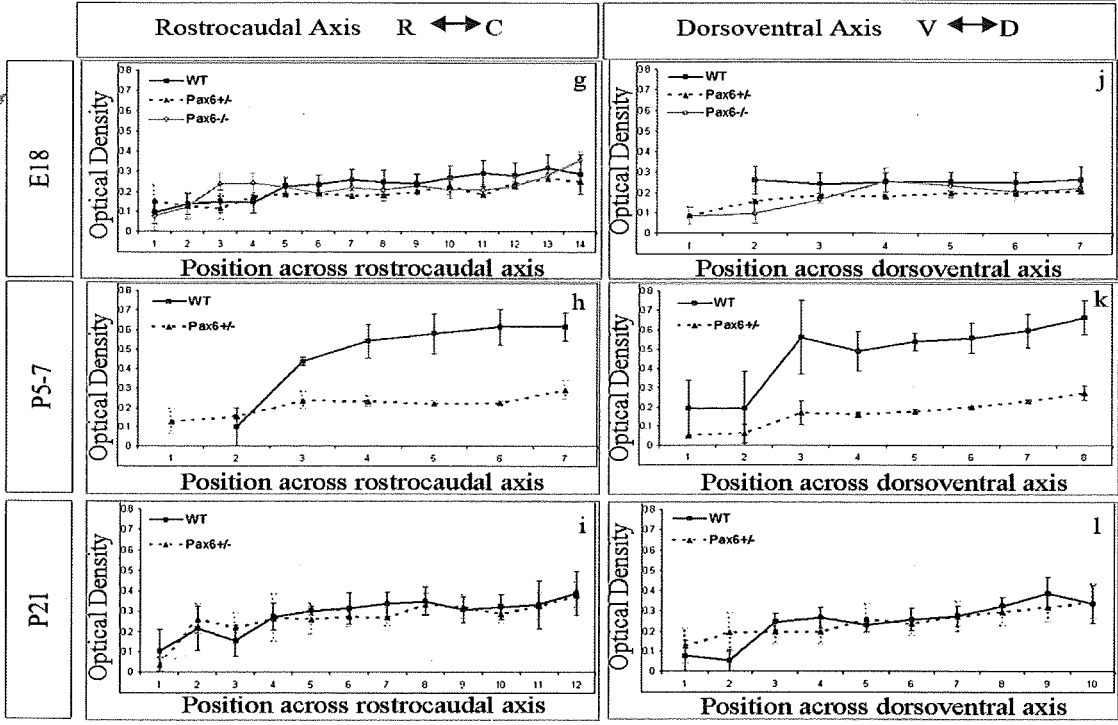
Table 5.1: Pax7$^+$ cell distribution in the superior colliculus of wildtype and *Pax6* mutant mice

Pax7$^+$ Cell Distribution							
R-C Axis		**^{a}Rostral**	**^{a}Mid**	**^{a}Caudal**	**$^#$R-M**	**$^#$M-C**	**$^#$R-C**
E18	+/+	19.00 ± 4.97	111.62 ± 21.95	25.38 ± 8.12	0.002	0.042	
	+/-	24.33 ± 4.09	90.67 ± 18.77	25.67 ± 4.21	0.044	0.005	
	-/-	24.83 ± 9.58	79.67 ± 8.09	13.33 ± 1.64	0.043	0.014	
P5-7	+/+	1.67 ± 0.44	96.33 ± 38.84	104.17 ± 25.72	0.016		0.014
	+/-	*38.00 ± 8.23	122.17 ± 13.85	82.33 ± 28.39	0.007		0.042
P21	+/+	14.00 ± 2.29	89.17 ± 6.08	97.67 ± 5.18	0.013	0.029	0.010
	+/-	41.83 ± 25.08	109.33 ± 13.71	91.17 ± 16.54			
D-V Axis		**^{a}Ventral**	**^{a}Mid**	**^{a}Dorsal**	**$^#$V-M**	**$^#$M-D**	**$^#$V-D**
E18	+/+	49.38 ± 4.76	138.75 ± 10.43	273.38 ± 37.40	0.003	0.011	0.001
	+/-	*14.33 ± 5.89	101.17 ± 1.67	251.50 ± 34.35		0.035	0.013
	-/-	*10.83 ± 2.68	118.83 ± 15.37	244.17 ± 10.75	0.009	0.024	0.004
P5-7	+/+	15.00 ± 1.04	62.33 ± 22.42	132.67 ± 33.16		0.020	0.019
	+/-	51.83 ± 18.19	110.00 ± 31.84	176.33 ± 29.61		0.041	
P21	+/+	12.67 ± 4.44	61.00 ± 4.44	178.50 ± 33.76	0.020	0.028	0.013
	+/-	14.50 ± 3.51	95.67 ± 29.75	206.17 ± 24.54	0.016	0.049	0.002

+/+: wildtype   +/-: *Pax6$^{+/-}$*   -/-: *Pax6$^{-/-}$*

^{a} values are mean cell numbers ± SEM (n = 3, except *Pax6$^{+/+}$* E18, where n = 4).

* denotes a statistically significant variance (ANOVA) when comparing an axis point in *Pax6* mutant mice to the equivalent axis point in wildtype mice. Corresponding significance values are indicated in the text.

$^#$ columns detail significant p values for paired *t* test assessing variation between axis points. Where p>0.05, values are omitted.

Dorsoventral axis (Figure 5.1d-f; Table 5.1)

At all stages of superior collicular development, the number of Pax7$^+$ cells is graded from ventral^{low} to dorsal^{high} (Figure 5.1d-f; E18, p=0.001; P5-7, p=0.019; P21, p=0.013; Table 5.1).

Pax6 mutant mice: Rostrocaudal axis (Figure 5.1a-c; Table 5.1)

Both homozygous $Pax6^{-/-}$ and heterozygous $Pax6^{+/-}$ mice demonstrate a curvilinear cell distribution at E18 (Figure 5.1a; Table 5.1). At P5-7 a rostral^{low} to caudal^{high} gradient is present (p=0.042), however there are more Pax7$^+$ cells in the mid region than caudally (Table 5.1; Figure 5.1b). This persists at P21, with a loss of graded expression evident (p>0.05; Table 5.1; Figure 5.1c).

Dorsoventral axis (Figure 5.1d-f; Table 5.1)

In embryonic and mature *Pax6* mutant mice there is a statistically significant ventral^{low} to dorsal^{high} distribution of Pax7$^+$ cells in the superior colliculus (E18, p=0.013$^{+/-}$, p=0.004$^{-/-}$; P21, p=0.002; Figure 5.1d,f; Table 5.1). However, in contrast to wildtype mice, the graded distribution at P5-7 is lost (p>0.05; Figure 5.1e; Table 5.1).

Comparison of Pax7 cell distribution between *Pax6* mutant and wildtype mice

It is interesting to note that at all stages examined, the numbers of Pax7$^+$ cells in the caudal superior colliculus of mutant and wildtype mice are similar (Figure 5.1a-c). By contrast, there are increased numbers of Pax7$^+$ cells rostrally in *Pax6* mutant mice, particularly at P5-7 (ANOVA: p=0.001). Postnatally, the cell distribution is highest in the mid region of this axis in *Pax6* mutant mice, in contrast to the caudal region in wildtype mice. This indicates a rostral shift in polarity which remains at P21, and subsequently the graded distribution across the rostrocaudal axis is lost. Moreover, there is a significant increase in the distribution of Pax7$^+$ cells spatially along the rostrocaudal axis at P5-7 (number of frames across the rostrocaudal axis: wt 5.33 ± 0.333; Pax6$^{+/-}$ 6.67 ± 0.333; ANOVA: p=0.048; Table 5.1). The overall spatial distribution of Pax7$^+$ cells across the dorsoventral axis of the superior colliculus at P5-7 does not change relative to wildtype littermates (number of frames analysed across the dorsoventral axis: wt 7.00 ± 1.00; *Pax6*$^{+/-}$ 7.00 ± 1.00; ANOVA: p>0.05). However, significantly fewer Pax7$^+$ cells are seen in the ventral superior colliculus at E18 in both *Pax6*$^{+/-}$ (ANOVA: p=0.013) and *Pax6*$^{-/-}$ (ANOVA: p=0.007) mice (Table 5.1).

Pax7 cellular protein levels

Wildtype mice: rostrocaudal axis (Figure 5.1g-i; Table 5.2)

At all stages examined, levels of Pax7 across the rostrocaudal axis are fairly uniform (Table 5.2). Pax7 levels are increased at P5-7 relative to E18 but decrease again at P21 (Figure 5.1g-i; Table 5.2).

Dorsoventral axis (Figure 5.1j-l; Table 5.2)

Similarly, there are uniform levels of Pax7 protein per cell across the dorsoventral axis at all stages, with increased levels noted at P5-7 relative to E18 and P21 (Figure 5.1j-l; Table 5.2).

Table 5.2: Pax7$^+$ cellular protein levels in the superior colliculus of wildtype and *Pax6* mutant mice

Pax7$^+$ Cellular Protein Levels						
R-C Axis	^{a}**Rostral**	^{a}**Mid**	^{a}**Caudal**	$^\#$**R-M**	$^\#$**M-C**	$^\#$**R-C**
E18 +/+	0.2305 ± 0.0424	0.2423 ± 0.0542	0.3148 ± 0.0665			
E18 +/-	0.2439 ± 0.0633	0.2338 ± 0.0539	0.3248 ± 0.0623		0.029	0.004
E18 -/-	0.1651 ± 0.0297	0.1434 ± 0.0215	0.2493 ± 0.0401			
P5-7 +/+	0.4061 ± 0.0153	0.5589 ± 0.0934	0.6138 ± 0.0691			
P5-7 +/‒	*0.1649 ± 0.0107	*0.2230 ± 0.0205	*0.2549 ± 0.0276			
P21 +/+	0.2644 ± 0.0307	0.3196 ± 0.0732	0.3600 ± 0.1137			
P21 +/-	0.2366 ± 0.0622	0.2723 ± 0.0562	0.3509 ± 0.0647		0.013	
D-V Axis	^{a}**Ventral**	^{a}**Mid**	^{a}**Dorsal**	$^\#$**V-M**	$^\#$**M-D**	$^\#$**V-D**
E18 +/+	0.2490 ± 0.0615	0.2490 ± 0.0460	0.2461 ± 0.0508			
E18 +/-	0.2060 ± 0.0574	0.2155 ± 0.0390	0.2410 ± 0.0412		0.008	
E18 -/-	0.1169 ± 0.0181	0.1512 ± 0.0279	0.1475 ± 0.0318			
P5-7 +/+	0.5096 ± 0.0627	0.5259 ± 0.0668	0.6288 ± 0.0924			
P5-7 +/-	*0.1649 ± 0.0002	*0.1841 ± 0.0052	*0.2504 ± 0.0385			
P21 +/+	0.2512 ± 0.0364	0.2662 ± 0.0512	0.3590 ± 0.0870			
P21 +/-	0.1917 ± 0.0477	0.2385 ± 0.0640	0.3308 ± 0.0793		0.040	

+/+: wildtype   +/-: *Pax6$^{+/-}$*   -/-: *Pax6$^{-/-}$*
^{a} values are mean optical density ± SEM (n =3, except *Pax6$^{+/+}$* E18, where n = 4).
* denotes a statistically significant variance (ANOVA) when comparing an axis point in *Pax6* mutant mice to the equivalent axis point in wildtype mice. Corresponding significance values are indicated in the text.
$^\#$ columns detail significant p values for paired *t* test assessing variation between axis points. Where p>0.05, values are omitted.

Pax6 mutant mice: Rostrocaudal axis (Figure 5.1g-i; Table 5.2)

Pax6 mutant mice demonstrate uniform Pax7 cellular protein levels across the rostrocaudal axis at all stages (Figure 5.1g-i; Table 5.2).

Dorsoventral axis (Figure 5.1j-l; Table 5.2)

Likewise, the dorsoventral axis contains uniform levels of Pax7 at all stages in *Pax6* mutant mice (Figure 5.1j-l; Table 5.2).

Comparison of *Pax7* cellular protein levels between *Pax6* mutant and wildtype mice

Pax6 mutant mice exhibit similar Pax7 protein levels to wildtype mice at E18. Remarkably at P5-7, when wildtype levels of Pax7 are increased in all cells across the rostrocaudal axis, $Pax6^{+/-}$ mice exhibit levels of Pax7 similar to those in the embryo and are thus dramatically reduced relative to the wildtype of the same age (ANOVA; rostral, p=0.0002; mid, p=0.025; caudal, p=0.009; ventral, p=0.005; mid, p=0.007; dorsal, p=0.019). At P21, this difference is no longer apparent, as wildtype levels decrease and remain slightly graded across the axis.

5.4.2 Quantification of *ephrin-A2* expression in the mouse superior colliculus at P5-7

It is well known that e*phrin-A2* is involved in mapping of the retinocollicular projection during mouse development, and like *Pax7* is expressed in rostral^{low} caudal^{high} and ventral^{low} dorsal^{high} gradients across the superior colliculus/tectum (Feldheim *et al.* 2000; Knoll *et al.* 2001; Marin *et al.* 2001). As the most substantial changes in *Pax7* expression were observed at P5-7 of mouse development, ephrin-A2 levels were quantified in mice at this time point.

Ephrin-A2 cell distribution in wildtype and *Pax6* mutant mice at P5-7 (Figure 5.2g-h)

In the superior colliculus of *Pax6* mutant mice, ephrin-A2$^+$ cell numbers caudally are similar to those of the wildtype (ANOVA: p>0.05; Figure 5.2g). Rostrally, the distribution is increased with a significantly expanded spatial distribution across the rostrocaudal axis

(number of frames across the rostrocaudal axis: wt 6.67 ± 0.33; Pax6$^{+/-}$ 8.67 ± 0.33; ANOVA: p=0.013) similar to that seen above for Pax7. *Pax6* mutant mice also show a significant increase in spatial distribution of cells ventrally (number of frames across the dorsoventral axis: wt 7.00 ± 0.58; Pax6$^{+/-}$ 11.00 ± 0.58; ANOVA: p=0.008; Figure 5.2h).

Ephrin-A2 cellular protein levels in wildtype and *Pax6* mutant mice at P5-7 (Figure 5.2i-j)

Across the rostrocaudal axis, ephrin-A2 cellular protein levels in *Pax6* mutant mice are similar caudally to that of the wildtype, however a significant variation occurs rostrally at point 3 of the axis (ANOVA: wt 0.0155 ± 0.0085; *Pax6*$^{+/-}$ 0.2201 ± 0.0650; p=0.035; arrowhead Figure 5.2i), with increased expression noted in *Pax6* mutant mice. This variation was the only significant difference in protein levels across the rostrocaudal or dorsoventral axes (Figure 5.2i-j).

Comparison between *ephrin-A2* expression profiles in *Pax6* mutant and wildtype mice at P5-7

There are considerable changes in *ephrin-A2* expression in *Pax6* mutant mice relative to wildtype mice, with changes similar to those observed for Pax7; rostrally, cell distribution is expanded and cellular protein levels are perturbed as the majority of ephrin-A2$^+$ cells demonstrate intermediate cellular expression levels similar to cells of the mid region. The dorsoventral axis shows a ventral expansion in ephrin-A2$^+$ cells (Figure 5.2j).

5.4.3 Co-localisation of Pax7 and ephrin-A2 in superior collicular cells

Confocal z-section imaging of Pax7/ephrin-A2 double immunofluorescent staining confirms cellular co-localisation of nuclear Pax7 and membrane bound ephrin-A2 in cells of the mouse superior colliculus at P5-7 (Figure 5.2c-d). All Pax7-positive cells were ephrin-A2-positive and the majority of ephrin-A2-positive cells were Pax7-positive (Figure 5.2c-d).

Ephrin-A2+ cell distribution in the mouse superior colliculus at P5-7

| Rostrocaudal Axis R ↔ C | Dorsoventral Axis V ↔ D |

Ephrin-A2+ cellular protein levels in the mouse superior colliculus at P5-7

| Rostrocaudal Axis R ↔ C | Dorsoventral Axis V ↔ D |
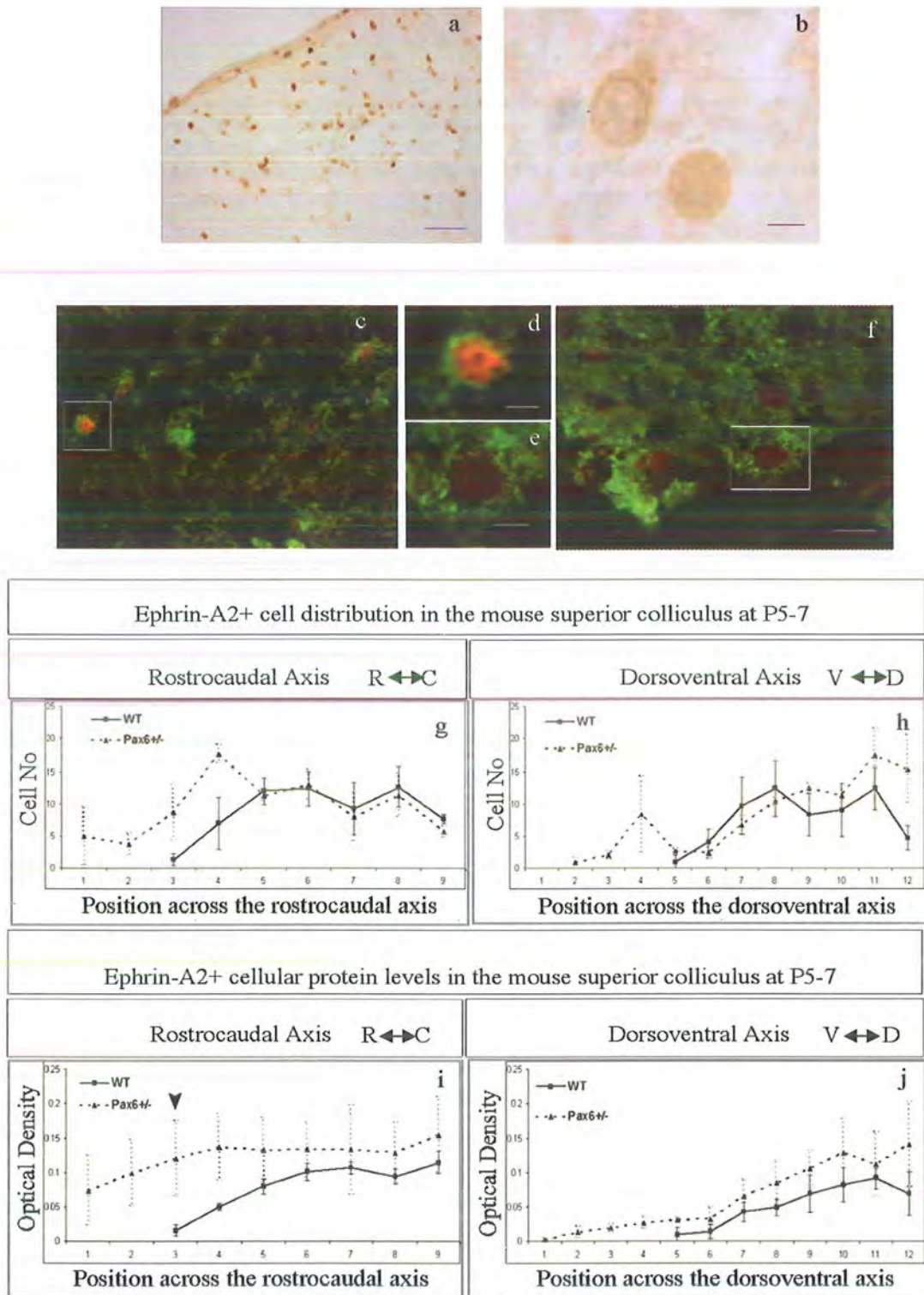
Figure 5.2. *Pax7 (a) and ephrin-A2 (b) immunohistochemical staining within the mouse superior colliculus at P5-7; (c-f) Confocal z-section micrographs of immunofluorescent staining identifying cellular co-localisation of nuclear Pax7 (red) with (c-d) membrane-bound ephrin-A2 (green) or (e-f) cytoplasmic and axonal βIII tubulin (green) within the mouse superior colliculus at P5-7 (n = 3 each); (g-j) Ephrin-A2$^+$ cell distribution and*

*cellular protein levels in the superior colliculus of **Pax6** mutant mice and wildtype littermates at P5-7, identifying rostrocaudal and dorsoventral expression patterns. Values are mean cell numbers ± SEM (g-h) or mean optical density ± SEM (i-j) (n = 3). Arrowhead denotes axis point with significant variation (ANOVA) in ephrin-A2$^+$ expression levels.*
Scale a) 50µm; b) 5µm; c) 20µm; d) [inset from c] 5µm; e) [inset from f] 5µm; f) 10µm.

5.4.4 Characterisation of Pax7$^+$ superior collicular cells as neurons

Co-localisation of Pax7 with the neuronal marker βIII tubulin (Figure 5.2e-f), which labels microtubules within the cytoplasm and processes, confirms that at P5-7 Pax7$^+$ cells are neurons.

5.5 Discussion

Our results illustrate that *Pax7* exhibits dynamic temporal and spatial expression patterns during superior collicular development, as previously identified for the developing chick tectum (Thomas *et al.* 2004). However, in the chick tectum, *Pax7* expression is graded across the rostrocaudal and dorsoventral axes throughout development, from initial stages of cell differentiation through to the more developed, post-proliferative stages (Thomas *et al.* 2004). Here we show that in the mouse, Pax7$^+$ cell distribution is curvilinear across the rostrocaudal axis in the embryo, and then significantly graded, rostral^{low} to caudal^{high}, p natally. These results are synonymous with a chronological maturation across the rostral (embryonic) to caudal (postnatal) axis, a phenomenon previously documented for the developing chick tectum (Cowan *et al.* 1984). This suggests that a functional role for *Pax7* in mouse superior collicular development extends further than initial specification, and may include determination of polarity and topography.

To determine the role of *Pax7* in regulation of superior collicular polarity, we investigated expression in *Pax6* mutant mice in which superior collicular topography (forebrain-midbrain boundary) (Mastick *et al.* 1997; Schwarz *et al.* 1999; Matsunaga *et al.* 2000) is altered. Altered boundary formation in *Pax6* mutant embryos is shown here to result in rostral/mid expansion of Pax7$^+$ cells, particularly at P5-7, indicating altered rostrocaudal

polarity. Similar expansion of *ephrin-A2* expression at this time confirms alterations to superior collicular polarity. Since Pax7 is known to regulate ephrin-A2 (Thomas *et al.* 2004), it is possible that Pax7 contributes to regulation of collicular polarity.

The concomitant alteration to both *Pax7* and *ephrin-A2* expression in *Pax6* mutant mice is not in itself conclusive evidence of a functional relationship. However this seems probable when these results are considered together with our demonstration of their cellular co-localisation, previous evidence of regulation of *ephrin-A2 in vitro* (initiated by *Pax7*-transfection and silenced by treatment with antisense oligonucleotides) and *in vivo* co-localisation in the chick (Thomas *et al.* 2004). We are currently assessing *ephrin-A2* expression in the superior colliculus of *Pax7* mutant mice to further elucidate this relationship.

Additionally, in both chick (Thomas *et al.* 2004) and mouse (this paper), Pax7 protein levels are dynamic during maturation of the superior colliculus/tectum. In the late embryonic stage, after most neurons have been born (DeLong and Sidman 1962), and RGC axons are entering the superior colliculus at the rostral region (Lund and Bunt 1976; Bunt *et al.* 1983; Godement *et al.* 1984; Edwards *et al.* 1986a), Pax7$^+$ cells across the rostrocaudal axis exhibit similar Pax7 levels. In stark contrast, during the first postnatal week when RGC axons are arborising and innervating the superior colliculus (Edwards *et al.* 1986a) and refinement of projection is occurring (Cowan *et al.* 1984), Pax7 cellular protein levels are dramatically increased, especially caudally. Consequently, it appears that refinements in *Pax7* expression occur in response to a variety of stimuli and thus in turn, Pax7$^+$ cell distribution and cellular levels may both be important for directing developmental processes.

It appears that initially a Pax7 cell distribution may contribute to superior collicular polarity and topography, and thus may act to direct incoming RGC axons to appropriate spatial locations. However, when axons are arborising and forming initial synapses, increased Pax7 levels may be a key cellular response to axonal input to direct additional cell functions/output. Furthermore, the summation of the graded cell distribution and upregulated cellular expression levels in the mouse provides a steep gradient of *Pax7* expression at this significant time point, which concurs with observations in the developing

chick tectum (Thomas *et al.* 2006), demonstrating differential regulation of *Pax7* throughout the developmental stages examined. In the developing chick tectum, Pax7 protein levels are already upregulated (Thomas *et al.* 2006) at the time that RGC axons reach the tectum at E6 (DeLong and Coulombre 1965), however this coincides with a peak in proliferation within the tectum (Cowan 1971; Scicolone *et al.* 1995), and as these proliferating cells are Pax7$^+$ at this time (Thomas *et al.* 2006), it is not possible to segregate the effects of RGC innervation on Pax7 levels from that of the proliferative phase.

Most importantly, dramatically reduced Pax7 protein levels at P5-7 in *Pax6* mutant mice relative to wildtype is strong evidence that *Pax7* levels are regulated at this time in response to RGC input. This is further supported by the obvious demarcation in the wildtype between high expression levels in the dorsal, retinorecipient region, where RGC axons are arborising, compared to reduced expression in the ventral region (Figure 5.1k). Taken together, co-localisation of Pax7 with the neuronal marker βIII tubulin, together with their positional placement within the retinorecipient laminae and the upregulated response after RGC input, provide strong evidence that *Pax7* participates in retinotopic mapping. Furthermore, recent experiments demonstrate that after optic nerve crush in adult rats, *Pax7* is upregulated, re-establishing the rostral^{low} to caudal^{high} gradient within neurons of the superior colliculus (Thomas *et al.* 2007). However, conclusive proof of synaptic activity between RGC axons and *Pax7*-expressing collicular cells remains to be determined.

The reduced number of *Pax7*$^+$ cells ventrally in *Pax6* mutant mice at E18 is perplexing. *Pax6* is expressed within the dorsal tegmentum, ventral to the superior colliculus (Agarwala *et al.* 2001), however it is not known what role this plays, if any, in setting *Pax7* levels in the superior colliculus. It may be more significant that the dorsal retinorecipient laminae connect with the ventral superior collicular cells (Jay and Sparks 1984; Rees 1996), and this circuitry may be altered due to reduced retinal input, resulting in perturbed *Pax7* levels ventrally. In support of this, previous research in the chick tectum at E14 following unilateral enucleation has demonstrated greatly reduced numbers of cells ventrally due to secondary transneuronal degeneration; deafferentation results in atrophic cells dorsally with subsequent degeneration in the ventral region (Cowan *et al.* 1984). Our results infer that this phenomenon may similarly occur in *Pax6* mutant mice, presumably to a milder extent due to reduced input dorsally, rather than withdrawal of input.

The significance of the persistent, graded *Pax7* cell distribution within the adult superior colliculus is yet to be determined. Further work will be required to characterise the *Pax7* expressing cells in the adult mouse superior colliculus to determine whether these cells represent a pool of "environmentally responsive" cells (Thomas *et al.* 2007) which may be functionally significant in terms of brain repair.

5.6 Conclusion

Our data demonstrate that polarity within the superior colliculus is altered in *Pax6* mutant mice. It is evident that a dosage-dependent relationship with *Pax6* is required for correct levels of *Pax7* within the mouse superior colliculus. Furthermore, the dramatic perturbation to Pax7 cellular protein levels in *Pax6* mutant mice at P5-7, as RGCs are innervating and arborising within this region, supports our suggestion that *Pax7* is involved in map formation, and regulation of Pax7 protein levels in the wildtype during this period occurs as a result of afferent input from the eye - that is, an axon-mediated response.

5.7 Acknowledgments

CHAPTER 6

PAPER 3

Pax7 is Requisite for Maintenance of a Subpopulation of Superior Collicular Neurons and Shows a Diverging Expression Pattern to *Pax3* during Superior Collicular Development

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed."

ALBERT EINSTEIN, 1879-1955.

6.1 Abstract

6.1.1 Background

Pax7 encodes a transcription factor well-established as an important determinant of mesencephalic identity and superior collicular development. *Pax7* mutant mice, however, present with no obvious morphological impairments to the superior colliculus. This finding is paradoxical and has been attributed to functional redundancy afforded by its paralogue *Pax3*. Here we utilise *Pax7* mutant mice to investigate the precise role of this important developmental regulator during superior collicular development and neuronal specification/differentiation. We also assess its spatiotemporal relationship with *Pax3* during embryonic development.

6.1.2 Results

Analysis of the superior colliculus of *Pax7* mutant and wildtype mice at a variety of developmental timepoints revealed that whilst correct initial specification is maintained, a subpopulation of dorsal mesencephalic neurons is lost at early postnatal stages. Moreover, a comparative analysis of embryonic *Pax3* and *Pax7* expression profiles indicate that *Pax3* expression overlaps extensively with that of *Pax7* initially, but their expression domains increasingly diverge as development progresses, coinciding spatiotemporally with neuronal differentiation and maturation of the tissue. Furthermore, *Pax3* expression is perturbed within the CNS of embryonic *Pax7* mutant mice.

6.1.3 Conclusion

In summary, these results demonstrate that during superior collicular development, *Pax7* is required to maintain a subpopulation of dorsal, mesencephalic neurons and partially regulates, spatiotemporally, *Pax3* expression within the CNS. The differential nature of *Pax7* and *Pax3* with respect to neuronal differentiation may have implications for future stem cell therapies aimed at exploiting their developmental capabilities.

6.2 Background

It is evident that *Pax7* is a multiplex contributor to correct CNS development. This is exemplified by dynamic spatiotemporal expression patterns, occurring from early development and persisting in restricted regions throughout adulthood. *Pax7* expression initially occurs in the neural tube and mesencephalon from very early stages (Basch *et al.* 2006; Otto *et al.* 2006) and is required for polarisation of the dorsoventral axis of the neural tube (Ericson *et al.* 1996) and specification of the superior colliculus/tectum from the mesencephalic alar plate (Kawakami *et al.* 1997; Nomura *et al.* 1998; Matsunaga *et al.* 2001; Thompson *et al.* 2007). In the developing superior colliculus, graded expression of *Pax7* establishes rostrocaudal and dorsoventral polarity. Expression of *Pax7* localises within superior collicular neurons as development proceeds. This expression is upregulated during retinal innervation and axonal arborisation but reduced in *Pax6* (*Sey*) mutant mice (Thompson *et al.* 2007), with reduced (20-30%) retinal innervation (Deiner and Sretavan 1999), confirming that Pax7-expressing cells are responsive to retinal input. Demonstrated colocalisation in superior collicular neurons with the mapping marker ephrin-A2 validates Pax7 participation in retinotopic mapping (Thompson *et al.* 2007). Continued, graded expression into adulthood is thought to maintain a small population of dorsal neurons in the mature colliculus (Stoykova and Gruss 1994; Thompson *et al.* 2007), although the functional requirement for this feature remains obscure.

Given the aforementioned importance of *Pax7* in mesencephalic and superior collicular development, the lack of gross defects in this region in *Pax7* mutant mice is surprising, and points to rescue by the paralogous *Pax3* gene which has overlapping expression domains (Stoykova and Gruss 1994; Mansouri *et al.* 1996b). Here, within the superior colliculus, we seek to determine the developmental role of *Pax7* in specification of neurons, and assess its spatiotemporal relationship with *Pax3*. We have analysed *Pax7* mutant mice (Mansouri *et al.* 1996b) relative to wildtype at key stages of development and results indicate that a subpopulation of neurons is lost during early postnatal stages. We show that this apparent loss of neurons is not due to aberrant specification or proliferation, or cell-fate switching/transdifferentiation to the astrocytic lineage, but rather appears due to the inability of *Pax7* mutant mice to maintain a subpopulation of dorsal superior collicular neurons.

Furthermore, analysis of *Pax3* expression in embryonic wildtype and *Pax7* mutant mice indicates crossregulation between paralogues, and illustrates a functional divergence during superior collicular development. We propose that within the superior colliculus initial overlapping *Pax3* expression ensures correct neuronal specification, and temporospatial separation of expression patterns leads to solitary expression of *Pax7* during a critical period of neuronal maturation, which abrogates the ability of *Pax3* to compensate, revealing the aberrant phenotype.

6.3 Methods

6.3.1 Mouse tissue

The generation and genotyping of *Pax7* mutant mice has been described previously (Mansouri *et al.* 1996b). Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, and animal experimental procedures conformed to National Health and Medical Research Council of Australia guidelines, with approval by the Animal Ethics Committee of Edith Cowan University. The production of transgenic animals at the Max Planck Institute for Biophysical Chemistry was performed with the approval of LAVES (Landesamt für Verbraucherschutz und Lebensmittelsicherheit) in Oldenburg.

Whole embryos (E12.5) or embryonic brains (>E15.5) from mutant and wildtype littermates were isolated and postfixed in 4% paraformaldehyde. Postnatal mice were deeply anaesthetised with Avertin and intracardially perfused with fixative. Brains were isolated and postfixed with 4% paraformaldehyde. Tissue was cryoprotected in 20% or 30% sucrose/PBS prior to sectioning. Embryos at E12.5 were sectioned whole. For all other stages, brain hemispheres were sectioned in the sagittal (left) or coronal (right) planes at 10-20μm, and slides were stored at -80°C until required. A minimum of three animals per group (+/+; +/-; -/-) per timepoint were analysed (with the exception of Pax7$^{-/-}$ animals at E18.5, where n=2).

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6.3.2 Immunohistochemistry

Whole embryo tissue from E12.5 mice was subjected to antigen retrieval by microwave heating in sodium citrate buffer pH6.0 prior to processing. All tissue sections were treated with 0.2% Triton-X100/PBS (10 min), 1.5% H_2O_2/PBS (2-3x10 min), blocked with 10% fetal calf serum/PBS (30 min) and incubated overnight at $4^{\circ}C$ with primary antibodies; Pax7 (1:20 or 1:10 (E12.5), mouse, monoclonal, DSHB, Iowa City, IA, USA); Pax3 (1:100 or 1:50 (E12.5), mouse, monoclonal, DSHB, Iowa City, IA, USA); Pax6 (1:100, mouse, monoclonal, DSHB, Iowa City, IA, USA); En-1 (1:50, mouse, monoclonal, DSHB, Iowa City, IA, USA); ephrin-A2 (1:500, rabbit, polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA); NeuN (1:100, mouse, monoclonal, Chemicon/Millipore, Billerica, MA, USA). Sections were then incubated with biotinylated anti-mouse/anti-rabbit IgG (1:3, Dako, Sydney, NSW, Australia) followed by streptavidin/HRP complex (1:3, Dako, Sydney, NSW, Australia) (20 mins each at room temperature). Visualization with diaminobenzidine (3% in substrate buffer, Dako, Sydney, NSW, Australia) preceeded processing through an ethanol series/xylene, and mounting in DePex. Control slides without primary antibodies were immunonegative.

6.3.3 Immunohistochemical quantification and analysis

Images were captured with a Leica DC300 camera attached to an Olympus BX41 microscope and analysed using Optimas 6.5 Digital Image Analysis software (Media Cybernetics, Bethesda, MD, USA). For cellular quantification at E15.5 and P18.5 images were captured within Optimas (100x magnification) and $Pax7^+$ cells were marked and counted. For optical density and cell distribution measurements at P5, the microscope was equilibrated prior to analysis, and microscope and image software settings were standardised. Measurements of Pax7 immunostaining were obtained on a frame-by-frame basis (400x magnification), and encompassed the entire $Pax7^+$ portion of the superior colliculus, producing a serial reconstruction of the region (Coggeshall and Lekan 1996). Each individual cell measurement was topographically replotted within Excel and values were normalised against immunonegative adjacent tissue. Results were subsequently

analysed producing maps of mean cellular distribution, or graphed as either mean cellular protein level or mean cell number at each axis point. Standard errors are shown. Refer to Appendix for more details of methodology (pp153-156).

6.3.4 Immunofluorescence

Tissue sections were blocked with 3% normal goat serum (NGS)/0.2% Triton-X100/TBS (TX-TBS) for one hour at room temperature and incubated overnight at 4°C with primary antibody diluted in 1% NGS/TX-TBS. Primary antibodies included Pax7 (1:10); Pax3 (1:50); activated-Caspase 3 (1:100, rabbit, polyclonal, Promega, Sydney, NSW, Australia); α-internexin (1:150, rabbit, polyclonal, Chemicon/Millipore, Billerica, MA, USA); βIII tubulin (TuJ1)(1:500, rabbit, polyclonal, Abcam, Cambridge MA, USA); Doublecortin (1:500, rabbit, polyclonal, Abcam, Cambridge, MA, USA); GFAP (1:1000, rabbit, polyclonal, Chemicon/Millipore, Billerica, MA, USA); Ki67 (1:150, rabbit, polyclonal, Abcam, Cambridge, MA, USA). Primary antibodies were visualised with conjugated anti-rabbit IgG-AlexaFluor488 (1:500; Molecular Probes-Invitrogen, Melbourne, VIC, Australia), or biotinylated goat anti-mouse IgG (1:500; Abcam, Cambridge, MA, USA), incubated overnight followed by streptavidin-AlexaFluor546 (1:500; Molecular Probes-Invitrogen, Melbourne, VIC, Australia), incubated for 2 hours at room temperature. Slides were counterstained with Hoechst (1/5000; Sigma Aldrich, Sydney NSW, Australia) and mounted in Fluorsave (Calbiochem, La Jolla CA, USA). Controls with primary antibodies omitted were immunonegative. Fluorescent micrographs and the confocal z-stack image (Figure 6.5) were obtained using Confocal Assistant software (Version 4.02) and the Biorad MRC 1000/1024 Confocal Microscope or Olympus BX41 microscope equipped with an Olympus DP71 camera.

6.3.5 Statistical analysis (Table 6.1)

To assess variation between wildtype and mutant mice at E15.5, P5 and P18.5, we compared the number of Pax7+ cells in wildtype mice with those in $Pax7^{+/-}$ mice using paired t test (two-tailed). For determination of graded expression at P5, mean values at three points across each axis (rostral, mid and caudal, or ventral, mid and dorsal) were

calculated by averaging cell numbers in two frames at each axis position. Graded expression across each axis was assessed by analysis of significant variation between axis points using the paired t test (two-tailed): rostral vs mid (R-M), mid vs caudal (M-C), rostral vs caudal (R-C) or ventral vs mid (V-M), mid vs dorsal (M-D), ventral vs dorsal (V-D). Corresponding significance values are reported in Table 6.1. All statistical analyses were conducted utilising SPSS Version 13.0 Statistical Software, with a 95% confidence interval.

6.4 Results

6.4.1 *Pax* gene expression patterns

The Pax7 antibody has previously been demonstrated to be suitable for use in mouse tissue by Western Blot analysis (Seale *et al.* 2004). The Pax3 antibody has been tested *in vitro* and *in vivo* for specificity in recognition of mouse Pax3 by Western Blot analysis (Venters *et al.* 2004).

Pax7 expression in wildtype mice

Within the anlage of the tectum, at the earliest embryonic stage examined (E12.5), *Pax7* expression occurs from the mesencephalic ventricular zone to the most superficial layer. Immunoreactive cells are most dense in the ventricular and subventricular zones, then decrease in the intermediate zone, which is larger rostrally compared to the caudal region at this stage (Fig 6.1a-b). *Pax7* expression can also be detected within the subthalamus, pretectum, pons, and in the ventricular zones of the cerebellar primordium, pons and myelencephalon (4th ventricle). This profile concurs with *in situ* hybridisation results reported previously at E13 (Stoykova and Gruss 1994), with the exception of expression detected at the 4th ventricle. At E15.5, a large number of immunoreactive cells are detected (Fig 6.2a) in the presumptive superior colliculus whilst expression declines in the caudal tectum, reflecting the emerging distinction of the tectum into the inferior and superior colliculi. $Pax7^{+}$ cells also recede from the mesencephalic ventricular zone, although remnants of expression can still be detected at the dorsal ventricular surface, with cells also noted at the ventral ventricular surface at certain mediolateral positions. $Pax7^{+}$ cells can be

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Pax7 Expression Profiles
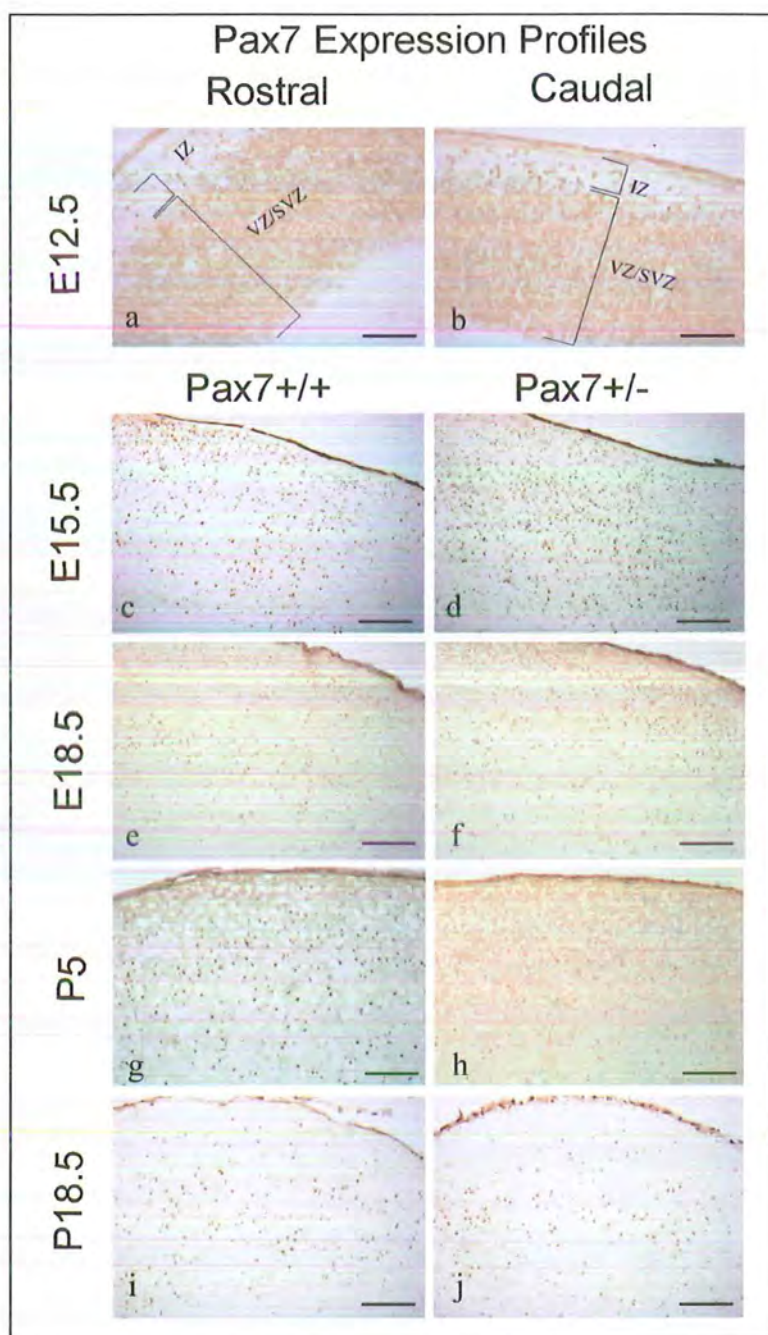
Figure 6.1. **Pax7 expression profiles in the developing superior colliculus of Pax7 mutant and wildtype mice.** *Comparative Pax7 expression profiles throughout development of the mouse tectum/superior colliculus in wildtype (a,b,c,e,g,i) and Pax7$^{+/-}$ mice (d,f,h,j). Note expression within the intermediate zone (IZ) in both the rostral (a) and caudal (b) regions of wildtype mice at E12.5. The embryonic Pax7 profile is comparable between wildtype and Pax7$^{+/-}$ mice at E15.5 (c-d) and E18.5 (e-f), however at P5 there is a paucity of Pax7$^+$ cells in the dorsalmost region of Pax7$^{+/-}$ mice relative to wildtype (g-h). At P18.5 Pax7 expression is similar between wildtype and Pax7$^{+/-}$ mice (i-j). Abbrev. IZ, intermediate zone; VZ/SVZ, ventricular zone/subventricular zone. Scale bar: a-b 100μm, c-j 200μm.*

detected up to the pial surface of the superior colliculus with the exception of the stratum zonale, which is now becoming evident (Fig 6.1c). Expression remains robust within the pons. *Pax7* expression at E18.5 is similar to that noted at E15.5 (Fig 6.1e), however immunoreactivity is no longer detected within the ventricular zone and expression within the subthalamus is waning. At P5, while a rostral[low] to caudal[high], ventral[low] to dorsal[high] gradient is maintained, Pax7^{+} cell numbers are reduced throughout the superior colliculus (Table 6.1; Fig 6.1g; Fig 6.2a,b,d,e; (Thompson *et al.* 2007)). Cellular protein levels are similarly graded (Fig 6.2f-g; (Thompson *et al.* 2007)). Expression is also reduced at the midbrain-hindbrain boundary, pons and subthalamus. Even fewer Pax7^{+} cells are detected throughout the juvenile superior colliculus (P18.5) (Fig 6.1i; Fig 6.2a). Expression persists at the midbrain-hindbrain boundary and rostral to the choroid plexus (4th ventricle), with weak expression in the subthalamus (n=3-5 at all stages).

Table 6.1. Pax7^{+} cell distribution in the superior colliculus of wildtype and *Pax7* mutant mice at P5.

Pax7[+] Cell Distribution							
D-V Axis		[a]Ventral	[a]Mid	[a]Dorsal	[#]V-M	[#]M-D	[#]V-D
P5	+/+	117.50 ± 8.29	311.50 ± 60.88	551.25 ± 106.79	.051	.025	.025
	+/-	126.25 ± 34.97	257.75 ± 56.98	317.25 ± 85.55	.025	>0.05	>0.05

+/+; wildtype +/-; *Pax7*^{+/-}
[a] values are Mean Cell Numbers ± SEM (n=4).
[#] indicates a significant p value from paired *t*-test assessing variation between axis points, within wildtype and *Pax7*^{+/-} mice.

Pax7 expression in *Pax7*^{+/-} mutant mice

In *Pax7*^{+/-} embryos, the *Pax7* expression profile is grossly indistinguishable from that of wildtype mice (Fig 6.1d,f); cell counts, wt = 2248.67 ± 94.75; *Pax7*^{+/-} = 2373.67 ± 206.88 (p>0.05; n=3; Fig 6.2a). However, at P5, there is a dramatic reduction in the number of Pax7^{+} cells in the most superficial region of *Pax7*^{+/-} mice relative to those of wildtype mice (Table 6.1; Fig 6.1h; Fig 6.2b-e; n=4 each). This manifests as a distinct area near the pial surface that is almost devoid of Pax7^{+} cells uniformly across the entire rostrocaudal (Fig 6.2d) and mediolateral (data not shown) axes. Moreover, the number of Pax7^{+} cells is

reduced throughout the dorsal half of the superior colliculus. Although the reduction in the total number of Pax7⁺ cells in $Pax7^{-/-}$ relative to wildtype mice at P5 does not reach statistical significance, this phenomenon results in the loss of graded cellular distribution across the dorsoventral axis (Table 6.1; Fig 6.2e; p>0.05). Concomitantly there is some variation in cellular protein levels ventrally creating a lack of graded expression medially (Fig 6.2g). Interestingly, at P18.5 we did not discern any differences in *Pax7* expression between heterozygous and wildtype mice; cell numbers, wt = 828.5 ± 108.97; $Pax7^{+/-}$ = 913.60 ± 69.77 (p>0.05, Table 6.1; Fig 6.1i-j; Fig 6.2a; n=3-5 at all stages).

Pax3 expression in wildtype and *Pax7* mutant mice

At E12.5, in wildtype and *Pax7* mutant mice *Pax3* expression can be detected from the pretectum to the tectum in the ventricular and subventricular zones, with rostral expression lower in intensity relative to the mid-caudal region (Fig 6.3a-b). Immunoreactivity is also evident in the ventricular zones of the cerebellar primordium, pons and myelencephalon, and at the midbrain-hindbrain boundary. By E15.5 *Pax3* expression within the superior colliculus of wildtype mice is primarily restricted to the dorsal ventricular zone, and the pons (Figs 6.3c,e). In $Pax7^{-/-}$ mutant mice at this stage, Pax3⁺ expression expands ventrally within the myelencephalon and pons (Figs 6.3d,f). In striking contrast to wildtype (Fig 6.3g) and $Pax7^{+/-}$ mice, $Pax7^{-/-}$ mice demonstrate large numbers of Pax3⁺ cells above the rostral ventricular zone (3ʳᵈ ventricle) (Fig 6.3h). Furthermore, Pax3⁺ cells can be detected at the ventral ventricular surface in *Pax7* mutant mice but not in wildtype mice (Figs 6.3i-j). At E18.5, in wildtype (Fig 6.3k) and heterozygous mice the pretectum and ventricular zone of the superior colliculus remain Pax3⁺, although only a remnant of expression within a small number of weakly stained cells remains within the superior colliculus and in the pons. By contrast, Pax3 immunoreactivity cannot be detected within the ventricular zone of $Pax7^{-/-}$ mice (Fig 6.3l) (n=2-4 at all embryonic stages). Pax3 expression could not be detected at P5 in wildtype or mutant mice (data not shown).
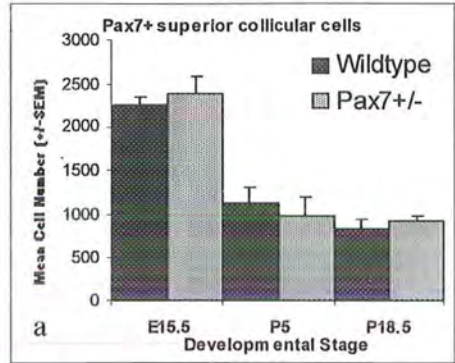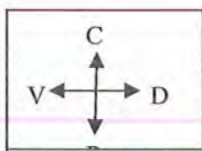
C
V ← → D

Pax7+ superior collicular cells

Map of Pax7⁺ cell distribution in the superior colliculus of wildtype and Pax7 mutant mice at P5

b   **Wildtype mice**   **Pax7+/- mutant mice**   c
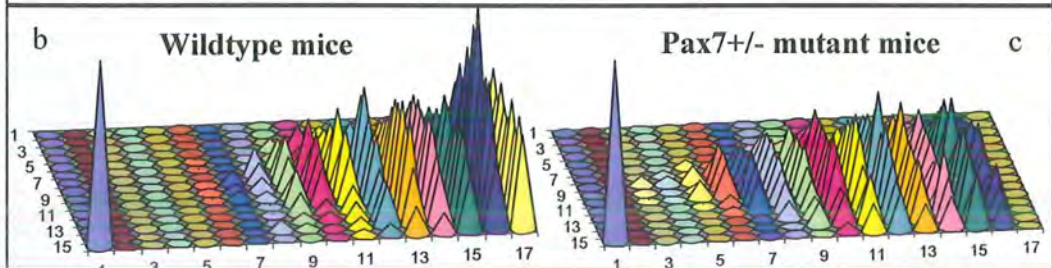
Pax7⁺ superior collicular cell distribution across rostrocaudal and dorsoventral axes at P5
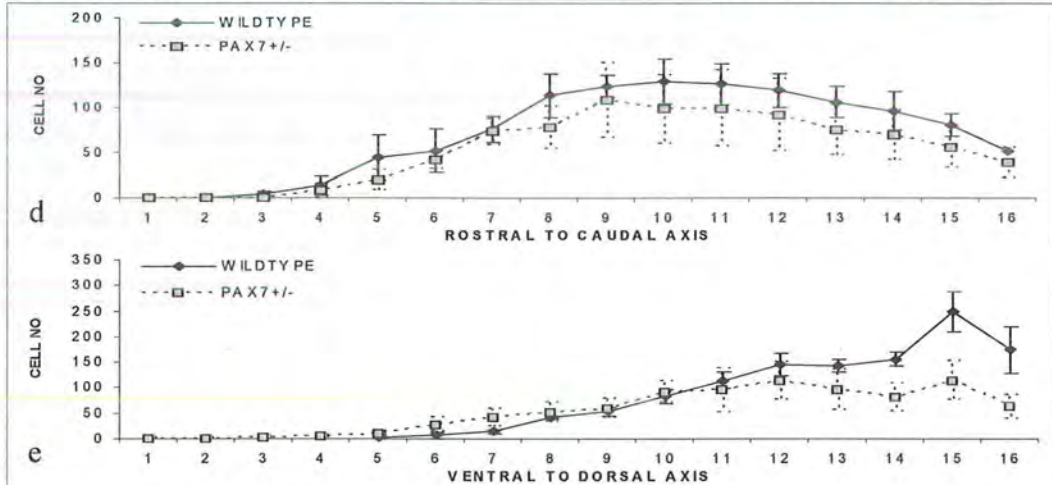
d   ROSTRAL TO CAUDAL AXIS

e   VENTRAL TO DORSAL AXIS

Pax7 protein levels in the superior colliculus of wildtype and Pax7 mutant mice at P5

f   ROSTRAL TO CAUDAL AXIS

g   VENTRAL TO DORSAL AXIS
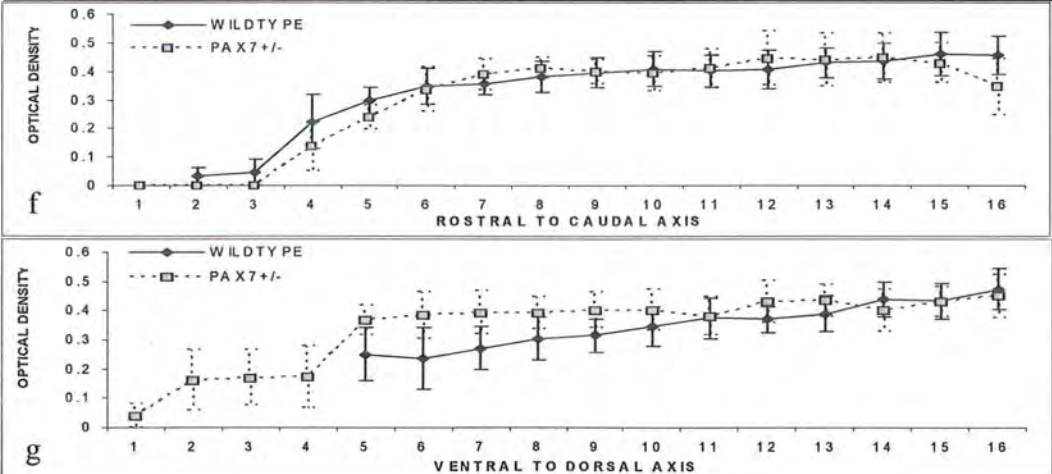
Figure 6.2. (Previous page): Quantification of Pax7 *expression in the superior colliculus of wildtype and* Pax7$^{+/-}$ *mice. (a)* Pax7$^+$ *cells in the superior colliculus of wildtype and* Pax7 *mutant mice at E15.5, P5 and P18.5 (n=3-5). (b-c) Map of* Pax7$^+$ *cell distribution in the superior colliculus of wildtype (b) and* Pax7$^{+/-}$ *mutant mice (c) at P5 showing reduced* Pax7$^+$ *cells in the superficial strata of* Pax7$^{+/-}$ *mice. The cone at position 1 represents positive control for relative calibration. (d-e) Mean (+/-SEM)* Pax7$^+$ *cellular distribution across rostrocaudal (d) and dorsoventral (e) axes. (f-g) Mean (+/-SEM)* Pax7 *protein levels across rostrocaudal (f) and dorsoventral (g) axes (n=4 each).*

6.4.2 Spatiotemporal assessment of neuronal proliferation and differentiation within the superior colliculus

To assess whether the loss of Pax7$^+$ cells in the dorsal superior collicular region of *Pax7* mutant mice was due to altered proliferation we explored *Ki67* expression at E12.5. We could not detect any variation in expression patterns between wildtype and mutant mice (data not shown), indicating that cellular proliferation proceeds normally, thus excluding neuronal precocity at this stage (n=3-4).

We next sought to relate observed differences in the *Pax7* and *Pax3* expression profiles to temporal differences in neuronal differentiation. We have shown previously that Pax7 co-localises with βIII-tubulin and ephrin-A2 in the mouse superior colliculus at P5 (Thompson *et al.* 2007). We therefore used these and other markers of early neuronal differentiation, α-internexin and Doublecortin as well as the mature neuronal marker, NeuN to analyse neuronal differentiation in wildtype and mutant mice.

The efficacy of βIII tubulin to discriminate differentiating neurons and tract formation in the mouse embryo has been demonstrated previously (Easter *et al.* 1993). At E12.5 we observed βIII tubulin$^+$ cells predominantly located within the intermediate zone at the pial surface of the tectum, identifying the emerging *stratum profundum* (SP) (Fig 6.4a), containing the first differentiating neurons of the superior colliculus (Edwards *et al.* 1986a).

Rostral	Mid-Caudal

Wildtype Mice	Pax7 Mutant Mice
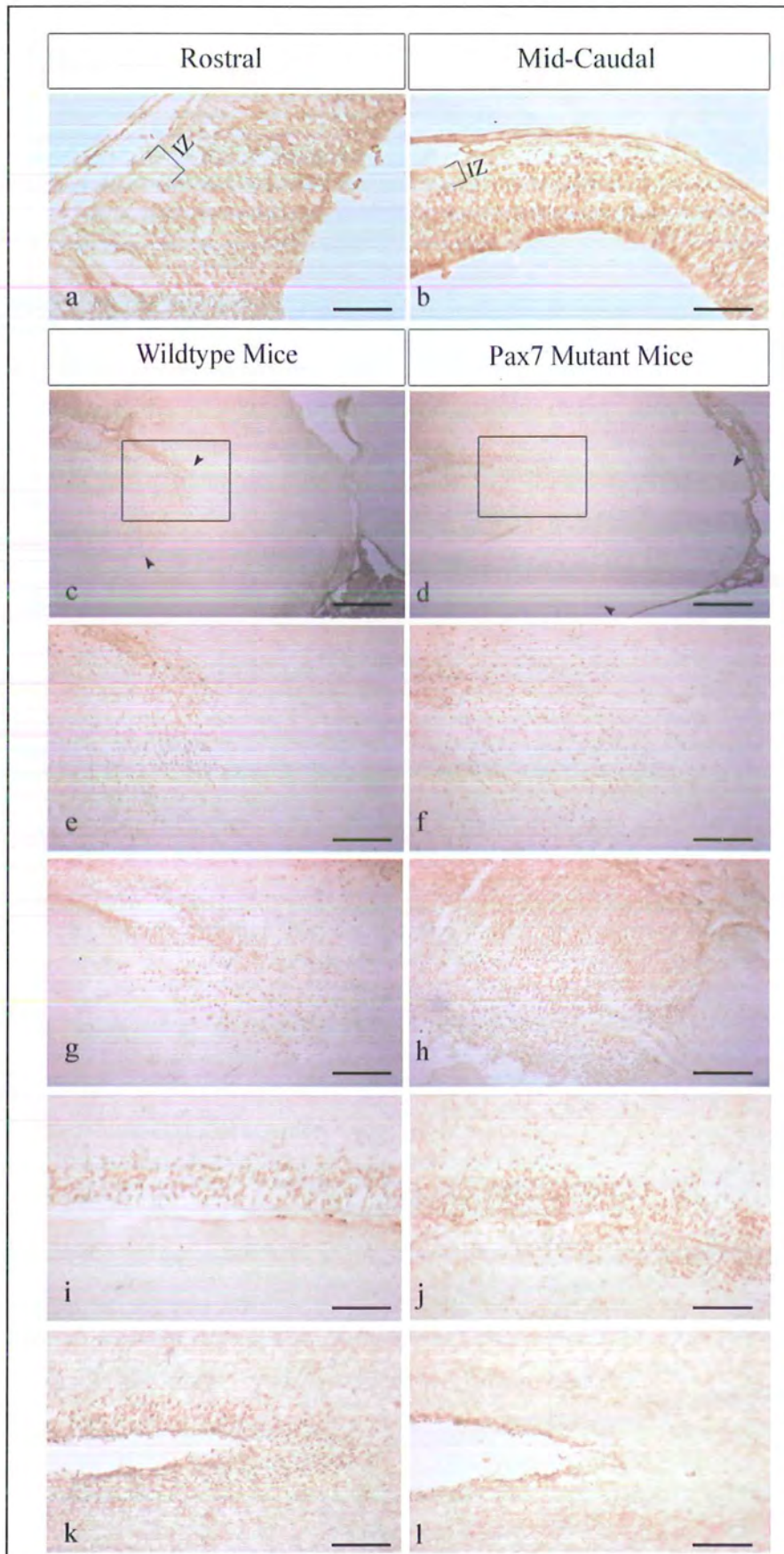
Figure 6.3. (Previous page): **Pax3** *expression profiles in the embryonic superior colliculus of* **Pax7** *mutant and wildtype mice.* *Comparative* Pax3 *embryonic expression profiles in wildtype (a,b,c,e,g,i,k) and* Pax7 *mutant mice (d,f,h,j,l). Note the decreased intensity of Pax3 in the rostral region (a) relative to the caudal region (b) of the ventricular/subventricular zones at E12.5, and lack of immunoreactivity within the intermediate zone. At E15.5, Pax3$^+$ cells are present in the hindbrain in wildtype mice (c, e [inset from c]) but are spatially expanded in* Pax7 *mutant mice (d,f [inset from d]). (Arrowheads denote spatial extent of expression). At the rostral mesencephalon at this timepoint there are increased numbers of Pax3$^+$ cells in Pax7$^{-/-}$ mice relative to wildtype (g-h) together with expanded expression at the ventral ventricular surface (j), whereas Pax3 immunoreactivity was only detected in the dorsal ventricular region in wildtype mice (i). Consequently, at E18.5, whilst Pax3$^+$ cells can be clearly detected in the ventricular zone of wildtype mice (k) they are no longer present in Pax7$^{-/-}$ mice (l). Abbrev. IZ, intermediate zone; VZ/SVZ, ventricular zone/subventricular zone. Scale bar: a-b, i-l 100 μm; c-d 500 μm; e-h 200 μm.*
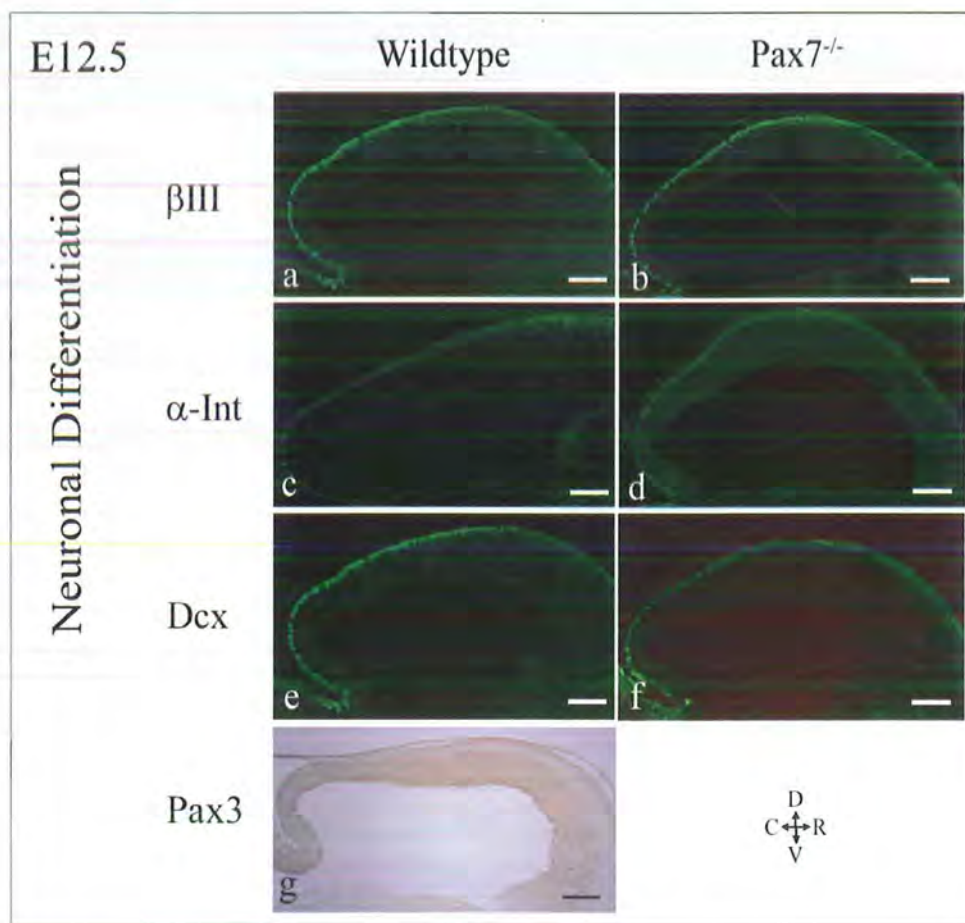
Figure 6.4. Comparative neuronal differentiation in the developing superior colliculus of **Pax7** *mutant and wildtype mice at E12.5.* *Neuronal differentiation in the developing mouse tectum/superior colliculus of wildtype (a,c,e) and* Pax7$^{-/-}$ *mice (b,d,f) at E12.5, as indicated by βIII tubulin (a-b), α-internexin (c-d) and Doublecortin (Dcx) (e-f) immunostaining. Comparison shows similar neuronal differentiation, with increased*

spatial expression rostrally relative to caudally, with the rostral region slightly variable at different positions across the mediolateral axis, and neuronal differentiation mid-caudally restricted to the intermediate zone at the pial surface. Compare to the complementary expression of Pax3 at E12.5 (g). Scale bar: 200 μm.

Immunolabelled cells were also observed further ventrally at the rostral tectum, but were reduced in number throughout the mid-caudal region, indicating a rostral to caudal cellular maturation, with the occasional immunoreactive cell seen in more ventral regions.

At E15.5 the SP is clearly visible in the ventral tectum, and immunolabelled, horizontally-oriented processes are now visible extending throughout the dorsorostral superior colliculus (Fig 6.5). These processes extend more caudally at E18.5 and most likely represent the incoming axons of retinal ganglion cells, the major afferent projection to the superficial superior colliculus (Edwards *et al.* 1986b).
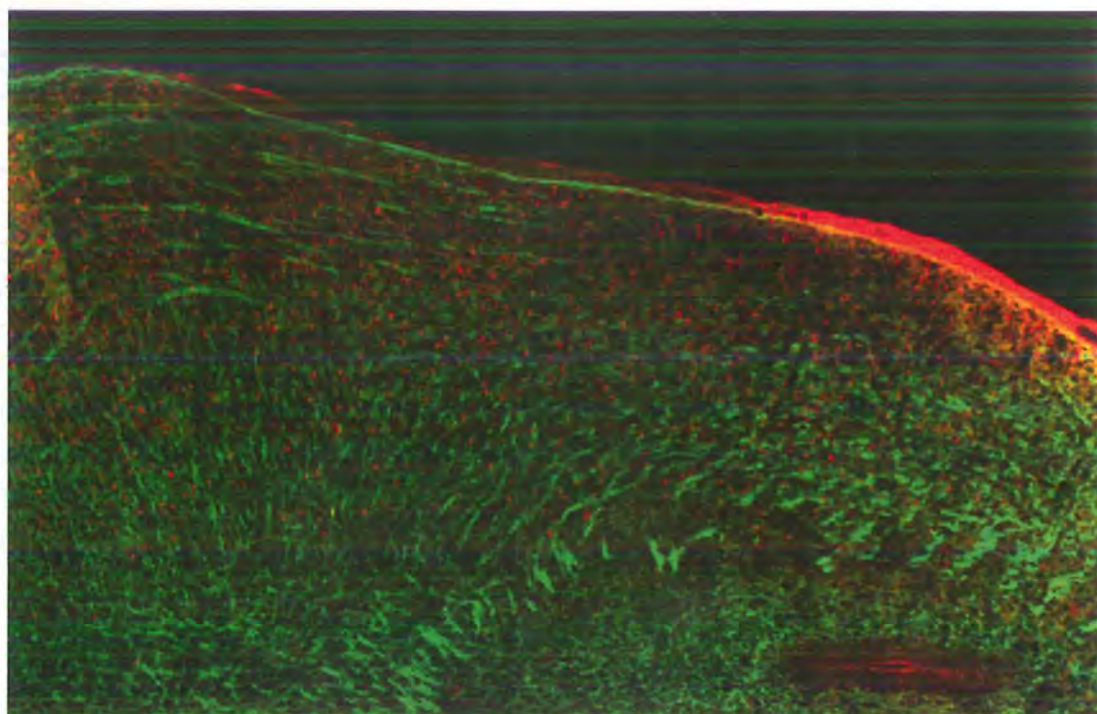
Figure 6.5. *Confocal* z-*section micrograph detailing* βIII tubulin *and* Pax7 *expression in the superior colliculus at E15.5.* *βIII tubulin (green) and Pax7 (red) immunostaining within the mouse superior colliculus at E15.5 (wildtype). Note the more dorsal positioning of the Pax7⁺ cells, and the rostrocaudally aligned axons penetrating through the rostral superior colliculus (left) towards the mid region, with superficial axons close to the pial*

surface penetrating more caudally (right). The neurons of the stratum profundum *are visible in the ventral regions, superior to the ventricle in the bottom right of the image. Scale bar; 150μm.*

When we compared *βIII tubulin* expression in the embryonic superior colliculus of wildtype mice to that of *Pax7* mutant mice we could not discern any differences in expression profiles (Fig 6.4a-b) (n=2-3 at all embryonic stages).

Similar results were obtained at E12.5 for α-internexin (Fig 6.4c-d; n=3), and Doublecortin (Fig 6.4e-f; n=3), early markers of postmitotic (Kaplan *et al.* 1990) and migrating and differentiating (Gleeson *et al.* 1999; Friocourt *et al.* 2003) neurons, respectively. Results confirm that in mutant mice (n=3 each) at E12.5, neuronal differentiation occurs normally within the intermediate zone close to the pial surface in a rostral[high] to mid-caudal[low] manner.

During all embryonic stages investigated, we could not detect any difference in the cellular distribution of NeuN[+] within the superior colliculus of *Pax7* mutant mice relative to that of wildtype mice (n=2-3). The tectum of wildtype mice at E12.5 contains a small number of postmitotic, NeuN^{+} cells within the intermediate zone, distributed in a rostral[high] to caudal[low] manner (Fig 6.6a-b). At all other embryonic stages examined, NeuN^{+} cells are distributed throughout the superior colliculus from ventral to pial surfaces (Fig 6.6c-d).

By contrast, at P5, the most superficial region of the superior colliculus of *Pax7* mutant mice is almost completely devoid of NeuN^{+} cells (Fig 6.6f) indicating dorsal neuronal loss at this important developmental time point. NeuN^{+} cells can be detected throughout the superior colliculus and are in close proximity to the pial surface in wildtype mice (Fig 6.6e) (n=3-6). Interestingly, within the adult mesencephalon (P18.5) *NeuN*, like *Pax7*, shows similar expression in both mutant mice and wildtype mice (n=2-5).
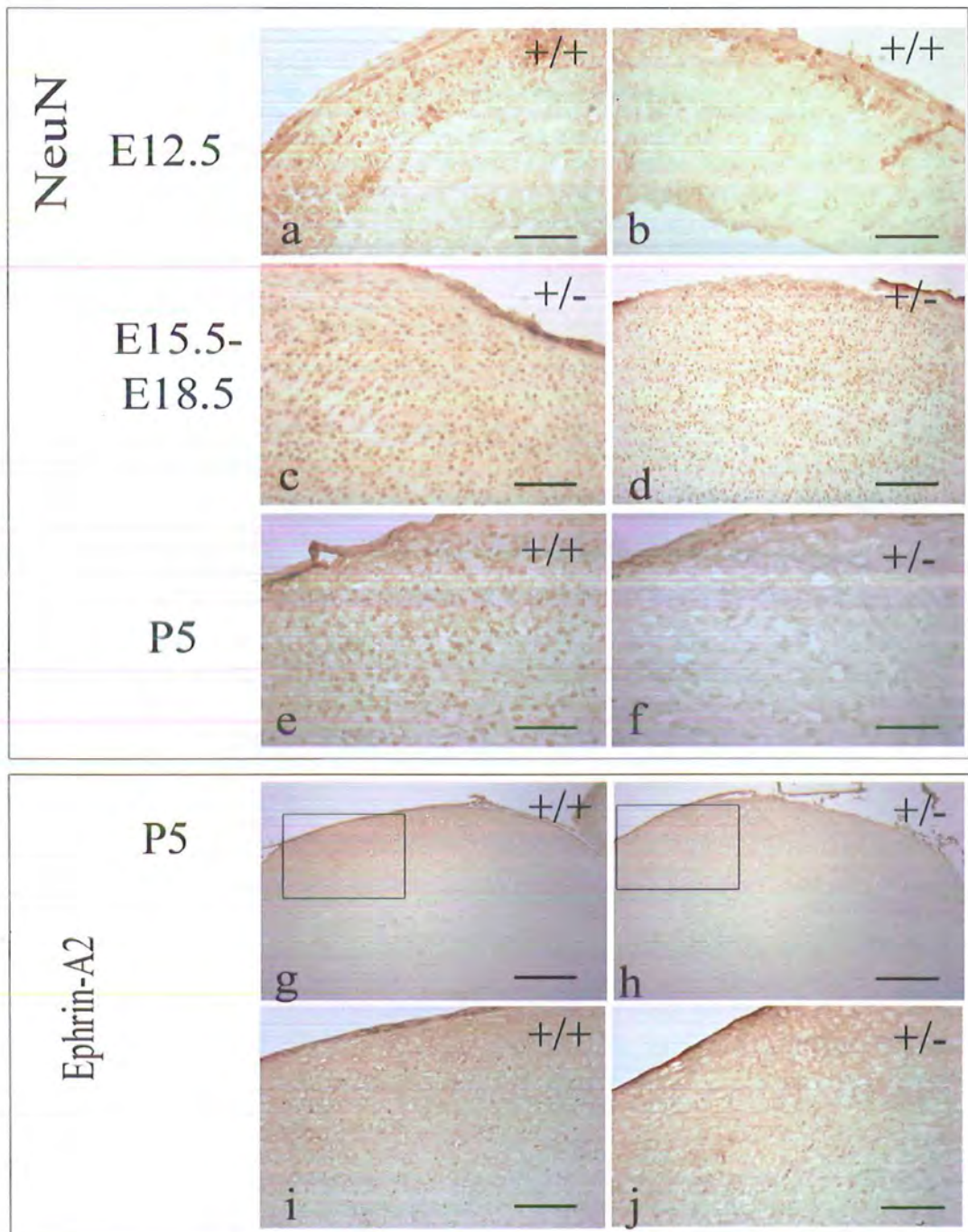
Figure 6.6. *Comparative* **NeuN** *and* **ephrin-A2** *expression profiles in the superior colliculus of* **Pax7** *mutant and wildtype mice.* NeuN *expression demonstrates increased rostral expression (a) compared to caudal expression (b) at E12.5, indicating that rostral maturation precedes that of the caudal region. Figures (c) and (d) illustrate a full complement of NeuN$^+$ cells dorsally at E15.5 and E18.5, respectively, in* Pax7 *mutant mice consistent with wildtype expression (data not shown). Comparison at P5 between wildtype (e) and* Pax7 *mutant mice (f) indicates a loss of neurons dorsally. Likewise, analysis of* ephrin-A2 *expression shows perturbation between wildtype (g,i) and* Pax7 *mutant mice (h,j) in the dorsalmost region. Scale bar: a-c,e-f 100 μm; d,i-j 200 μm; g-h 500 μm.*

6.4.3 Alterations to superior collicular polarity in *Pax7* mutant mice

Loss of Pax7⁺ cells and neurons in the dorsal superior colliculus at P5 (Fig 6.1g-h; Fig 6.2a-e; Fig 6.6e-f), when retinal ganglion cell axonal innervation and arborisation is occurring (Edwards *et al.* 1986b), would be expected to impact on superior collicular polarity and retinotopic mapping. We therefore assessed *ephrin-A2* expression in *Pax7* mutant mice relative to expression in wildtype littermates. Consistent with the altered *Pax7* and *NeuN* profile, at P5 ephrin-A2⁺ cells are missing in the most superficial region (Fig 6.6h,j), in contrast to wildtype expression displaying immunoreactivity close to the pial surface (Fig 6.6g,i), providing further evidence of neuronal loss and changes to polarity.

6.4.4 Neuronal loss is not due to astrocytic cell-fate switching or transdifferentiation

To assess the likelihood of astrocytic cell-fate switching or transdifferentiation, we analysed GFAP expression in postnatal stages as the majority of gliogenesis occurs within the first postnatal week (DeLong and Sidman 1962). We could not detect co-expression of *Pax7* and *GFAP* at P5 or P18.5 in either wildtype or *Pax7* mutant mice, consistent with previous results determined in the chick tectum (Thomas *et al.* 2007). GFAP⁺ processes can be seen extending from cells located at the pial surface, however the dorsal regions of the superior colliculus in *Pax7* mutant mice are similar to wildtype mice and are not populated by GFAP⁺ cells within the region of perturbation at P5 or P18.5 (Fig 6.7a-d), indicating that cell fate switching or transdifferentiation to the astrocytic lineage is not responsible for the reduced dorsal expression of *Pax7*, *NeuN* and *ephrin-A2* at this stage (n=3-4).
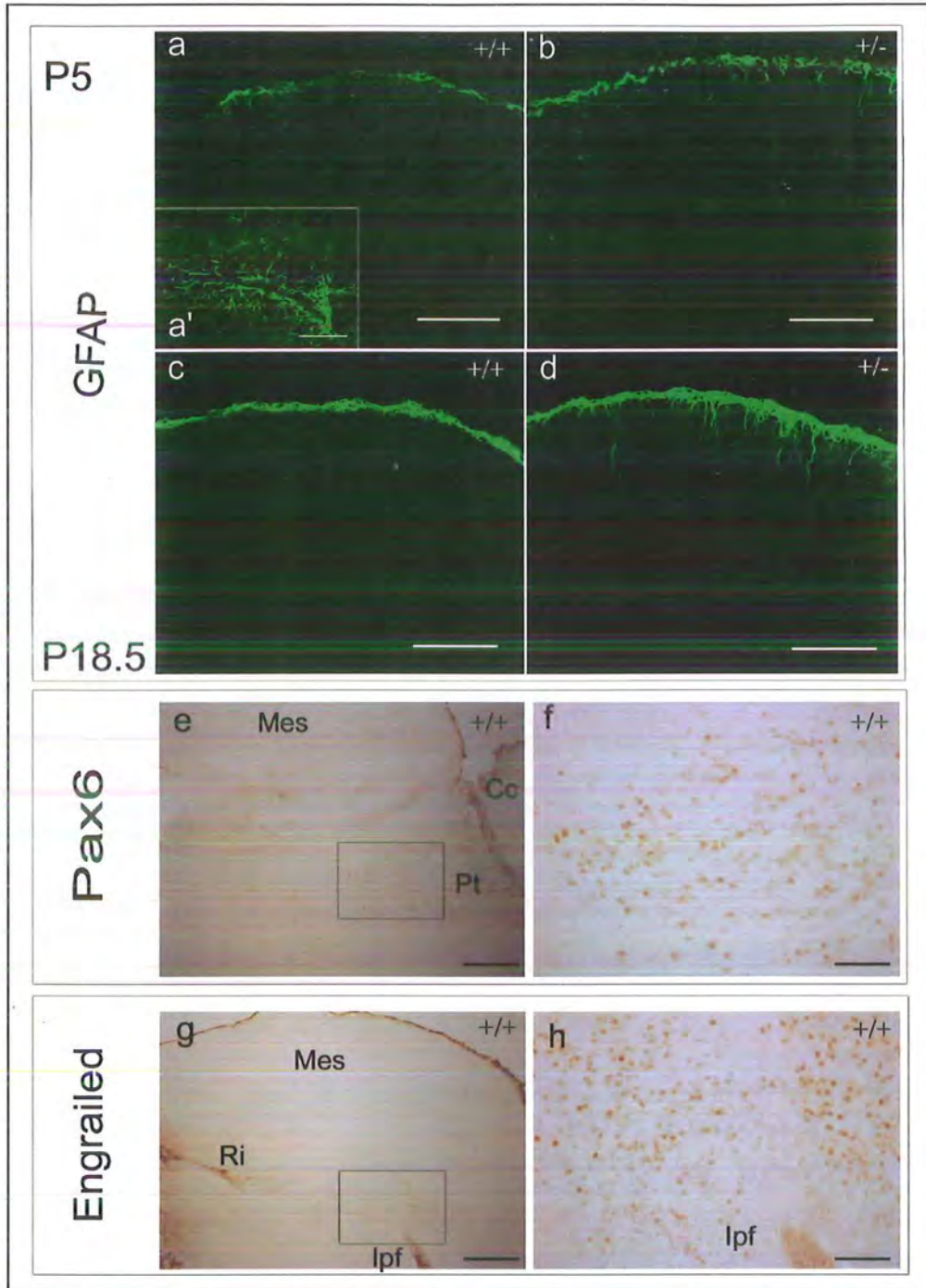
Figure 6.7. **Comparable astrocytic profile in the dorsal superior colliculus of wildtype and Pax7 mutant mice.** *Immunofluorescent detection of GFAP indicates a normal astrocytic profile at P5 (a-b) and P18.5 (c-d) for wildtype (a,c) and Pax7 mutant mice (b,d). (a') Positive control indicating GFAP expression in astrocytes of the myelencephalon. GFAP⁺ processes extend from cells located at the pial surface, however the dorsal half of the superior colliculus in Pax7⁻ᐟ⁻ mice, like that of wildtype, is not populated by GFAP⁺ cells. Therefore, cell fate switching and/or transdifferentiation towards the astrocytic lineage does not account for the reduction in Pax7⁺ cells dorsally.* **Immunohistochemical detection of Pax6 (e, f, [inset from e]) and Engrailed (En-1) (g, h [inset from g]) was utilised to examine mesencephalic boundary formation, which appear**

morphologically unaffected in **Pax7** *mutant mice. Abbrev. Cc, cerebral cortex, Ipf, interpeduncular fossa; Mes, mesencephalon; Pt, pretectum; Ri, rhombencephalic isthmus. Scale bar: a-d,f,h 100 μm; e,g 500 μm.*

6.4.5 Neuronal loss may be attributable to cellular regression

To evaluate whether neuronal loss was due to apoptosis, we investigated activated-*Caspase 3* expression from E15.5 to P5, however we did not detect either co-expression with *Pax7* at any stage, or increased numbers of cells in mutant mice relative to wildtype (data not shown). This marker, however, proved difficult to analyse as elevated and widespread immunoreactivity detected at the earlier embryonic stages tested may be due to the emerging role of *Caspase 3* in neuronal differentiation (Fernando *et al.* 2005). We therefore utilised Hoechst as an indicator of pyknotic nuclei, and assessed co-expression with activated-*Caspase 3* as confirmation of the apoptotic status of the cells, but only a few apoptotic cells were detected at any given time point. The identification of apoptotic cells would, however, require knowledge of the exact timeframe in which the neurons were lost, due to rapid neuronal degeneration noted previously (Hughes 1961).

6.4.6 Mesencephalic boundary formation

To explore the effects of perturbed *Pax7* expression on formation of superior collicular boundaries, ie between the diencephalon and mesencephalon and between the superior colliculus (dorsal) and tegmentum (ventral mesencephalon), we assessed *Pax6* expression at the rostral and ventral boundaries of the superior colliculus (Stoykova and Gruss 1994; Kawakami *et al.* 1997; Agarwala *et al.* 2001). At E12.5, there are a few weakly-stained Pax6$^+$ cells superior to the interpeduncular fossa. At E15.5 in wildtype mice, Pax6$^+$ cells can be detected dorsally in the pretectum (Fig 6.7e,f) sometimes spreading to the rostral margin of the ventricular zone. Pax6 immunoreactivity could not be detected within the pretectum at E18.5 in either wildtype or mutant mice. We could not detect any abnormality in the expression domain of *Pax6* in *Pax7* mutant mice at these midgestational stages (n=3).

To investigate alterations to mesencephalic-metencephalic boundary formation, we assessed the expression of the marker *En-1* (Martinez and Alvarado-Mallart 1990) from E15.5 to P18.5. En-1$^+$ cells can be detected at the midbrain-hindbrain boundary, from the interpeduncular fossa to the rhombencephalic isthmus (Fig 6.7g,h) and expression wanes as development proceeds until P18.5, when *En-1* expression in the midbrain is usually restricted to a cluster of cells superior to the interpeduncular fossa. Throughout all stages examined, we could not detect any difference between wildtype and mutant mice (n=2-3).

6.5 Discussion

In order to understand the role of *Pax7* in superior collicular development we investigated the superior colliculus of *Pax7* mutant mice relative to wildtype at a variety of developmental stages. Whilst both *Pax3* and *Pax7* are known to define the early tectum subsequent to mesencephalic determination, and the ability to form ectopic tectum subsequent to misexpression within the diencephalon and ventral mesencephalon testifies to their critical nature in specification of tectal identity (Matsunaga *et al.* 2001), individual roles during further development have remained elusive. Here we show that loss of Pax7 dramatically alters *Pax3* expression domains and disturbs the neuronal profile in the dorsal layers of the developing superior colliculus.

6.5.1 *Pax7* and *Pax3* have separate roles during neuronal differentiation in the superior colliculus

Since *Pax7* and *Pax3* have similar expression domains in the developing superior colliculus at mid-gestation (Stoykova and Gruss 1994), we undertook a thorough, comparative analysis of their expression patterns in wildtype and mutant mice. It was anticipated that this analysis would highlight any changes caused by loss of Pax7 and shed light on their individual roles during superior collicular development.

Results indicate that as the superior colliculus emerges and the dorsal laminae are formed by cellular migration and differentiation, *Pax7* expression becomes progressively more

refined towards the upper, developing strata whereas *Pax3* expression becomes increasingly restricted towards the proliferative region (Fig 6.8). At E12.5, *Pax7*- and *Pax3*- expressing cells overlap in the ventricular and subventricular zones, but not in the emerging, differentiating intermediate zone.

At E15.5 tectal *Pax7* expression within the ventricular zone decreases, and is no longer evident at E18.5, with expression persisting within the upper developing primary layers (*strata profundum, intermedium and superficiale*). However, *Pax3* expression becomes restricted to the ventricular zone, with a few scattered cells within the intermediate region at E15.5 decreasing at E18.5. Postnatally, expression of *Pax7* is detected throughout the superior colliculus, numerically declining with maturation yet persisting at all developmental stages examined, whilst *Pax3* expression is extinguished.

The divergent *Pax3/Pax7* expression profiles, as demonstrated in this paper, suggest a dichotomy of function with respect to neuronal differentiation. The *Pax7* expression profile, without co-expression of *Pax3*, appears spatially associated with neuronal differentiation and maturation of the superior colliculus, whilst *Pax3* expression appears to be negatively associated with these characteristics. A role for *Pax3* in maintaining the undifferentiated phenotype has been demonstrated previously, both *in vitro* and *in vivo* for neuronal cells (Reeves *et al.* 1998; Reeves *et al.* 1999), for Schwann cells (Kioussi *et al.* 1995) and for melanoblasts (Galibert *et al.* 1999; Lang *et al.* 2005). Conversely, transfection of *Pax7* into P19 mouse embryonal cells *in vitro* directs these cells along a neuronal pathway (Ziman *et al.* 2001b). This dichotomy may, therefore, represent an important functional divergence between paralogues which may have implications for future stem cell therapies designed to treat midbrain disorders.
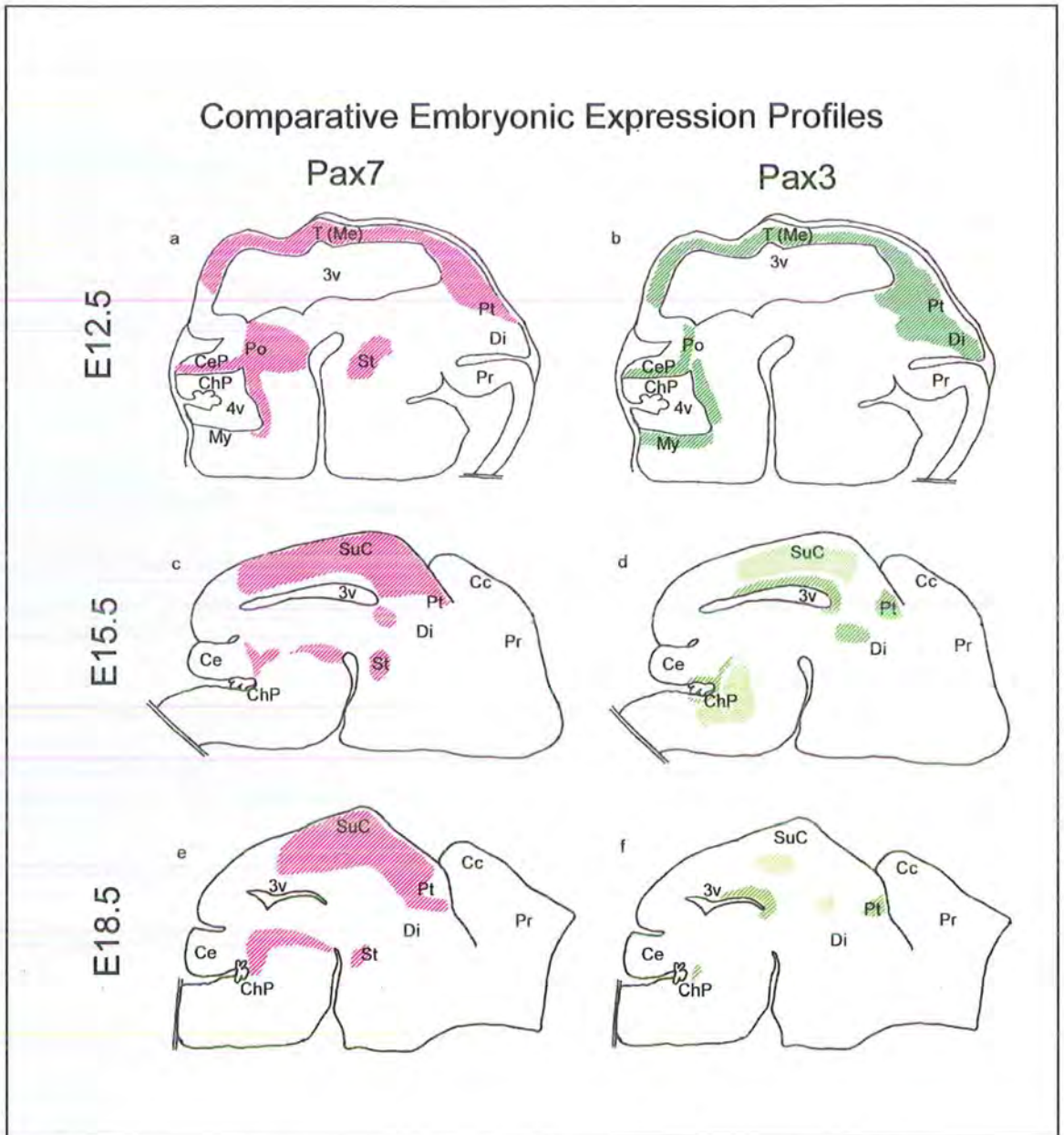
Figure 6.8. *Schematic illustration of divergent* Pax7 *and* Pax3 *expression profiles in the embryonic mouse superior colliculus.* *Divergent* Pax7 *(a,c,e) and* Pax3 *(b,d,f) embryonic expression profiles in wildtype mice at E12.5 (a-b), E15.5 (c-d) and E18.5 (e-f). The lighter pattern for Pax7 and Pax3 indicates low levels of expression compared to other regions (such as developing upper strata or ventricular zone, respectively) showing darker staining. Abbrev. Cc, cerebral cortex; Ce, cerebellum; CeP, cerebellar primordium; ChP, choroid plexus; Di, diencephalon; My, myelencephalon; Po, pons; Pr, prosencephalon; Pt, pretectum; St, subthalamus; SuC, superior colliculus; T (Me), tectum (mesencephalon); 3v, 3rd ventricle; 4v, 4th ventricle. Illustrations are not to scale.*

To investigate this further, we performed a comparative assessment of *βIII tubulin, α-internexin, Doublecortin* and *NeuN* expression at E12.5, just prior to the normal birthdate of the superficially located neurons. At this timepoint, the intermediate zone appears as an expanded region rostrally (which varies slightly at different positions across the mediolateral axis) diminishing to a thin region at the pial surface mid-caudally, indicating a rostral to caudal maturity, and is characterised by the expression of *Pax7, βIII tubulin* (somatic staining), *α-internexin, Doublecortin* and *NeuN*, and morphologically by increased internuclear distances. By contrast, *Pax3* expression cannot be detected within the intermediate zone. We could not discern any evidence of precocious neuronogenesis at this time, a function previously demonstrated for *Pax6* within the eye of *Sey* mutant mice (Philips *et al.* 2005). However, a more thorough investigation at closer temporal increments from E13 would be required to thoroughly preclude this possibility, as precocious or more rapid neuronogenesis may occur between the time frames investigated in this study. Furthermore, the perturbation to *Pax3* expression within the ventricular zone at the later embryonic stages investigated, suggests that the early processes of specification and/or migration may be accelerated somewhat with insufficient Pax7 levels. The unaltered proliferation/specification of tectal cells does not preclude a role for *Pax7* in these processes *per se*, but may indicate compensation via *Pax3*.

The observed perturbations in *Pax3* expression in *Pax7* mutant mice may indicate that *Pax7* acts to limit the expression domain of *Pax3*. This is supported by the rostral, ventral and caudal expansion of Pax3⁺ cells in the presence of reduced or absent *Pax7* expression. These regions of expanded expression occur where *Pax7* expression would normally be encountered, and suggests that *Pax7* acts to dorsalise (or at least positionally constrain) *Pax3* expression. A cell autonomous relationship between *Pax7* and *Pax3* has been determined whereby misexpression of one paralogue represses expression of the other, and a balancing mechanism may exist to produce correct total expression levels (Matsunaga *et al.* 2001). The exact mechanism behind this relationship is ambiguous, however it may be significant that the expression of *Pax7* precedes that of *Pax3* within the headfold and primitive fold ectoderm in the developing chick (Otto *et al.* 2006). Moreover, it is clear that this relationship is also dosage-sensitive, as perturbations occur in the haploinsufficient state.

6.5.2 Role for *Pax7* in timing of neuronal specification

Expression analysis and cellular quantification have demonstrated that *Pax7* haploinsufficiency does not alter the initial proliferation steps in Pax7^{+} cells during mid embryonic stages. The only notable disparity to *Pax7* expression occurs at P5, where a subpopulation of Pax7^{+} cells, situated in the dorsal half of the superior colliculus, is no longer detectable, presenting with an obvious margin lacking Pax7^{+} cells superficially. Sagittal and coronal sections have identified that this perturbation occurs across the dorsal surface of the entire rostrocaudal axis and most of the mediolateral axis.

To understand the significance of this anomaly we reviewed previous studies, which indicate that the majority of superior collicular neurons are born throughout the period E11 to E13 (DeLong and Sidman 1962; Edwards *et al.* 1986a) and distinct temporal and spatial migration patterns culminate in dictation of the correct cytoarchitecture of the developing superior colliculus (Fig 6.9). Cells generated at E11 initially migrate to populate the superior colliculus, and are subsequently divided dorsoventrally by cells generated at E13 (Edwards *et al.* 1986a). The cells that eventually reside within the most superficial region of the superior colliculus undergo their final postmitotic division at E13 while in the ventricular/subventricular zones, and migrate to subdivide their predecessors in the intermediate zone at E15. Thereafter, a subpopulation assume their final position superficially at E17 (Edwards *et al.* 1986a) which coincides with retinal ganglion cell innervation of both superficial and intermediate zones (Beckstead and Frankfurter 1983; Edwards *et al.* 1986b). The *stratum superficiale* thus contains cells generated at both E11 and E13, with the latter cells being more superficially placed In contrast, the ventral regions of the superior colliculus, associated with auditory and motor responses, undergo earlier development than the superficial regions and are thus populated by earlier-born neurons (Edwards 1980; Stein 1984; Edwards *et al.* 1986a). This temporal specification of cells is similarly observed in the chick tectum, where two populations of cells are generated, each with distinct laminar fates, with later born neurons expressing *Pax7* fated to reside in the more dorsal laminae (Fedtsova *et al.* 2008). It is therefore evident that the timely migration of cells from the ventricular zone contributes to the correct placement of cells within the superficial laminae, and this temporal-based specification of neurons

ensures that placement of cells within laminae coincides with their maturation and initiation of circuitry.

Region	Cells Generated	Cells Migrated
SS	E13	E17
	E11	E15
SI	E13	E15
SP	E11	E15

Note: The figure area also contains a diagram with axes labeled SS (Dorsal), SI, SP (Ventral) along the right, and E11, E13 along the left with curved arrows.
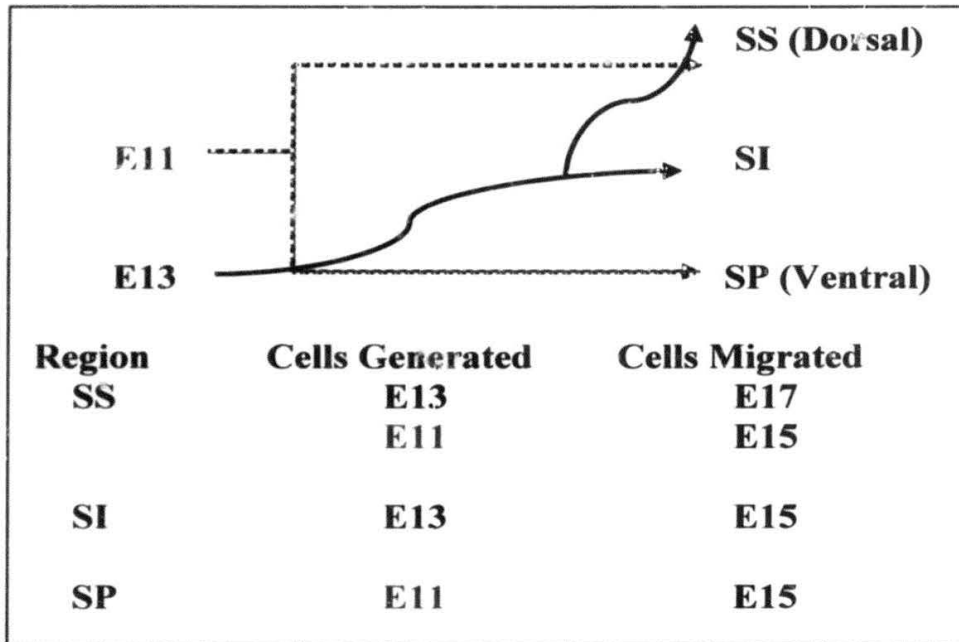
Figure 6.9. *A diagrammatic representation of the primary layers of the developing superior colliculus. The laminae of the developing superior colliculus contain two populations of cells that populate the upper (SS), mid (SI) and lower (SP) regions. At E15, cells generated at E11 populate the SS and SP, whereas cells generated at E13 intersect these regions to populate the SI, with a subsequent migration of a subpopulation upwards to the SS by E17. Thus, the SS consists of two different populations of cells, with the most recently generated cells residing in the more superficial regions. (Adapted from Edwards et al. 1986a). Abbrev. SS, strata superficiale; SI, intermedium; SP, profundum. "Cells Migrated" column relates to the developmental timepoint when the cells have migrated to their final destination.*

Overall, our results tend to suggest that *Pax7* may partially contribute to the correct timing of neuronogenesis by regulating migration of Pax3^{+} cells from the ventricular zone into the mid-collicular region in a timely and orderly fashion, a characteristic that is lost in *Pax7^{-/-}* mice between E15.5 and E18.5, at the time when wildtype cells destined to reside in the more superficial strata are migrating and assuming their final destination.

6.5.3 A putative anti-apoptotic role for *Pax7* in postnatal superior collicular development

As earlier quantification did not detect any numerical changes in the *Pax7* cellular profile in *Pax7$^{+/-}$* mice, it became apparent that these cells had either altered their differentiation profile or had regressed within the first postnatal week. We assessed *GFAP* expression postnatally and as there was no change in the cellular distribution of GFAP$^+$ cells in *Pax7* mutant mice we excluded cell fate switching or transdifferentiation to the astrocytic lineage as a causative factor for the reduced number of Pax7$^+$ cells dorsally. We were, however, unable to detect increased apoptosis within the superior colliculus using activated-*Caspase 3*, cresyl violet or Hoechst staining. A more detailed examination commencing from birth would be required to determine the exact time interval when perturbation occurs in order to conclusively demonstrate apoptotic mechanisms. Apoptotic labelling methods such as TUNEL or caspase 3, concomitant with Pax7 immunolabelling, would be required to address this issue, however rapid neuronal degeneration coupled with a progressive rostrocaudal maturation to the tissue which is likely to result in a punctuated apoptotic phenomenon, will likely impede the quantitative nature of the analysis required to differentiate between normal (wildtype) and increased (mutant) apoptosis. However, the loss of neurons evidenced in *Pax7* mutant mice indicates a loss of cells, rather than simply extinction of *Pax7* expression.

Relating phenotype to the specification of cells during superior collicular development (Edwards *et al.* 1986a) suggests that the second population of neurons, generated at E13, may be less capable of long-term survival than their earlier-produced counterparts, and as such are sensitive to gene dosage (Fig 6.10). Whilst the differential placement of late-born neurons identified in previous studies (Edwards *et al.* 1986a; Fedtsova *et al.* 2008) coincides spatially with the perturbed neuronal profile of *Pax7* mutant mice, further experimentation is required to distinguish a link between the generation of subpopulations of cells and their relationship with *Pax7* and postnatal cytoarchitecture. Furthermore, differential sensitivity to neuron death has been suggested for different functional populations of neurons within the superior colliculus (Giordano *et al.* 1980). Anti-apoptotic roles for *Pax* genes are well-documented to-date (Bernasconi *et al.* 1996;

Muratovska *et al.* 2003; Zhang *et al.* 2004; Park *et al.* 2006; Yi and Sommer 2007) and, importantly, the ciliary neurotrophic factor receptor has recently been demonstrated as a downstream target of Pax7 within the mouse embryo (White and Ziman 2008).
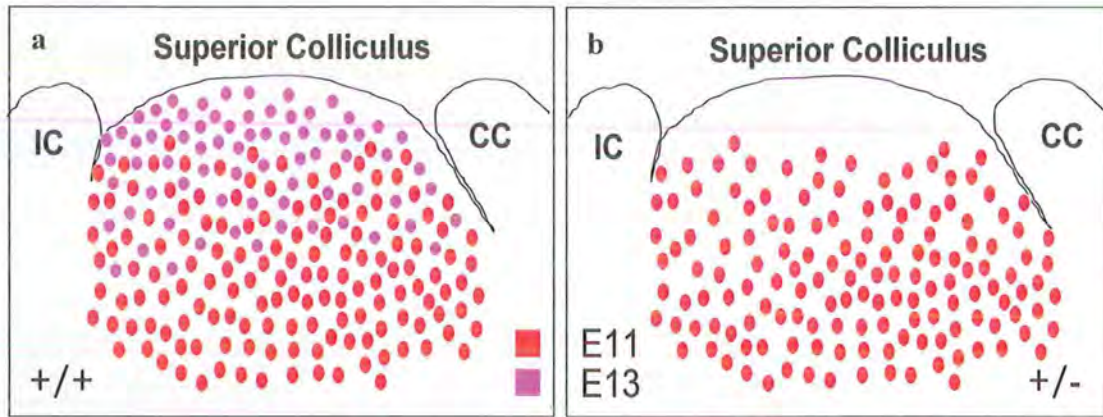
Figure 6.10. *A diagram detailing differential placement of separately generated populations of Pax7$^+$ cells at P5. A diagram of Pax7$^+$ cells in the superior colliculus of wildtype (a) and* Pax7 *mutant (b) mice at P5. Cells generated at E11 (red) and E13 (purple) show overlapping (medial) and unique (ventral [E11]/dorsal [E13]) expression patterns (a). This thesis proposes that cells generated at E13 show reduced capacity for long term maintenance in* Pax7 *mutant mice (b), generating a phenotype whereby a superficial region is absent of Pax7$^+$ cells, and the region immediately ventral to this exhibits a reduction in the number of Pax7$^+$ cells (refer Fig 2e). Abbrev. CC, cerebral cortex; IC, inferior colliculus.*

6.5.4 Apparent neuronal recovery at P18.5

We have demonstrated altered Pax7$^+$ and neuronal profiles in *Pax7* mutant mice at P5, however at P18.5 we can no longer detect these variations between *Pax7* mutant and wildtype mice. Whilst it is feasible that the similar number of Pax7$^+$ cells in *Pax7$^{+/-}$* mice relative to wildtype at P18.5 occurs because Pax7$^+$ cells in the adult do not normally include the subpopulation of cells which are missing at P5 in *Pax7* mutant mice, the apparent recovery of the neuronal profile by P18.5 has proven enigmatic. We have drawn from established studies of CNS and superior collicular development to address this issue.

It is clear that early postnatal stages of superior collicular development are tumultuous. The colliculus becomes invaded by retinal afferents just prior to birth and this continues within the first postnatal week, with formation of collaterals giving rise to axonal

arborisation and commencement of synaptogenesis (Godement *et al.* 1984; Edwards *et al.* 1986b; Sachs *et al.* 1986). During this process, a dramatic transformation occurs in which almost half of all fibre bundles are removed from the upper half of the *stratum superficiale* (the region including the perturbation where Pax7$^+$ cells are essentially absent) (Edwards *et al.* 1986b; Sachs *et al.* 1986). This process coincides with naturally occurring neuron death in the superior colliculus (Giordano *et al.* 1980; Finlay *et al.* 1982), indicating neuronal formation in excess of adult requirements. Excessive neuron formation is a recurrent theme in CNS development (Hughes 1961; Prestige 1965; Cowan 1971). Therefore it is likely that excessive neuron formation masks the loss of the more susceptible, Pax7-deficient neurons, with sufficient cells remaining to satisfy functional requirements. Subsequently, displacement of cells due to fibre invasion and the ensuring maturation of the tissue could possibly account for the apparent phenotypic equivalence of NeuN$^+$ cells noted at P18.5. It should be noted that functional studies have not been performed on *Pax7* mutant mice to-date to assess visual acuity.

6.6 Further studies

A possible cause for the loss of superficial neurons in early postnatal stages could be altered circuitry. Neurons of the superficial superior colliculus communicate with the deeper collicular regions through superficial neuronal axons (Valverde 1973; Mooney *et al.* 1988), or via dendrites from neurons of the deep layers (Mooney *et al.* 1984) which in turn contain a variety of efferent projections from regions such as the cortex, retina, zona incerta (region surrounding subthalamus) (Stein 1978; Berson and McIlwain 1982; Beckstead and Frankfurter 1983; Kolmac *et al.* 1998) and indirectly from the subthalamus via the substantia nigra pars reticulata or enteropeduncular nucleus (Benazzouz *et al.* 1995; Benazzouz *et al.* 2000; Bressand *et al.* 2002). Taking this into consideration, an interesting finding from this research is the persistent *Pax7$^+$/Pax3$^-$* expression profile of the subthalamus throughout all timepoints examined. Providing *Pax3* is not expressed in this region at an earlier developmental stage than examined in this study, the subthalamus may represent a novel opportunity to further dissect the functional repertoire of *Pax7* from that of *Pax3*. Additional work would therefore be required to characterize the subthalamus of *Pax7* mutant mice to address this issue, as this important brain region is a target of deep

brain stimulation to treat disorders such as Parkinson's Disease and epilepsy (Bressand *et al.* 2002; Fawcett *et al.* 2005).

Further studies on the role of *Pax7* in superior collicular development would benefit from investigation into the axonal projection and circuitry of Pax7^{+} cells, which may be elaborated by the generation of *Pax7 tau*-GFP knockin X *Pax7* mutant mice. Cell lineage tracing experiments in *Pax7* mutant mice would be required to trace the progression of cells generated at E11 and E13 to conclusively resolve the questions related to the early postnatal perturbation and the apparent neuronal recovery in the adult. Moreover, functional studies assessing the ability of *Pax7* mutant mice to evoke a co-ordinated response to stimuli will be required to assess the integrity of the mature superior colliculus.

6.7 Conclusion

In summary *Pax7*, while not required for neuronogenesis or neuronal differentiation at early stages of superior collicular development, is absolutely requisite in a dosage-dependent manner for long term maintenance of a subpopulation of dorsal mesencephalic neurons. This characteristic may well impart prophylactic properties to stem cells utilised in future replacement therapies to enhance treatment of neurodegenerative diseases of the midbrain. Furthermore, comparative expression analyses indicate a functionally divergent role for *Pax7* and *Pax3* during neuronal differentiation within the superior colliculus.

6.8 Authors' contributions

AM initially generated and currently maintains the *Pax7* mutant mouse line, and critically reviewed the manuscript. AZ performed handling, genotyping, perfusion and initial processing of *Pax7* mutant mice, and revision of the manuscript. JT participated in the design of the study and further processed the tissue, performing all subsequent experimentation and analysis, including drafting of the manuscript. MZ participated in the design and coordination of the study and editing of the manuscript. All authors read and approved the final manuscript.

6.9 Acknowledgments

CHAPTER 7

DISCUSSION

"I have a conviction that it's only when you are put at full stretch that you can realize your full potential."

Sir Edward "Weary" Dunlop, 1907-1993.

CHAPTER 7: DISCUSSION

The primary objective of the research undertaken for this project was to decipher the role of *Pax7* during formation of the murine superior colliculus. Several animal models were used in this study to explore the ability of *Pax7* to determine superior collicular traits, specifically regional identity, boundary formation, cellular specification and polarity.

Pax7 mutant mice display normal formation of the superior colliculus with boundary formation and cellular specification as per wildtype. Expanded mesencephalic territory (resulting from a shift in the placement of the diencephalic-mesencephalic boundary) noted in *Pax6* mutant mice cannot be attributed to a role for *Pax7* in boundary formation, as *Pax6* (causal in this regard) is a known contributor to formation of this boundary (Mastick *et al.* 1997; Schwarz *et al.* 1999). The results presented in this thesis demonstrate that altered *Pax7* levels do not alter superior collicular boundary formation in *Pax7* mutant mice, and concurs with the currently accepted paradigm that *Pax7* participates in mesencephalic development subsequent to boundary formation (reviewed in Nakamura and Watanabe 2005). However, it still remains possible that *Pax7* plays a role in diencephalic-mesencephalic boundary formation in mutant animals (eg where the isthmus is ablated) by specifying mesencephalic identity (perhaps by participation in the feedback loop), as this boundary still forms when other factors known to be involved in diencephalic-mesencephalic boundary specification are absent (Brand *et al.* 1996; Pfeffer *et al.* 1998; Scholpp and Brand 2003; Scholpp *et al.* 2003).

The role of *Pax7* during cellular specification within the developing superior colliculus of *Pax7* mutant and wildtype mice was examined and results indicate that *Pax7* is expressed throughout cellular specification within the ventricular regions. Analysis during mid-late embryogenesis reveals that formation of Pax7$^+$ cells proceeds normally in *Pax7*$^{+/-}$ mice relative to wildtype, and is therefore not sensitive to gene dosage. However, the similar spatiotemporal expression of *Pax3* during neuronogenesis may functionally compensate during cellular specification processes.

Quantification of *Pax7* expression within the superior colliculus of wildtype mice revealed graded expression of *Pax7* at all developmental stages examined, from late embryogenesis

not yet been conducted in *Pax7* mutant mice to assess visual acuity or the ability to evoke a coordinated response to visual stimuli. Whilst it appears superficially that in adult *Pax7* mutant mice the superior colliculus overcomes the loss of neurons observed during the initial mapping stages, functional studies are required to test the veracity of the mature superior colliculus to assess whether the apparent conservation in neuron maturation has produced a brain region which has functionally recovered.

Any investigation into the role of *Pax7* in superior collicular development would not be complete without a concomitant examination of *Pax3* expression. Individual roles for *Pax3* and *Pax7* have been an unresolved area of research which has proven enigmatic to date. Previous research indicated overlapping expression domains (Stoykova and Gruss 1994), at the final stages of neuronogenesis within the mesencephalic ventricular zone (DeLong and Sidman 1962; Edwards *et al.* 1986a). Here we show that *Pax3* and *Pax7* expression profiles diverge between mid to late stages of embryogenesis coinciding temporally and spatially with neuronal differentiation and maturation of the superior colliculus. It appears that this spatial divergence in expression culminates in reduced maintenance of a subpopulation of dorsal mesencephalic neurons in *Pax7* mutant mice, possibly because of an inability of *Pax3* to functionally compensate. Alternately, it may be that *Pax3* does not render a protective effect on superior collicular cells, as it has been shown to be deficient in this regard in skeletal muscle satellite cell cultures, where it cannot replace the anti-apoptotic function of *Pax7* (Relaix *et al.* 2006). *Pax7* may, therefore, act to divert the cells from apoptotic pathways that occur after excessive neuron formation. Nevertheless, it indicates a role for *Pax7* in maintenance of a subpopulation of dorsal superior collicular neurons. When this is considered together with evidence that $Pax7^+$ cells respond to retinal input (this thesis), it suggests that the upregulated, axon-mediated *Pax7* response may act to preserve the correctly innervated target cells from the normal process of apoptosis designed to cull excess neurons, thereby reinforcing correct axonal mapping and circuit formation.

Our observation of altered embryonic expression of *Pax3* in *Pax7* mutant mice, in both the mesencephalon and rhombencephalon, indicates that cross-talk between paralogues occurs for correct specification of brain regions, and indicates for the first time that abherrant superior collicular neuronal patterning occurs in *Pax7* mutant mice. The exact nature of this relationship has not been resolved, but it appears that *Pax7* partially constrains the

Pax3 expression domain in specific areas. Cross-talk between *Pax7* and *Pax3* has previously been identified in a recent investigation identifying upregulated *Pax7* levels in the developing neural tube of *Pax3* hypomorphic mice (Zhou *et al.* 2008) but **reduced** *Pax7* expression (whole embryo) in *Pax3* **null** mice, indicating that this cross-talk is differentially affected by gene dosage. Further investigation into *Pax7* expression in *Pax3* mutant mice might resolve this issue, however, investigation into *Splotch (Pax3⁻/⁻)* mutant mice is limited to early embryonic development (E14) prior to embryonic lethality resulting from absence of Pax3 (Auerbach 1954).

Any future attempt to unravel individual functions for *Pax7* and *Pax3* during superior collicular development would require conditional knockout models, activated at key developmental timepoints to tease out functional divergences. For instance, *Pax3* and/or *Pax7* conditional knockouts, activated after neural tube closure and prior to neuronal differentiation could be of benefit in understanding their respective roles in cellular specification and migration within the superior colliculus. This may also be effectively achieved with the use of siRNA via a lentiviral delivery system. It would be interesting to discover whether superior collicular proliferation and/or neuronogenesis proceeds in the absence of both genes subsequent to correct neural tube formation.

A substantial body of work is emerging that identifies the importance of *Pax6* levels (in a dosage-dependent manner) for controlling the timing of processes such as progenitor cell proliferation, cell cycle progression, differentiation and neurogenesis in cortical development (Estivill-Torrus *et al.* 2002; Berger *et al.* 2007; Quinn *et al.* 2007). Thus it appears that *Pax* expression may serve to regulate the timing of cellular differentiation, migration and proliferation. In agreement with this suggested role, the altered temporal migration of Pax3⁺ cells from the ventricular zone of *Pax7* mutant mice infers roles for these *Pax* genes in dictating temporal patterns of neuron specification and/or differentiation within the mesencephalon. Conditional knockout mice would also allow exploration into the different functions of *Pax3* and *Pax7* with respect to delamination and migration.

Delamination of cells exiting the ventricular zone and correct migration of postmitotic cells within the tissue is required for normal development (Cowan 1971; Edwards *et al.* 1986a). Once migrating cells have reached their destination in the superior colliculus, they reside in

the appropriate lamina based on their cellular characteristics, which in turn dictate retinal topography (Yamagata et al. 1995; Yamagata et al. 2006). *Pax3* has been shown to be required for delamination processes during limb formation (Franz and Kothary 1993; Bober et al. 1994; Goulding et al. 1994). In contrast, the mature superior colliculus, which contains Pax7$^+$ but not Pax3$^+$ cells, is characterised by its laminated structure.

Additionally, identification of non-overlapping regions of *Pax7* and *Pax3* expression is critical to understanding differences in functional capacities without the confounding influences of functional redundancy. This research identified the subthalamus of the ventral diencephalon as *Pax7* positive/*Pax3* negative at all stages examined. We cannot preclude that *Pax3* may be expressed within this region at a timepoint earlier than those examined within this project, therefore an in-depth investigation of subthalamic development in wildtype and *Pax7* mutant mice would be recommended to directly target this research question. The popular nature of the subthalamus as a target for deep brain stimulation in the treatment of diseases such as Parkinson's and epilepsy (Bressand et al. 2002; Fawcett et al. 2005) warrants conduction of such a project.

As the subthalamus indirectly influences the superior colliculus, via the *substantia nigra pars reticulata,* (Benazzouz et al. 1995; Benazzouz et al. 2000; Bressand et al. 2002), an investigation into their relationship in both wildtype and *Pax7* mutant mice may shed light on the cause of neuronal regression detected in *Pax7* mutant mice. It would be prudent to take into consideration that the cause of premature death observed in *Pax7$^{-/-}$* mice, which generally coincides with the time of weaning (Mansouri et al. 1996b) and eye opening (Edwards et al. 1986b), has not been resolved to-date. An investigation into the circuitry of Pax7$^+$ cells would require generation of mice in which projections of Pax7$^+$ cells are labeled to assess their wildtype projection, augmented with an investigation of the same in *Pax7*-labelled X *Pax7* mutant mice to assess changes in circuitry, and to generate a more holistic understanding of the role of *Pax7* in development of the superior colliculus and other brain regions.

CHAPTER 8

CONCLUSION

"Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life."

Marcus Aurelius, 121-180.

CHAPTER 8: CONCLUSION

In summary, this research has demonstrated that *Pax7* is requisite, in a dosage-dependent manner, for the maintenance of a subpopulation of dorsal mesencephalic neurons. The maintenance of dorsal neurons, together with graded expression rostrocaudally and dorsoventrally, indicates a role for *Pax7* in polarization of the superior colliculus and retinocollicular mapping. Similar alteration to graded expression of *Pax7* and *ephrin-A2* in *Pax6* and *Pax7* mutant mice confirm this. Furthermore, heightened *Pax7* expression levels in wildtype mice during retinal innervation together with reduced Pax7 levels subsequent to optic nerve hypoplasia (*Pax6* mutant mice) demonstrates a response of Pax7$^+$ cells to retinal innervation. Taken together, our results suggest that *Pax7* expression affords protection to the innervated cells of the superior colliculus as RGC axon collaterals are forming, major refinements to mapping are occurring and synaptogenesis is commencing (Godement *et al.* 1984; Edwards *et al.* 1986a; Sachs *et al.* 1986) Anti-apoptotic roles for *Pax* genes have previously been established (Bernasconi *et al.* 1996; Cai *et al.* 2005; Relaix *et al.* 2006) and are a well-accepted feature of this key family of developmental regulators. However, further work is required to decipher the exact nature of protection afforded to the dorsal cells by *Pax7* expression. Our results also suggest a role for these genes in temporal regulation of cell proliferation, differentiation and migration.

FUTURE DIRECTIONS

This study assessed for the first time the possibility of precocious neuronogenesis due to *Pax7* insufficiency. The rationale for this investigation came from the observation that *Pax6* regulates timing of neuronogenesis within the eye (Mastick *et al.* 1997) and the cerebral cortex (Estivill-Torrus *et al.* 2002; Berger *et al.* 2007; Quinn *et al.* 2007). This investigation was, however, limited to a specific subset of developmental stages. It is recommended that a more thorough investigation at closer temporal increments from E10 to E13 be conducted to completely exclude the possibility that perturbation to the timing or speed of neuronogenesis may be masked by the time frames investigated in this study. Our demonstration of *Pax3* perturbation within the ventricular zone at later embryonic stages validates the theory that alteration to early processes of specification and/or migration may occur due to insufficient *Pax7* levels.

The significance of altered *Pax3* expression within the ventricular zone may be important, as *Pax3* appears to be associated with undifferentiated cells, whereas *Pax7* (in the absence of *Pax3*) appears associated with differentiation of emerging neurons. If this difference represents a functional variation between paralogues this may have important implications for future stem cell therapies. The role of *Pax7* in this regard may be associated with maintaining naïve neuronal cells in a suspended state, allowing for a temporal discrepancy between initial differentiation of the cells and receipt of axonal input, perhaps by providing neurotrophic support. In support of this, the ciliary neurotrophic factor receptor has recently been revealed as a downstream target of Pax7 (White and Ziman 2008).

The observations reported in this thesis have led us to form an inference as to the possible roles of *Pax7* and *Pax3* during neuronal proliferation, specification and maturation. During early stages of superior collicular development, *Pax3* may regulate the "switch" from proliferation towards differentiation, hence this process appears undisturbed in *Pax7* mutant mice. Should *Pax7* in turn regulate *Pax3* during the timed specification, proliferation and exit of neuroblasts from the ventricular zone, together with the suggested role in maintenance of trophic support to neurons awaiting retinal input, this would satisfy the phenotype of the *Pax7* mutant mice. Furthermore *Pax3 (Splotch)* mutant mice, which die

midgestationally after neural tube closure and immediately after the peak proliferative period (E14), present with excessive neural growth within the mesencephalon (and other brain regions) (Auerbach 1954). If our hypothesis is correct, then neuron formation and ventricular exit would be accelerated somewhat in *Pax3* mutant mice, with precocious cells perhaps unable to maintain integrity for extended periods of time without neurotrophic support from projecting cells.

Therefore, it will be important to investigate the *Pax7* expression profile in *Pax3* mutant mice, together with neuronal and cell cycle markers to evaluate this relationship, and to assess Pax7 and Pax3 co-localisation.

Finally, the role of *Pax7* in adult neurons is perplexing (Stoykova and Gruss 1994; Bernasconi *et al.*; Shin *et al.* 2003). As injury to the wildtype adult rat optic nerve results in an increase in Pax7$^+$ cells in the mesencephalon (Thomas *et al.* 2007), it would be interesting to assess the mesencephalic response to injury of the optic nerve or brain in *Pax7* mutant mice. Whilst it may not be possible in *Pax7*$^{-/-}$ mice due to premature lethality (experimentation would need to be conducted in early postnatal stages in the immature brain), *Pax7*$^{+/-}$ mice may provide important information regarding the ability of *Pax7* to orchestrate a post-trauma response within the developed mammalian brain, as *Pax7*-driven maintenance functions are also compromised in the heterozygous state. This information may address the exact nature of Pax7$^+$ neurons in adult mice.

LIMITATIONS OF THE STUDY

➤ As this project did not detect increased apoptosis in the superior colliculus of *Pax7* mutant mice, this research would profit from further investigation aimed at studying wildtype and *Pax7* mutant mice at closer temporal increments to answer two important questions identified in these results; precocious or rapid neuron formation (E10-E13) and pinpointing the timing of regression of dorsal superior collicular neurons (between birth and P5). However, due to rapid degeneration of regressing neurons, naturally occurring cell death in the first postnatal week and the punctuated nature of apoptosis across the rostrocaudal axis, the latter may not be possible.

➤ The monoclonal antibody to Pax7 recognises an epitope within the transactivation domain of the Pax7 protein product, and as such, it detects all isoforms of Pax7 (Kawakami *et al.* 1997). This thesis, therefore, does not allow for variation in function related to specific isoforms. It is possible that specific isoforms may have different functions. Differential isoform functions have been identified recently for *Pax6*, where overexpression of variable isoforms either inhibited (paired-less) or promoted (paired domain+) adult neuronogenesis (Ninkovic *et al.* 2007).

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APPENDIX

QUANTIFICATION METHODOLOGY

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After immunohistochemical staining (Figure A.1), extensive microscopic analysis was undertaken to ensure comparison of similar regions of the superior colliculus along the mediolateral axis. The area explored was close to the medial extent of the hemisphere, delineated by the longitudinal cerebral fissure. Superior collicular shape was also assessed. Caudal, rostral, dorsal and ventral points were chosen as follows: caudal by the morphological boundary between colliculi, rostral by the extinction of *Pax7* expression, dorsal by the pial surface, and ventral was designated as the opposite region of dorsal. The term "mid" is used to define the point midway across each axis. Rostral, caudal, dorsal, and ventral points were examined in sections equivalently situated across the mediolateral axis.

Pax7 expression was quantified at the chosen region utilising Optimas Digital Image Analysis of images imported through a microscope/camera. As light decays, the microscope was equilibrated for 20 minutes to regulate light levels, thereafter microscope settings were optimised for a clear, representative image. Images were not sharpened or artificially enhanced in any manner, and white/black/gamma and light levels were noted and used uniformly for all analyses conducted, together with all other microscope parameters such as the image size and resolution. Images were taken at high magnification (400x) to ensure an increased number of frames to maximise resolution of results. The term "frame" relates to one field of view within Optimas.

Quantification commenced in the most caudodorsal portion of the superior colliculus, delineated by the morphological boundary between the superior and inferior colliculi in the caudal extent, and the pial surface of the tissue in the dorsal extent. Cells were marked within frames, and care was taken when advancing the frame to ensure precise movement. Every cell was quantified in each frame by drawing an area that captured the nuclear immunohistochemical staining with a computer mouse. Entire staining of the whole frame was also measured for a comparative analysis. After all cells were examined for specificity and marked, the data were imported into a spreadsheet environment which automatically designated the frame with a number, and the frame was carefully advanced. This procedure

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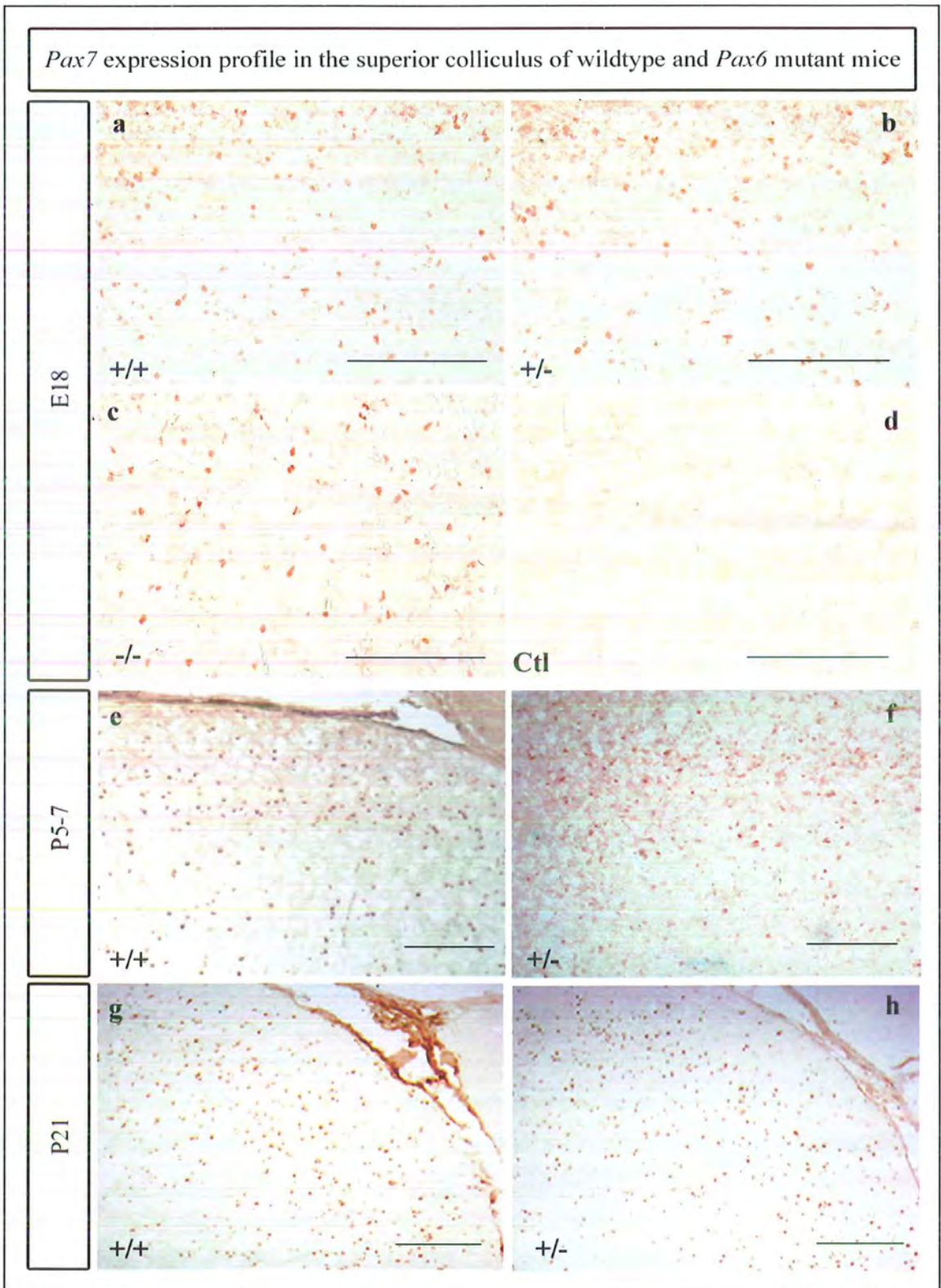
Figure A.1: Pax7 immunohistochemical staining in the superior colliculus of wildtype (a,e,g) and Pax6 mutant (b,c,f,h) mice at E18 (a-c), P5-7 (e-f) and P21 (g-h). Figure 2d shows a control section, where primary antibodies were omitted. Wildtype mice: +/+. *Pax6* mutant mice genotypes: +/- heterozygotes; -/- homozygotes. Scale bar = 100μm.

was repeated until every cell in the entire superior colliculus was quantified, designated by *Pax7* expression as a marker of the superior colliculus. A hand-drawn map was produced to recapitulate the position of each frame within the tissue. This procedure was exhaustive and thorough, and allowed an enhanced view of the cell for correct identification of staining. Adjacent, unstained tissue was quantified and used to normalise measurements to account for background (non-specific) staining. Ephrin-A2 quantification was performed as above, with the exception that three points were measured on the immunostained portion of each cell membrane (at 1000x magnification to detect protein present only on soma), and analysis utilised 1 in every 3.5 frames across the rostrocaudal axis and 1 in every 4 frames across the dorsoventral axis.

Within the spreadsheet environment, the digital results were plotted back into a two-dimensional map, representing the rostrocaudal and dorsoventral extent of the superior colliculus, and values were normalised. The "number of cells" and the "average amount of protein per cell" for each individual frame was plotted in its two-dimensional position (similar to a Cartesian grid) and was used to produce cone charts showing cellular distribution and protein levels throughout the tissue. Each individual cell protein measurement was then reinstated in its topographical position in another map to explore expression at each position across the rostrocaudal or dorsoventral axes. This additional mapping was performed in order to average ACTUAL cell protein measurements at axis positions, rather than taking an average of AVERAGE cell protein measurements, which were utilised for the cone charts. To produce maps and graphs representing average expression for each animal class (wildtype/heterozygotes/homozygotes), values for each individual brain section were consolidated within their respective groups.

This precise method of quantification was adopted because we were interested in showing a complete map of expression, rather than choosing to quantify and report a selection of points only across each axis. Although much quicker to perform, the latter would only effectively demonstrate a trend in expression rather than detailed expression in its entirety. Furthermore, utilizing every frame of analysis, which represents the entire tissue, also allowed us to use the results as a quantitative measure of tissue size, and highlighted the expanded mesencephalic region in *Pax6* mutant mice. Due to the expanded size of the

mesencephalon in *Pax6* mutant mice it would not have been possible to accurately compare a "chosen point" in the tissue between genotypes. Rather, we showed differential *Pax7* expression and size variation between wildtype and mutant mice by comparing their differential expression side-by-side. This was also necessary to show the entire graded nature of the expression.