

2004

## Analysis of expression patterns of ScL and Pax7 in the mouse brain for potential concomitant function within the mesencephalon

Julie Stampalia  
*Edith Cowan University*

Follow this and additional works at: [https://ro.ecu.edu.au/theses\\_hons](https://ro.ecu.edu.au/theses_hons)



Part of the [Medical Neurobiology Commons](#)

---

### Recommended Citation

Stampalia, J. (2004). *Analysis of expression patterns of ScL and Pax7 in the mouse brain for potential concomitant function within the mesencephalon*. Edith Cowan University. [https://ro.ecu.edu.au/theses\\_hons/371](https://ro.ecu.edu.au/theses_hons/371)

This Thesis is posted at Research Online.  
[https://ro.ecu.edu.au/theses\\_hons/371](https://ro.ecu.edu.au/theses_hons/371)

# Edith Cowan University

## Copyright Warning

You may print or download ONE copy of this document for the purpose of your own research or study.

The University does not authorize you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site.

You are reminded of the following:

- Copyright owners are entitled to take legal action against persons who infringe their copyright.
- A reproduction of material that is protected by copyright may be a copyright infringement. Where the reproduction of such material is done without attribution of authorship, with false attribution of authorship or the authorship is treated in a derogatory manner, this may be a breach of the author's moral rights contained in Part IX of the Copyright Act 1968 (Cth).
- Courts have the power to impose a wide range of civil and criminal sanctions for infringement of copyright, infringement of moral rights and other offences under the Copyright Act 1968 (Cth). Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

**ANALYSIS OF EXPRESSION PATTERNS OF  
*Scl* AND *Pax7* IN THE MOUSE BRAIN  
FOR POTENTIAL CONCOMITANT FUNCTION  
WITHIN THE MESENCEPHALON**

By

**Julie Stampalia**

Supervised by:

Dr. Anke van Eekelen & Dr. Mel Ziman

A Thesis Submitted in Partial Fulfilment of the Requirements  
for the Award of

**Bachelor of Science, Honours (Human Biology)**

In the Faculty of Communications, Health and Science,  
Edith Cowan University, Joondalup

DATE OF SUBMISSION:

10<sup>th</sup> November 2004

## USE OF THESIS

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

## ABSTRACT

Investigation of the cascade of events that leads to brain formation during embryogenesis allows an understanding of the processes involved that may well be applicable to future neurodegenerative therapies. Two genes at work during embryonic neurogenesis in a brain region called the superior colliculus are *Scf*, a member of the basic helix-loop-helix factor transcription factor family and *Pax7*, a paired box transcription factor containing a homeobox domain. The superior colliculus forms part of the visual system in mammals and in separate studies both the *Scf* and *Pax7* genes have been shown to be present in this region of the mouse brain from early neurogenesis onwards continuing into adulthood. This study was undertaken to ascertain whether both genes were operating within the same cells suggesting that they may be part of the same cascade of gene regulatory events during development or co-function in cells of the mature superior colliculus. Mice were analysed at seven different timepoints from embryonic day (E) 12.5, E15.5, E18.5 to the day of birth, postnatal day (P) 5, at weaning (P21) and in the adult brain. Their dissected brains were cryosectioned and processed for (immuno)histochemistry. Preliminary results were generated with LacZ staining to detect *Scf* expression and immunoperoxidase staining to detect *Pax7*. Confocal microscopy of (immuno)fluorescent stained sections confirmed co-expression.

Interestingly, expression of the *Scf* and *Pax7* transcription factors was found to overlap in a large subset of *Pax7* and *Scf* positive cells in the superficial layers of the mature superior colliculus and co-localisation in these cells was confirmed. By contrast, *Pax7* and *Scf* did not co-localise in these cells during embryonic neurogenesis. Compared with a range of neurogenic transcription factors that are down-regulated after embryonic neurogenesis, the continued expression of *Scf* and *Pax7* in the adult brain suggests a possible change in function from neuronal differentiation before birth to a possible concomitant maintenance of the cellular phenotype function in the adult brain. The lack of co-expression in the embryonic brain does not support the hypothesis that *Pax7* and *Scf* are involved in the same developmental cascade of gene regulation. However, the finding that *Pax7* and *Scf* co-localise in mature neurons of the superior colliculus does allow for co-function of these two transcription factors.

## 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;
- (ii) contain any material previously published or written by another person except where due reference is made in the text; or
- (iii) contain any defamatory material.

Signature \_\_\_\_\_

Date \_\_\_\_\_

17/01/05

## ACKNOWLEDGMENTS

Firstly, I would sincerely like to thank my supervisors, Dr. Anke van Eekelen and Dr. Mel Ziman for their continuing support, patience and guidance throughout the year. If it were not for their exceptional knowledge and expertise this thesis would not have been able to be completed to such a high standard.

Special thanks must go to Elena Takano, my proxy lab supervisor. Elena has displayed extraordinary patience from a barrage of questions and has always been ready to help out when needed. Her own work remains as motivation to always strive for excellence.

Thanks must also go to the rest of the Cancer Biology team at the Telethon Institute for Child Health Research. It has been a great pleasure to work with such an outstanding group of people. Special thanks to Kelly Becher for all the genotyping that she has performed for me throughout the year and to Lan Doan for her continual good humour and support. I wish all in the lab continued success.

Fortunately, I have also been a part of another group of exceptional students and I would like to thank my ECU buddies for their help, support and down-to-earth advice over the year. The work they perform is first class and as a collective, the group is one of Edith Cowan University's greatest assets. It has been an honour to be a part of this team and I wish them all great success in the future.

To my family and friends who have barely seen me this year, thank you for your support, encouragement and patience as I put the rest of my life on hold. Special thanks to my husband Mark and children, Luke and Jake who have had to put up with the greatest inconveniences and are still so proud of my efforts at the end of the day. I love you! And of course, to my Mum and Dad who were the inspiration behind the journey. God Bless.

# TABLE OF CONTENTS

	Page
Abstract	3
Declaration	4
Acknowledgements	5
Table of Contents	6
<b>1. INTRODUCTION</b>	<b>7</b>
1.1. BACKGROUND TO THE STUDY	8
1.2. SIGNIFICANCE OF THE STUDY	9
1.3. HYPOTHESIS	9
1.4. AIM	9
<b>2. LITERATURE REVIEW</b>	<b>10</b>
2.1 THE <i>SCL</i> GENE	11
2.1.1. <i>Scl</i> is an oncogene in T-cell Acute Lymphoblastic Leukaemia (T-ALL)	11
2.1.2. <i>Scl</i> encodes a bHLH transcription factor	11
2.1.3. Characterisation of enhancer sequences in the <i>Scl</i> gene	12
2.1.4. The function of <i>Scl</i> in haematopoiesis	12
2.1.5. The function of <i>Scl</i> in the CNS	13
2.2. TRANSGENIC MOUSE MODELS	14
2.2.1. The Cre/loxP System	14
2.2.2. Reporter genes	15
2.3. THE <i>PAX7</i> GENE	18
2.3.1. Pax7 is a transcription factor with a paired domain	18
2.3.2. Neural enhancers of <i>Pax7</i>	20
2.3.3. The function of <i>Pax7</i> in the musculo-skeletal system	20
2.3.4. The function of <i>Pax7</i> in the CNS	21
2.4. TRANSCRIPTION FACTORS AND BRAIN DEVELOPMENT	22
2.4.1. The midbrain/hindbrain boundary development	22
2.4.2. The midbrain/forebrain boundary development	24
2.4.3. Development of the superior colliculus	24
2.4.4. Summary	26
<b>3. MATERIALS AND METHODS</b>	<b>28</b>
3.1. ETHICAL CONSIDERATIONS	29
3.2. EXPERIMENTAL PROCEDURES	29



3.2.1.	Mouse Models	29
3.2.2.	Timed pregnancies	30
3.2.3.	Harvesting embryos	30
3.2.4.	Tamoxifen Injections	30
3.2.5.	Cross fostering of tamoxifen treated embryos	31
3.2.6.	Anaesthetic	31
3.2.7.	DNA Isolation	32
3.2.8.	Genotyping of genetically manipulated mice using Real-time PCR	32
3.2.9.	Transcardial Perfusion for the fixation of brain tissue	35
3.2.10.	Ice Hypothermia and transcardial perfusion of mouse pups	35
3.2.11.	Frozen Blocks	35
3.2.12.	LacZ histochemistry	36
3.2.13.	Immunohistochemistry of mouse brain tissue for antibodies raised in mice	36
3.2.14.	Immunofluorescence for Pax7	37
3.2.15.	Microscopic Analysis	38
3.2.16.	Research Design	38
<b>4.</b>	<b>RESULTS</b>	<b>41</b>
4.1.	LACZ STAINING	42
4.2.	PAX7 STAINING	42
4.3.	FLUORESCENT AND CONFOCAL MICROSCOPY	43
4.4.	NEURONAL MARKERS	43
4.5.	MAPPING OF EXPRESSION PATTERNS IN THE BRAIN	46
4.6.	E12.5 TIMEPOINT	48
4.7.	E15.5 TIMEPOINT	48
4.8.	E18.5 TIMEPOINT	50
4.9.	P0 TIMEPOINT	51
4.10.	P5 TIMEPOINT	54
4.11.	P21 AND ADULT TIMEPOINT	56
<b>5.</b>	<b>DISCUSSION</b>	<b>59</b>
5.1.	NEW FINDINGS IN THIS STUDY	60
5.2.	CHANGE OF FUNCTION OVER TIME FOR PAX7 AND SCL	60
5.3.	DYNAMIC EMBRYONIC EXPRESSION PATTERNS OF PAX7 AND SCL	62
5.4.	GENERATION OF AN EXPRESSION MAP FOR PAX7	63
5.5.	APPLICATION OF GENETICALLY MANIPULATED REPORTER MICE FOR SCL EXPRESSION	64
5.6.	FUTURE RESEARCH	65
<b>6.</b>	<b>REFERENCES</b>	<b>67</b>

## **1. INTRODUCTION**

## 1.1 BACKGROUND TO THE STUDY

The Stem Cell Leukaemia (*Scf*) gene encodes a basic helix-loop-helix transcription factor that was originally identified through a translocation of the gene to the T-cell receptor locus in acute lymphoblastic leukaemia. The gene is essential for the early development of the haematopoietic and vasculogenic system (Begley and Green, 1999). Studies indicate that *Scf* functions as a cell fate determinant in the haematopoietic system by regulating the proliferation of multipotent haematopoietic stem cells (Green et al., 1991; Elefanty et al., 1997), directing the differentiation of erythroid cells, megakaryocytes and mast cells (Green et al., 1991) and developing the yolk sac vessels (Robb et al., 1995). *Scf* expression has also been found in the central nervous system and the expression pattern of *Scf* in the brain has been mapped using an *Scf LacZ* knockin reporter mouse. The expression mapping shows a high degree of expression in the dorsal superficial layers of the superior colliculus, the pretectal area, the tegmental regions and moderate to low density expression throughout the midbrain, hindbrain and caudal thalamus (van Eekelen et al., 2003). A recent *Scf* gene deletion study has indicated that *Scf* is required for normal mid/hindbrain development, suggesting a cell fate determinant role for *Scf* in neurogenesis (data to be submitted for publication by van Eekelen et al.).

The Pax7 transcription factor is encoded by a member of the paired box gene family first identified in *Drosophila* (Bopp et al., 1986). The expression pattern of the *Pax7* gene suggests a functional role in development of neural crest, skeletal muscle and the central nervous system (Mansouri et al., 1996). Pax7 appears to be a significant factor during brain development, functioning in regionalisation and polarity of the superior colliculus. Pax7 has also been implicated with refining the optic nerve innervation to the dorsal layers in the superior colliculus during early postnatal development (Thomas et al., 2004). The adult *Pax7* expression pattern is similar to that of the late embryo with prominent expression found in the dorsal superficial layers of the superior colliculus (Stoykova and Gruss, 1994).

The *Scf* and *Pax7* genes display a high level of expression during embryonic neurogenesis particularly in the mesencephalon (midbrain) during the formation of the superior colliculus. Both factors continue to be expressed in the dorsal layers of the superior colliculus in postnatal and adult brain (Stoykova and Gruss, 1994;

Nomura et al., 1998; Matsunaga, 2001; van Eekelen et al., 2003; Thomas et al., 2004). This spacio-temporal overlap in expression suggests that *Scl* and *Pax7* may be involved in related functions during brain development and in the adult brain. Expression of *Scl* and *Pax7* throughout embryonic neurogenesis, implies a function in determining cell fate and/or differentiation, particularly in the superior colliculus (Stoykova and Gruss, 1994; van Eekelen et al., 2003). In addition, with the expression of both factors in regional organisational centres of the developing brain, a regionalisation function is suggested (Kawakami et al., 1997; Matsunaga, 2001; van Eekelen et al., 2003) and continued expression in post-mitotic cells after birth also implies a role in the maintenance of the cellular phenotype of *Scl* and *Pax7* expressing neurons (Stoykova and Gruss, 1994; van Eekelen et al., 2003).

## **1.2 SIGNIFICANCE OF THE STUDY**

This study will help determine more accurately the roles of *Scl* and *Pax7* in neuronal function and contribute to the understanding of factors involved in embryonic and adult neurogenesis. This is essential for future development of possible therapies for neurodegenerative diseases based on the endogenous generation of new neurons in the adult brain, rather than by transplantation methods or gene therapy.

## **1.3 HYPOTHESIS**

*Pax7* and *Scl* co-localise within cells of the mesencephalon from embryonic neurogenesis to adulthood indicating a co-ordinated neurogenic function of both transcription factors in developing and mature neurons.

## **1.4 AIM**

The aim of this study is to investigate the co-expression of the *Scl* and *Pax7* genes as a basis for a common functional role of the two genes in embryonic, postnatal and adult brain. To do this a detailed morphological analysis of *Pax7* and *Scl* co-localisation in the brain at different stages of embryonic neurogenesis as well as after birth, at weaning and in the adult brain will be performed. This detailed analysis includes the generation of an accurate but so far not reported map on the expression pattern of *Pax7* during embryonic neurogenesis and postnatal development.

## **2. LITERATURE REVIEW**

## 2.1 THE *Scl* GENE

### 2.1.1. *Scl* is an oncogene in T-cell Acute Lymphoblastic Leukaemia (T-ALL)

The *Scl* gene, also referred to as *Tcl-5* and *Tal-1*, was originally identified through a translocation from chromosome 1 into the T-cell receptor locus on chromosome 14. At the new locus, aberrant production of the transcription factor caused the gene to become oncogenic leading to T-ALL. Further, deletions at chromosome 1 have put the *Scl* gene under the control of the ubiquitous *Sil* promoter also resulting in T-ALL oncogenic function. The mechanism involved in transforming the cells into a leukemic state is unknown but *Scl* is thought to increase proliferation and decrease cell death (Begley and Green, 1999).

### 2.1.2 *Scl* encodes a bHLH transcription factor

Basic helix-loop-helix (bHLH) transcription factors play a major role during embryogenesis in determining neuronal subtype specification and differentiation (Guillemot et al., 1993). The bHLH structure contains two important binding domains with the basic domain facilitating DNA binding while the helix-loop-helix domain binds other proteins to form heterodimers. *Scl* is a class B HLH protein known to bind with class A proteins such as E2A, that acts as a transcriptional activator, to form a heterodimer that binds DNA at the E-box of downstream gene enhancers, regulating developmental pathways (Hsu et al., 1991). *Scl* binds to CAGGTG E-boxes with a higher binding affinity if the E-box is followed by a GATA motif or with a lower binding affinity if an upstream CAGATG E-box is nearby (Cohen-Kaminsky et al., 1998). The HLH domain can bind with other proteins to form a larger protein complex. The individual proteins involved in complexes may have no singular effect on downstream target genes but may all be necessary components in order to activate the synergistic complex in an all or nothing manner (Lecuyer and Hoang, 2004). The specific protein interactions allow for a precise spacio-temporal activation of the target gene. Specific complexes will differentially activate each target gene. A protein change within a multifactorial complex may make for a more efficient activator to certain downstream genes (Lecuyer and Hoang, 2004). There are a number of *Scl* isoforms that may well vary the activational function of a complex and create diversity between cell types, regions and stages of differentiation. (Begley and Green, 1999; Calkhoven et al., 2002).

### 2.1.3. Characterisation of enhancer sequences in the *Scl* gene

The *Scl* gene is expressed in the haematopoietic system, endothelial cells and the brain and specific enhancers within the *Scl* locus have been identified to direct *Scl* expression specifically to these functionally different cell types. A 3' *Scl* enhancer has been described that targets the haemangioblast, a precursor for both haematopoietic stem cells and endothelial cells (Gottgens et al., 2002) and a 5' endothelial enhancer has also been demonstrated. Of concern to this project is the 5' *Scl* neural enhancer (940bp) used in the construct of a transgenic mouse model that directs transgene expression under this regulatory gene sequence stretching from -0.9kb to Exon 3 (0.9E3). This enhancer is important for *Scl* expression within the midbrain, hindbrain and spinal cord. Exon 1a forms part of the promoter and is essential for directing expression to the midbrain (Sinclair et al., 1999).

### 2.1.4. The function of *Scl* in haematopoiesis

Early research on *Scl* has shown that the *Scl* gene is essential for the early development of the haematopoietic and vasculogenic systems (Begley and Green, 1999). Functional studies of the haematopoietic system have found *Scl* expressed in primitive haematopoietic stem cells, progenitor cells and differentiated erythrocytes, megakaryocytes and mast cells (Hall et al., 2003). *Scl* regulates multi-potent haematopoietic cell proliferation, facilitates erythroid differentiation and inhibits myeloid differentiation (Kallianpur, 1994; Begley and Green, 1999). A conserved upstream open reading frame acts as a translational control mechanism regulating differing *Scl* isoforms. The isoforms vary in the translation of the N terminus while all retain the bHLH domain. Specific *Scl* isoforms determine lineage commitment with the truncated *Scl* isoform promoting the erythroid lineage while the full length *Scl* protein follows the megakaryocyte lineage (Calkhoven et al., 2002). Evidence that *Scl* is a critical factor within haematopoiesis is displayed in the knockout model by the failure of embryos to survive beyond E9.5 with death occurring from hypoxia following on from absolute anaemia and yolk sac failure (Robb et al., 1995) at the stage when foetal circulation is meant to commence. While embryonic blood vessels develop typically in the knockout model, vessels within the yolk sac display no patterning and fail to develop without *Scl* (Elefanty et al., 1997). To functionally assess *Scl* in the adult haematopoietic system, conditional *Scl-null* mice based on the

*Mx-Cre/loxP* system have been developed that knock out the *Scl* gene upon interferon administration at a chosen timepoint. These studies have shown that *Scl* has a function in adult haematopoiesis by maintaining megakaryocyte production and erythropoiesis. Macrophages, granulocytes and myeloid progenitors are unaffected by the *Scl* deficiency even though *Scl* expression is found within the myeloid progenitor (Hall et al., 2003).

#### 2.1.5. *The function of Scl in the CNS*

*Scl* expression in the central nervous system has been mapped using a *Scl LacZ* knockin reporter mouse (van Eekelen et al., 2003). *Scl* is expressed in regions of the foetal brain known to differentiate into the *Scl* expression domains of the adult brain. Expression is found in the diencephalon (caudal thalamus of the forebrain), the mesencephalon (midbrain) including the superior colliculus and substantia nigra and in metencephalon (hindbrain) (van Eekelen et al., 2003). *Scl* does not express in glial cells but it does in post-mitotic neurons. In general, *Scl* expressing cells are found evenly scattered over different brain regions suggesting that only a percentage of cells express *Scl* (van Eekelen et al., 2003). Embryonic *Scl* expression surrounds the aqueduct in the intermediate and marginal zone of the neuroepithelium and the fourth ventricle in the ventricular zone suggesting an involvement in the differentiation and possible neuronal subtype specification of migrating neurons from neural stem cells during development (van Eekelen et al., 2003).

The most prevalent *Scl* staining in the adult brain is seen in the superior colliculus with a decreasing expression gradient observed over the seven layers from dorsal to ventral. There is a high density of *LacZ* staining in the dorsal zonal and superficial grey layers of the superior colliculus, a moderate density in the optic layer and a low density in the intermediate and deep layers. Low density *Scl* expression is seen in the cortex of the inferior colliculus (van Eekelen et al., 2003). *Scl* expression in the superior colliculus particularly implies a potential role in the oculomotor, pupillary and retinotectal visual pathway. Functional studies on the role of *Scl* in the central nervous system are currently being undertaken using conditional gene knockout mouse models that knockout the gene within the brain only. Preliminary results show that the spatial *Scl* knockout in the brain results in a runt animal with 25% of animals dying at birth and a further 35% at weaning age and are growth retarded. The



remaining animals display hyperactive behaviour. Furthermore, a subset of neurons that express *Scf* in the normal brain are absent in the mid and hindbrain of the conditional knockout and suggest a crucial role for *Scf* during brain development. (*Personal communication from JAM van Eekelen at the Telethon Institute for Child Health Research*).

## 2.2 TRANSGENIC MOUSE MODELS

In order to make functional hypotheses for specific genes, researchers have been manipulating the pluripotent embryonic stem cells of mice to make gene knockout mouse models by homologous recombination. A phenotypic analysis of loss of function may be made by replacing the gene sequence with an alternative construct, deleting the gene from the genome or knocking in a different gene, to give an indication of function (Garcia and Mills, 2002). The complete removal of some genes from the genome results in a lack of offspring because the gene concerned is necessary for the development and survival of the animal. Such is the case for the *Scf* gene, which is imperative to the development of the haematopoietic system rendering an embryo unviable beyond E9.5 (Robb et al., 1995). By using the full *Scf* knockout model it is impossible to study *Scf* function in the brain. Conditional gene models have been designed to circumvent the problems of lethality during embryogenesis allowing genes to be deleted in both a spatial and temporal manner (Garcia and Mills, 2002).

### 2.2.1 The Cre/loxP System

*Cre recombinase* (*Cre*) is an enzyme produced in the bacteriophage P1 that recombines DNA at specific recognition sites called loxP sites. The loxP sites have two 13bp inverted repeat regions that bind *Cre* and a central 8bp spacer where cleavage and recombination occur. When bound by *Cre*, the DNA between two loxP sites is cleaved (Sternberg and Hamilton, 1981). Genetically manipulated mice are inter-crossed to produce offspring that carry two loxP sites on either side of a pertinent area of the gene in question (floxed) and also carry the *Cre* sequence within its genome. When *Cre* is expressed it will recombine the loxP sites by cleaving the gene sequence in between (Fig. 2-1.) (Garcia and Mills, 2002).

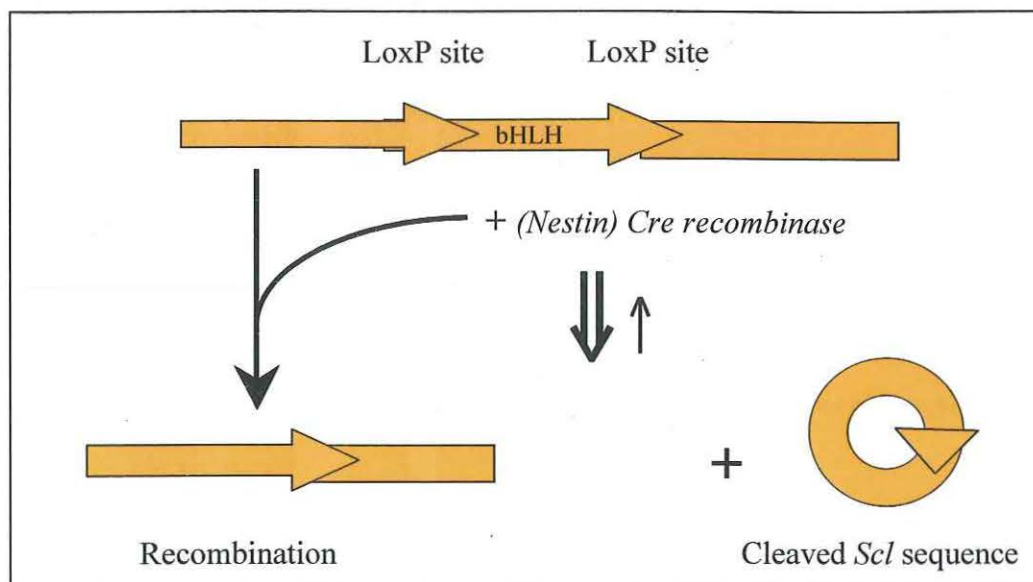
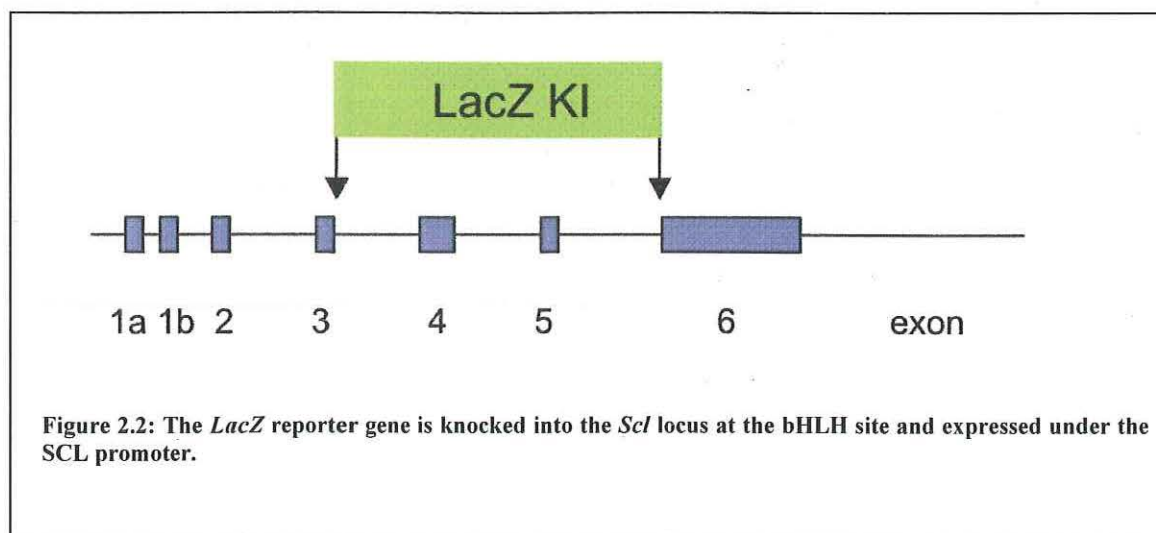


Figure 2-1. The conditional *Scl* knockout mouse. *Cre recombinase*, produced under the neural-specific *Nestin* promoter, will bind two LoxP sites, cleave and circularise the DNA in between and recombine the original strand.

The previously mentioned conditional *Scl* knockout model has been generated such that, *Cre* expression is regulated by the promoter and neural tissue-specific enhancer of the proneural gene, *Nestin* (Tronche et al., 1999). LoxP sites have been inserted into *Scl* flanking the region encoding the bHLH domain (Fig. 2.1). Cleaving of the gene is spatially restricted to the brain because *Cre* is only expressed in the neural progenitor cells.

### 2.2.2. Reporter genes

In most cases where knockout models have been generated, the gene of interest has been replaced by a knockin gene being a reporter gene placed under the control of the regulatory sequences of the gene of interest. This has been done for *Scl* and has provided an excellent *Scl-LacZ* knockin reporter mouse to study the expression pattern of *Scl* in the brain. The *LacZ* reporter gene has been knocked-in to the *Scl* locus (Fig. 2.2) in the region encoding the translational sequence of the *Scl* gene and is expressed under the *Scl* endogenous promoter in all *Scl* expressing cells.



The *LacZ* reporter mouse is extremely useful for gene expression studies within the brain because the *Scl* monoclonal antibodies that can be used for Western blotting are ineffective in the immunohistochemical studies on brain sections. Once the  $\beta$ -galactosidase enzyme assay has been completed on tissue sections, a blue reaction is visualised (Hatton and Lin, 1992) where *Scl* is normally expressed. To avoid creation of a knockout mouse, heterozygous mice are used, which express the *LacZ* reporter gene from one allele while the normal expression of *Scl* still occurs from the other.

Unfortunately, the *LacZ* signal is not strong enough for immunofluorescent histochemistry using an antibody against the X-gal protein. As an alternative, a mouse model containing the *enhanced Yellow fluorescent protein (eYfp)* reporter has been developed. *eYfp* is knocked-in to the *Rosa26* locus, which is a housekeeping gene ubiquitously expressed and resulting in a high level of fluorescence (Srinivas et al., 2001). Moreover, a stop sequence flanked by LoxP sites precedes the reporter gene in this knockin mouse resulting in *eYfp* expression only if the stop sequence is recombined/deleted by *Cre* (Fig. 2.3); (Srinivas et al., 2001).

In the group supervised by Dr. van Eekelen at ICHR, a *Cre* transgenic mouse has been generated in which *Cre* is expressed under the control of the 5' *Scl* neural enhancer (0.9E3) and is produced only in *Scl* expressing neurons. Thus until the

expression of *Cre* no eYfp will be produced in *Scl*-expressing neurons because of the presence of a stop sequence at the start of the *eYfp* gene. However, upon *Cre* expression, the stop sequence is cleaved and eYfp is produced.

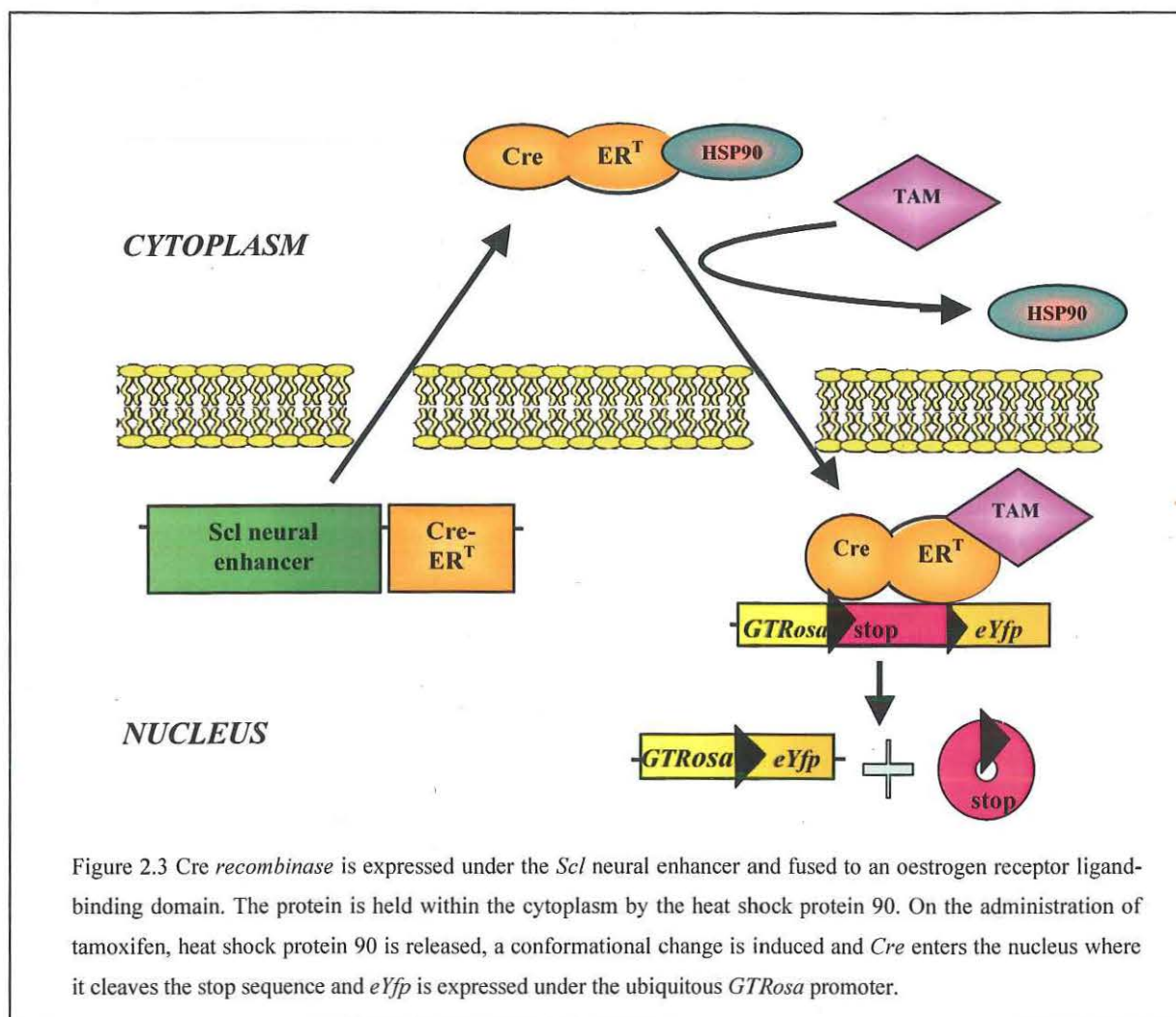


Figure 2.3 *Cre recombinase* is expressed under the *Scl* neural enhancer and fused to an oestrogen receptor ligand-binding domain. The protein is held within the cytoplasm by the heat shock protein 90. On the administration of tamoxifen, heat shock protein 90 is released, a conformational change is induced and *Cre* enters the nucleus where it cleaves the stop sequence and *eYfp* is expressed under the ubiquitous *GTRosa* promoter.

To add temporal control over deletion of the floxed gene of interest (*R26R-EYfp* in this study), the 0.9E3 *Cre* transgenic mouse was generated as an inducible *Cre* mouse with *Cre* fused to a mutated oestrogen receptor ligand-binding domain constructed to mimic the nuclear hormone receptor (Brocard et al., 1997). When the hormone binds to the receptor, the protein is translocated into the nucleus for DNA binding (Fig 2.3). Without hormonal binding the protein remains in the cytoplasm bound to heat shock protein 90. The introduced oestrogen receptor ligand-binding domain is mutated such that *Cre* can only be activated by tamoxifen (Fig. 2.3). The three mutations within the



receptor prevent the binding of endogenous oestrogen (Brocard et al., 1997). Taken together, *eYfp* expression in the brain will reflect *Scl* neural expression upon tamoxifen administration.

The different models discussed are of relevance to this study as the *Scl* antibody is ineffective when immunostaining brain tissue. The *Scl-LacZ* knockin model has been designed as a reporter for *Scl* expression, displaying good morphology and suitable for light microscopy while the Brain-*Cre*  $\times$  *GTRosa26-eYfp* model has been valuable for fluorescent and confocal microscopy. These conditional mouse models allow us to perform several experiments that enhance or further our understanding of the role of *Scl* in the CNS.

## 2.3 THE *Pax7* GENE

### 2.3.1. *Pax7* is a transcription factor with a paired domain

The *Pax7* gene encodes a transcription factor that contains a paired DNA binding domain first identified in the paired protein of *Drosophila melanogaster* (Bopp et al., 1986). The *Pax7* gene is one of nine family members that encode a DNA binding domain from a gene region called the paired box (Pax). The encoded paired domain forms six  $\alpha$ -helices grouped into two helix-turn-helix motifs, is 128 amino acids long, and in close proximity to the amino terminus of the protein. In addition, Pax7 contains a 61 amino acid paired type homeodomain (Jostes et al., 1990; Stoykova and Gruss, 1994) as well as a conserved octapeptide sequence (Fig 2.4). Pax proteins, like other transcription factors are thought to bind enhancer sequences of downstream genes with their DNA binding domains.

The Pax proteins have been grouped into different classes according to their structural differences and Pax7 has been classified as a Class II protein as it contains the paired box DNA binding domain, the full length homeodomain encoding region and the conserved octapeptide (Ziman et al., 2001b). Pax7 also contains a transactivation domain (Fig. 2.4) binding other proteins to induce downstream gene transcription (Ziman et al., 2001a).

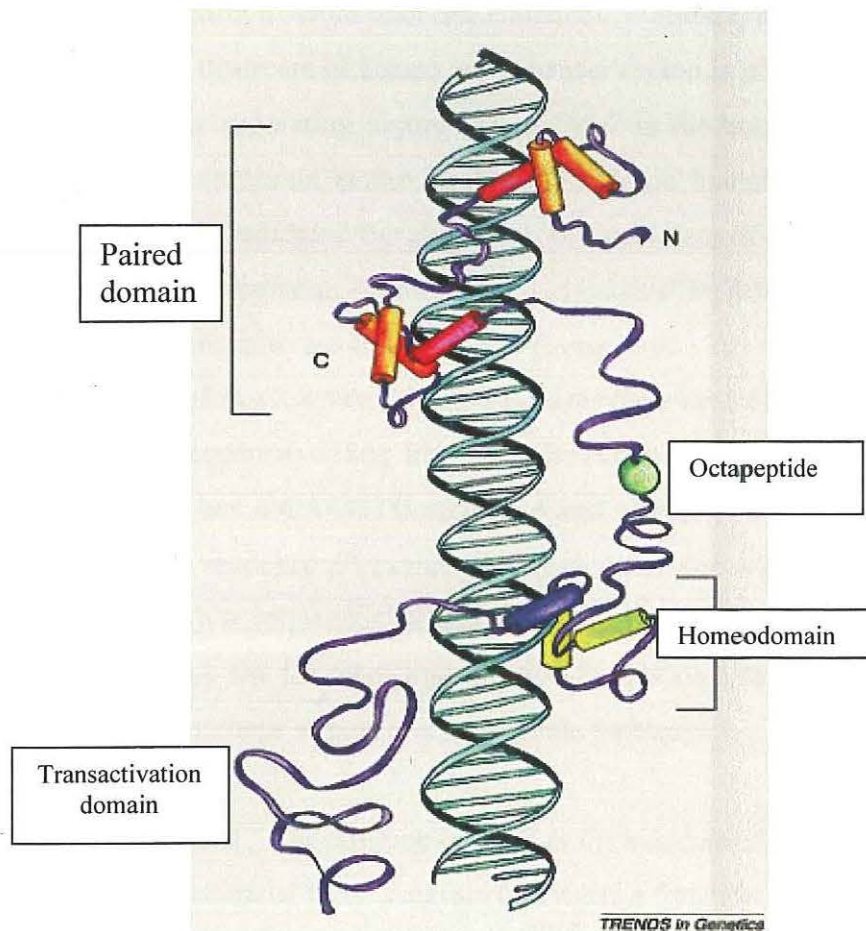


Figure 2.4 *Pax7* protein schematic showing the subdomains of the paired domain, each containing a helix-turn-helix motif, an octapeptide, the helix-turn-helix homeodomain and the protein-binding transactivation domain (*Chi and Epstein, 2002*).

Another Class II protein homologous to Pax7 is Pax3, with a structure closely resembling that of Pax7 and thought to offer partial redundancy where the expression domains overlap (Mansouri et al., 1996). The diversity in function of Pax7 throughout development is thought to be explained by the identification of different protein isoforms. Alternate splicing regions within the intron-exon boundaries of the *Pax7* paired box encode different proteins thought to operate in specific spatial and temporal domains. The alternate splicing within the paired box region may modify the proteins tertiary structure, thus altering DNA binding and activating different pathways (Ziman et al., 2000; Ziman et al., 2001a). Further, six different PAX7 allelic forms have been identified.

### 2.3.2. Neural enhancers of *Pax7*

The distinct expression patterns of *Pax7* within the central nervous system are under the regulatory control of more than one enhancer. While the promoter region of *Pax7* is located -2.1kb upstream of Exon1, an enhancer region is positioned within the first intron of the gene regulating expression of *Pax7* in the brain region, the pons. By contrast, *Pax7* expression at the midbrain/hindbrain boundary and the cerebellar ventricular zone is regulated by elements -4kb upstream of the gene. An enhancer situated within the upstream -10kb of *Pax7* regulates the roof plate, cephalic neural crest and mesencephalic tectal expression (Lang et al., 2003). Within the upstream promoter region of *Pax7*, seven different E-boxes have been observed. At -2073 there is a CACCTG sequence, -1861 has a CAGGTG sequence, -1393 has a CATCTG sequence, -1051 has a CACGTG sequence and -956, -570 and -280 all have the CAGCTG E-box sequence (Personal communication Rob White, Centre for Human Genetics). The E-box sequences located within the promoter region upstream of *Pax7* make an opportune site for other transcription factors such as *Sc1* to bind and induce *Pax7* expression perhaps as part of a neurogenic pathway.

### 2.3.3. The function of *Pax7* in the musculo-skeletal system

The expression pattern of *Pax7* suggests that it has a function in cephalic neural crest cells and the development of skeletal muscle as well as the central nervous system (Gruss and Walther, 1992). From E9, early dorso-ventral somite patterning is thought to be regulated by *Pax7* and *Pax3*. *Pax7* expression is then restricted to the dermamyotome. Later it is observed in skeletal muscle progenitors (Kawakami et al., 1997) and in the intercostal muscles (Jostes et al., 1990). A myogenic progenitor cell termed a satellite cell is found as a quiescent muscle precursor normally adjoining mature muscle fibres. During muscle regeneration, satellite cells proliferate and fuse to form new muscle fibres. Persistent *Pax7* expression maintains the precursors in an undifferentiated state, remaining expressed when cells are activated but disappearing on terminal differentiation. Satellite cells are absent in the *Pax7* knockout mice and a runted animal results, deficient in postnatal skeletal muscle growth (Seale et al., 2000).

#### 2.3.4. The function of Pax7 in the CNS

The *Pax* genes are implicated in a variety of functions based on temporal and spatial restriction of expression in the brain from early embryogenesis through to early postnatal development. *Pax7* initially expresses in the neural tube at embryonic day 8 along the dorso-ventral axis during neural tube development and before cellular differentiation (Jostes et al., 1990 ; Stoykova and Gruss, 1994; Nomura et al., 1998). Once the neural tube has closed, the neural epithelium becomes a mitotically active ventricular zone generating stem cells for the developing central nervous system. At this time, *Pax7* persists in the dorsal ventricular zone until the ventricular zone reduces reducing its mitotic activity (E14) (Jostes et al., 1990). *Pax* genes then display a role in regional differentiation of the embryonic brain by expressing within or between regions that correlate to anatomical borders (Stoykova and Gruss, 1994). *Pax7* and *Pax3* are part of the cascade that delineates the midbrain from the forebrain (Stoykova and Gruss, 1994) while the homologues *Pax2* and *Pax5* are expressed at the midbrain/hindbrain boundary (Stoykova and Gruss, 1994; Bouchard et al., 2000).

From the delineation of anatomical borders, *Pax7* is then involved in establishing regionalisation and polarity of the superior colliculus (optic tectum). This has been demonstrated by the misexpression of *Pax7* in the diencephalon inducing the formation of an ectopic tectum showing innervation and lamination by retinal axons (Matsunaga, 2001). During embryogenesis, *Pax7* expressing cells migrate from the ventricular zone to the *stratum griseum superficiale*, a region of the superior colliculus that later receives retinal ganglion cell input. This implies a later specification and/or differentiation role for *Pax7*. *Pax7* displays an expression gradient of rostral<sup>low</sup> - caudal<sup>high</sup> and dorsal<sup>high</sup> - ventral<sup>low</sup> within the tectum throughout development implying a role in the polarity of the superior colliculus (Thomas et al., 2004). Co-expression of *Pax7* with *ephrin-A2* during early postnatal development, suggests a role for *Pax7* in establishing the retinotectal map and refining optic nerve innervation. *Pax7* achieves this in part by regulating *ephrin-A2* expression (Thomas et al., 2004). In the adult brain, *Pax7* expression is found along the rostro-caudal axis of the brain with particularly high expression levels within the dorsal superior colliculus similar to expression patterns in the embryonic brain (Stoykova and Gruss, 1994).



The *Pax7* knockout mouse survives to term but usually dies prior to weaning while the heterozygous animal develops and reproduces normally. Phenotypically, the knockout exhibits severe growth retardation with a shortened maxilla, smaller serous gland tubules and a pointed snout. Derivatives from areas of overlapping temporal expression of *Pax3/7* in the neural tube appear normal in the *Pax7* knockout model. This suggests that the paralogue gene *Pax3* has played a compensatory role for the loss of *Pax7* (Mansouri et al., 1996).

## 2.4 TRANSCRIPTION FACTORS AND BRAIN DEVELOPMENT

In general, brain development i.e. neurogenesis and gliogenesis, relies on a cascade of events driven by changes in gene expression profiles of proliferating and differentiating neuroepithelial cells. These events are steered by transcription factors which themselves are developmentally regulated. The many known families of transcription factors involved in developmental processes each have their specific properties like the structure of the DNA binding domain e.g. the helix-loop-helix motif that determines the interaction at the DNA groove. Transcription factors may be activated by specific intracellular pathway signalling as a response to cell membrane receptors and extracellular signalling interactions. On the other hand, they may be dependent on the binding to other nuclear proteins to influence their activity. In turn however, all transcription factors alike bind to enhancer and promoter sequences of downstream genes in a pattern that results in the activation or repression of the genes. During early neurogenesis, the differences in patterning direct cells to become committed to the neuronal fate and specified to those cellular phenotypes required for a particular region. The best studied example of this occurs at the midbrain/hindbrain boundary after neural tube closure from E8.5 to E12.5.

### 2.4.1. The midbrain/hindbrain boundary development

The area designated at the midbrain/hindbrain boundary is known as the isthmus. During neurogenesis, the isthmus is considered a mid-hindbrain organiser that forms at the junction of the expression domains of the transcription factors *Otx2* and *Gbx2* (Fig. 2.5) (Broccoli et al., 1999; Joyner et al., 2000) and the anterior domain of *Fgf8* expression (Shamim et al., 1999). *Otx2* is found expressed in a decreasing gradient toward the isthmus from the midbrain while *Gbx-2* is expressed in a decreasing gradient from the hindbrain (Joyner et al., 2000). In a developmental cascade *Otx2*

induces *Pax2* that activates the expression of *En1*, which enters a feedback loop with the signalling molecule *Fgf8* that regulates the later expression of *Pax5* (Liu and Joyner, 2001). *Pax5* upregulates *En2* with a positive feedback loop developing between the two genes. *En1* and *En2* expression overlaps that of *Pax2* and *Pax5* across the isthmus (Joyner, 1996) and *En1*, *En2* and *Pax5* expression is found throughout the mid/hindbrain boundary region (Liu and Joyner, 2001). The cascades ultimately result in the formation of the superior and inferior colliculi (as discussed in section 2.1.14), posterior midbrain, cerebellum and possible ventral brainstem (Trokovic et al., 2003).

Signalling molecules from one region have been demonstrated to repress the signalling molecules from the abutting region with the overlap found at the isthmus. The *Otx2* and *Gbx2* border is required to position the expression of the signalling molecules *Wnt1* and *Fgf8*. Initially, *Wnt1* and *Fgf8* are found outside the mid/hindbrain boundary in the entire midbrain and rhombomere 1 of the hindbrain respectively but on neural tube closure, *Wnt1* (posterior midbrain) and *Fgf8* (anterior hindbrain) expression becomes restricted to adjacent narrow rings at the isthmus until embryonic day 12.5 (Liu and Joyner, 2001).

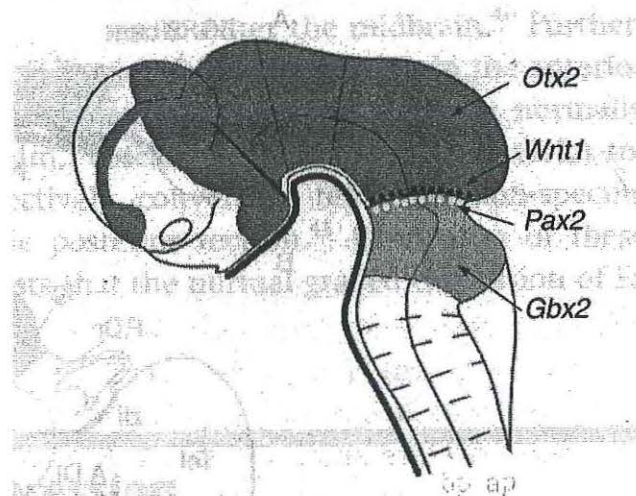


Figure 2.5

Expression patterns at the isthmus of embryonic mouse brain. After neural tube closure, the transcription factor *Otx2* expresses in the presumptive midbrain and the signalling molecule *Wnt1* is expressed in a narrow ring at the isthmus. The transcription factors *Gbx2* and *Pax2* express in the presumptive hindbrain with *Pax2* abutting that of *Wnt1* expression (Brown et al., 2001).

The important elements involved at the mid/hindbrain boundary are associated with a regulatory feedback loop that maintains the expression of each other (Shamim et al., 1999). Gradients of opposing and overlapping expression act by mutual repression to maintain each regional identity. This is also observed at the mid/forebrain boundary around the same period of neurogenesis.

#### 2.4.2. *The midbrain/forebrain boundary development*

Although the mid/forebrain boundary is not as well described as the mid/hindbrain boundary, it can serve as another example of how the specific expression patterns of transcription factors and their interplay influence brain development. The caudal forebrain is divided into three compartments being the pretectal region, the dorsal thalamus and the ventral thalamus. Between the midbrain and pretectum lies the posterior commissure. On the induction of *Pax7* in the midbrain there is a simultaneous suppression of *Pax6* and *Pax6* expression is found in the diencephalon abutting that of *Pax7*. The anterior boundary of the midbrain is defined by the rostral *Pax7* and the caudal *Pax6* expression found in the posterior commissure of the pretectum (Stoykova and Gruss, 1994). Expression patterns of *Pax6* and *Pax7* in the pretectum suggest that they are expressed in different cell populations (Kawakami et al., 1997; Nomura et al., 1998). The *En* genes are thought to be transcriptional repressors, and with a decreasing expression gradient across the midbrain from caudal high to rostral low, these factors are also thought to repress forebrain genes at the mid/forebrain boundary. *Pax2* is also thought to repress *Pax6* at the mid/forebrain boundary (Stoykova and Gruss, 1994). *Otx2* and *Emx2* are factors suggested as having a developmental function in the forebrain although not in the posterior commissure (Suda et al., 2001).

#### 2.4.3. *Development of the superior colliculus*

Patterning of the early midbrain then allows these committed and specified neuroepithelial cells to migrate to their final destination and take part in the organisational phase of specific brain region development. The basic formation of the superior colliculus from single neuroepithelium occurs primarily between E11-E14. The mature superior colliculus is composed of seven layers with alternating layers of fibres and cells, while the formative superior colliculus consists of three primary layers, the stratum superficiale, the stratum intermedium and stratum profundum

(Edwards et al., 1986b). From E11, cells generated in the ventricular zone of the mesencephalic neuroepithelium, containing the germinal epithelial cells, migrate dorsally to the intermediate and marginal zones. The superficial layer is formed after the deep layers but prior to the completion of the intermediate layer. Superficial and deep layers are formed from independent cell populations (Edwards et al., 1986b). Peak cell generation for the superficial layers of the superior colliculus occurs from E12 (stratum opticum) to E13 (stratum griseum superficiale) while the deeper layers are formed at E11-E13 (Edwards et al., 1986b). Lamination or the layering of neurons and fibres in the intermediate and deeper layers begins at E14. By E15, a few retinal ganglion cell projections have reached the superficial layers and by E17 a large population of retinal ganglion cell axons penetrate all superficial layers concurrent with the near completion of cell migration (Edwards et al., 1986b).

The lamination process continues until the late embryonic and early postnatal periods accompanied by a considerable radial growth. At birth there are dense fibre bundles through all superficial layers but for the first 6 postnatal days these are removed from the *stratum griseum superficiale* (superficial grey layer) while those in the *stratum opticum* (optic layer) remain (Edwards et al., 1986b). Simultaneously, collaterals from axons in the *stratum opticum* extend and arborise the *stratum griseum superficiale* (Edwards et al., 1986a). The topography of retino-tectal projections has predominantly come from studies performed in chick (Matsunaga, 2001; Nakamura, 2001; Thomas et al., 2004). Generally, axons from the temporal retina project to the contralateral anterior superior colliculus while axons from the nasal retina project to the contralateral posterior superior colliculus (Bahr and Wizenmann, 1996). The topography of the tectum (the homologue to the superior colliculus in lower vertebrates) is thought to be formed from signalling molecules expressed as gradients on retinal axons with opposing gradients on tectal neurons. The gradient pattern is thought to be induced by a graded expression of transcription factors e.g. Pax7, Engrailed, inducing the cell surface Eph and ephrin family to play a predominant role in axon guidance (Knoll et al., 2001).

#### 2.4.4. Summary

The Stem Cell Leukaemia (*Scl*) gene encodes a basic helix-loop-helix transcription factor that functions as a cell fate determinant in the haematopoietic system. *Scl* expression has been found in the central nervous system, predominantly in the mesencephalon but also in the diencephalon and metencephalon. A recent *Scl* gene deletion study has indicated that *Scl* is required for normal mid/hindbrain development. The *Scl-LacZ* knockin reporter mouse has been developed for light microscopy analysis study the expression pattern of *Scl* in the brain. Further, a conditional yellow fluorescent (*eYfp*) reporter mouse for *Scl* expression has been generated using the *Cre-loxP* system. The mouse is an inducible model that generates fluorescence in *Scl* expressing cells upon tamoxifen administration. This mouse is bred for fluorescent and confocal analysis.

The *Pax7* gene encodes a transcription factor that contains a DNA binding domain called the paired box (Pax). *Pax7* also contains a homeodomain and conserved octapeptide sequence as well as a transactivation domain. *Pax3*, with a structure closely resembling that of *Pax7* is thought to offer partial redundancy where expression domains overlap. The expression pattern of *Pax7* suggests a functional role in the development of the neural crest, skeletal muscle and the central nervous system. During neurogenesis, *Pax7* is part of the cascade that delineates the midbrain from the forebrain and *Pax7* expressing cells are found in the ventricular zone where they migrate dorsally to the superior colliculus.

Throughout embryogenesis, the expression of transcription factors is developmentally regulated to maintain differences in specific cell types and tissues. These factors bind to enhancer and promoter sequences of downstream genes to activate or repress gene expression. Gradients of opposing and overlapping expression act by mutual repression to maintain regional identity. This has been observed at the mid/hindbrain boundary and the mid/forebrain boundary from E8.5 to E12.5. Patterning differences specify cell commitment and cascades ultimately result in the formation of specific regions, as the superior colliculus does from the mid/hindbrain boundary.

The *Scl* and *Pax7* genes encode transcription factors that express in the brain during embryonic neurogenesis, suggesting they may play some organisational role in brain

development. Both factors continue to express in the dorsal layers of the superior colliculus in postnatal and adult brain. The spacio-temporal overlap in expression suggests that *Scf* and *Pax7* may be involved in related functions during brain development and in the adult brain. This study was undertaken to ascertain whether *Scf* and *Pax7* were operating within the same cells during early neurogenesis through to adulthood, which then would suggest that they might be part of the same cascade in the development and/or maintenance of the mesencephalon.

### **3. MATERIALS AND METHODS**

### 3.1 ETHICAL CONSIDERATIONS

Ethical approval was obtained for this study from the Edith Cowan University Animal Experimentation Ethics Committee, approval code: 04-A13 and from the Institute for Child Health Research Animal Experimentation Ethics Committee, approval code: AEBC #133.

### 3.2 EXPERIMENTAL PROCEDURES

#### 3.2.1. *Mouse Models*

- The *Scl LacZ* knockin mouse model on a C57BL/6J background has the *LacZ* gene knocked in to the *Scl* locus so that the expression pattern of *Scl* can be visualised for light microscopy once sections have undergone the  $\beta$ -galactosidase enzyme assay.
- The 0.9E3 *Cre* ER(T) transgenic mouse model on a C57BL/6J background has *Cre recombinase* fused to the 5' *Scl* neural enhancer (0.9E3) as well as an oestrogen receptor ligand-binding domain. *Cre* is then produced on the administration of tamoxifen only in *Scl* expressing neurons. This inducible model is crossed with the *Rosa26 - eYfp* knockin mouse and the double-positive offspring are used for fluorescent and confocal microscopy analysis.
- The *Rosa26 - eYfp* knockin mouse model has the *enhanced Yellow fluorescent protein (eYfp)* knocked-in to the *Rosa26* locus, a ubiquitous housekeeping gene, to achieve a high level of fluorescence in all *Scl* expressing cells. A stop sequence flanked by LoxP sites precedes the *Rosa26-eYfp* gene in this knockin mouse. The stop sequence is cleaved once *Cre* binds the loxP sites and *eYfp* is expressed. By crossing the 0.9E3 *Cre*ER(T) mouse with the *Rosa26 - eYfp* knockin mouse, a strong yellow fluorescent signal is produced in *Scl* expressing neurons. Double-positive offspring are used for fluorescent and confocal microscopy analysis.

All mice were bred and cared for at the Telethon Institute for Child Health Research on a 12h/12hr light/dark cycle with water and food available ad libitum.



### 3.2.2. *Timed pregnancies*

To set up all timed pregnancies, female mice at least 6 weeks old received intraperitoneal (i.p.) injections of serum gonadotropin (Folligon; 2 units/0.1ml PBS, Intervet Australia) 2 days before mating and with chorionic gonadotropin (Chorulon; 2 units/0.1 ml PBS, Intervet Australia) 48 hours later. Fertile males were then added to the cage for one night. The following morning an external examination to detect a plug was performed on the females and the males were removed. For the *LacZ* timed pregnancies, both males and females were of heterozygote *LacZ*/wt genotype and heterozygote offspring were utilised for experiments. When results were analysed by fluorescent and confocal microscopy, a 0.9E3 *CreER*(T) transgenic mouse was crossed with a homozygous *Rosa26* – *eYfp* knockin mouse. Offspring with a double positive genotype were required to show *Scf* expression as yellow fluorescence because in these mice the fluorescent gene *eYfp* is activated upon tamoxifen administration.

### 3.2.3. *Harvesting embryos*

To harvest embryos at specific timepoints, pregnant mice were euthanased at the required time of pregnancy by cervical dislocation. Embryos were then removed and placed in PBS, followed by dissection from the uterine horn and yolk sac. The embryo was decapitated and the head was immersed in 4% paraformaldehyde (Sigma) fixative for 2 hours on ice and the lower body was used for genotyping. After fixation of the head, the embryonic tissue underwent three washes in PBS for 15 minutes, followed by immersion in 30% sucrose solution overnight at 4°C. Tissue was then cryofrozen the following morning in optimum cutting temperature formulation (OCT Compound; Sakura Finetek, USA) by immersion in liquid nitrogen.

### 3.2.4. *Tamoxifen injections*

To induce the expression of *eYfp*, 4-hydroxy-tamoxifen (Sigma) in corn oil was administered by intraperitoneal injection. Adult mice were given 2mg tamoxifen/ 0.2 ml in corn oil each day for 5 consecutive days. A week was then allowed for the expression of *eYfp* to accumulate before perfusion of the adult mouse. For the P21 timepoint, mice were given 0.5mg tamoxifen/0.5 ml in corn oil for 3 consecutive days from postnatal day 16. For earlier timepoints, the pregnant female received the

tamoxifen injections at a concentration of 0.5mg tamoxifen/0.5 ml in corn oil administered at E10.5 and 1mg/0.1ml at E11.5 for the E12.5 timepoint. At the E15.5, E18.5, E20.5 and P5 timepoints an amount of 1 mg/0.1 ml tamoxifen was given on E12 and 2 mg/0.2 ml was given on E13.

### 3.2.5. *Cross fostering of tamoxifen treated embryos*

To increase the survival of unborn pups from a tamoxifen injected female mouse during pregnancy, the embryos were born by Caesarean on E20.5 for the P5 timepoints. The pregnant female was euthanased by cervical dislocation to prevent anaesthetic accumulation in the embryos. The unborn pups were harvested separately, assisted with the commencement of breathing and wet with excrements from a foster mother so as to be accepted by the new mother. These new foster mothers were from an ARC-Swiss background with white fur colour to facilitate recognition/identification from the cross-fostered pups with a dark fur colour in the new litter.

Timed pregnancies were set up for the foster and the transgenic females so that a lactating foster mother would be available when the transgenic litter was due to be born. As the ARC Swiss foster mothers produce large litters they tend to give birth on E19.5 so the ARC Swiss pregnancies were set up a day earlier than the transgenic mice. At the day of birth, about half of the ARC Swiss litter were culled or given to other ARC Swiss mothers and replaced by the newborn pups of the transgenic female. The neonatal care given by the foster mother was monitored for thirty minutes and followed by regular checks throughout the day.

### 3.2.6. *Anaesthetic*

Anaesthetics (0.1ml/10gr body weight) were prepared using Ketamine 100mg/ml (Troy Laboratories) in conjunction with Xylazil 100mg/ml (Troy Laboratories) and 0.9% saline at a 4:1:5 ratio respectively. Ketamine is a dissociative anaesthetic while Xylazil provides sedation and analgesia. The anaesthetic was administered using a needle and syringe by the intraperitoneal (i.p.) route prior to perfusion.

### 3.2.7. DNA Isolation

Genotyping was performed on adult *Scl LacZ* knock-in and 0.9E3 *CreER*(T) mice at weaning age with DNA extracted from a tail tip. Mice were anaesthetised with the inhalant Methoxyflurane containing an analgesic, for tail tipping. 1-2ml of Methoxyflurane was placed on a cotton wool pad in a bell jar and allowed to vaporise. The mouse was then placed inside the bell jar until fully anaesthetised. For the earlier postnatal timepoints, a tail tip was taken after the administration of the ketamine/xylazil anaesthetic and just prior to perfusion. The lower body of the embryo was used for genotyping following dissection from the uterine horn.

The tail or embryo sample was placed in an ependorf tube and 0.5ml lysis buffer was added, made up from 0.1M Tris-HCl (pH8.5), 5mM EDTA (pH8.5), 0.5% sodium dodecyl sulfate, 0.2M NaCl and 82.5% autoclaved MilliQ H<sub>2</sub>O containing 20mg/ml Proteinase K (Roche). The tail sample was then digested overnight at 55°C by shaking at 1200rpm in the ependorf thermomixer. The following morning, digested tails were centrifuged at 13200 rpm for 15 minutes to pellet undigested material. The supernatant was transferred to a new sterile tube and 0.5ml isopropanol added to each supernatant/tube. Tubes were mixed by inversion for 20-30 mins until a fine white pellet was seen floating in solution and then centrifuged at 13200rpm for 14sec. The isopropanol was poured off and 1ml 70% ethanol added. The inversion step was repeated for 20-30mins and centrifuged at 13200rpm for 14sec. The ethanol was poured off and any remaining ethanol carefully pipetted out of the tube. 100ul of 10mM Tris-EDTA buffer made up from 10mM Tris-Cl and 1mM EDTA (pH 7.4) was added to each tube and the bottom of the tube was gently flicked about 4 times to dislodge the pellet from the side of the tube. Samples were shaken at 55°C at 1200rpm in thermomixer for about 1.5-2hours and then frozen at -20C until PCR was performed.

### 3.2.8. Genotyping of genetically manipulated mice using Real-time PCR

Amplified DNA was analysed by Real-time PCR assay using molecular beacons that are DNA hybridisation probes. The beacons have a stem and loop structure with the loop region being complementary to the target sequence and the stem region containing complementary arm sequences. Attached to one arm is a fluorescent

marker and to the other arm a quencher that dissipates energy from the fluorescent marker (Bustin, 2000). In this study the separate molecular beacons contain probes with complementary sequences to the *LacZ* gene (Fig. 3.1) and to the estrogen receptor region of *CreER(T)*. The real-time primers amplify 100bp products for both Real-time PCRs.

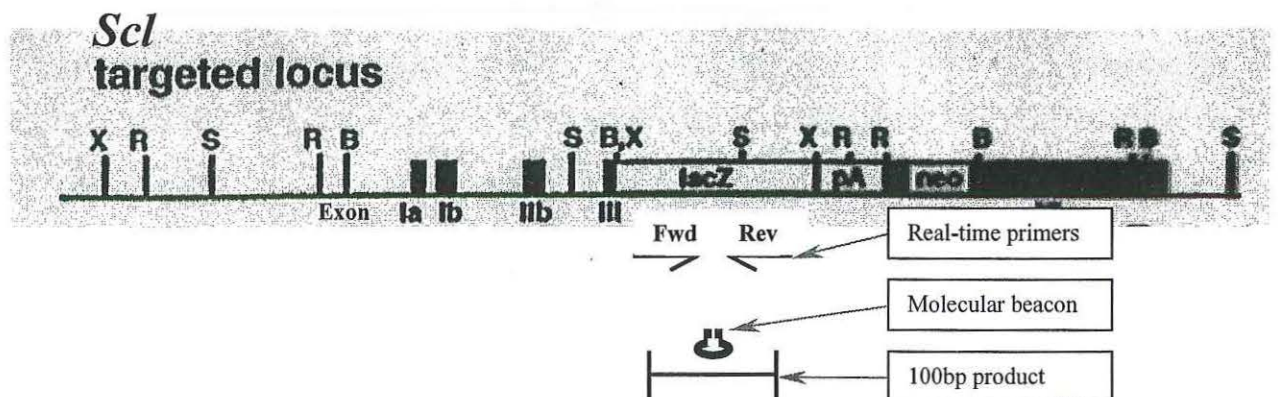


Figure 3.1 Real-Time PCR primers amplify a 100bp product within the *LacZ* cassette that has been knocked into the *Scl* gene. The molecular beacon hybridises to a sequence within the 100bp product and undergoes a conformational change that results in a fluorescent signal.

The *Cre* and *LacZ* Real-time PCR assays included 35 cycles of activation (95° for 600 seconds), denaturation (95°C for 15 seconds), annealing (55°C for 60 seconds) and extension (72°C for 15 seconds). During the annealing cycle, the molecular beacon loop underwent a conformational transition while hybridising with the target (Fig. 3.2B). Hybridisation forced the stem apart thus separating the fluorophore and quencher and so creating a fluorescence signal that was detected and quantified during each cycle by the Real-time PCR machine. The amount of fluorescence correlated with the amount of amplified product and the genotype was determined using a threshold value. The threshold value for the *LacZ* molecular beacon ranged from 0.05 - 0.15 fluorescence with an average of 0.1 while the *Cre* molecular beacon ranged from 0.02 - 0.05 fluorescence. The threshold value was reached during the exponential phase of amplification (>20 cycles), being significantly different from



background values (Bustin, 2000). If the fluorescence level was beyond that of the threshold value then the sample was positive for the specific transgene while a lower than threshold value meant that the sample was negative for the gene.

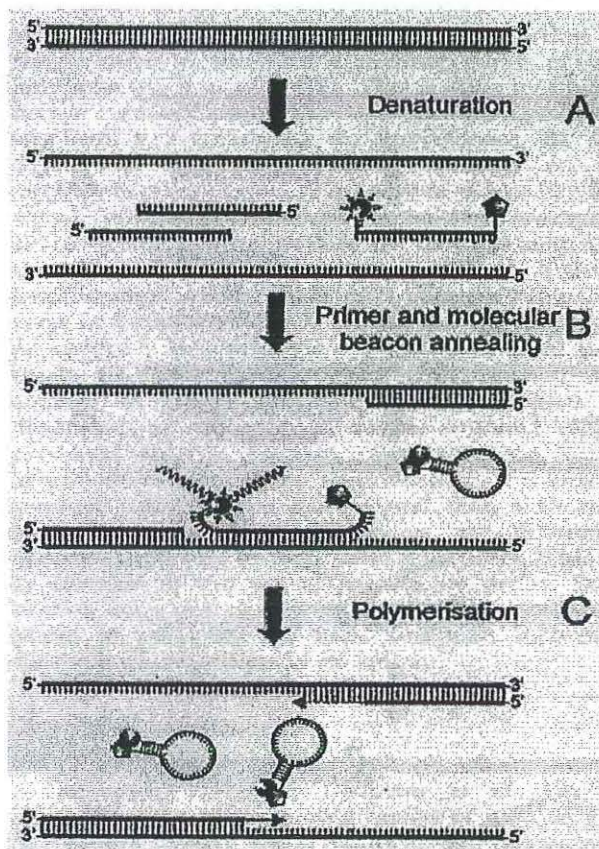


Figure 3.2 Real time PCR assay using a molecular beacon. (A) During the denaturation step the complementary arm sequences of the molecular beacon are denatured and the molecule fluoresces. (B) During the annealing phase the complementary stems will reanneal and the fluorophore will be quenched. However, in the presence of the target sequence, molecular beacons will also bind to the target and continue to fluoresce. (C) During polymerisation, the molecular beacon detaches from the target sequence, the arms reanneal and the fluorescence is again quenched. The intensity of the fluorescence is measured at the end of each cycle.

Analysis of the *LacZ* positive animals was done using the molecular beacon (MB) (MWG Biotech) for *LacZ* (5' CAC GCG GTG GTC GGC TTA CGG TGA TTT CGC GTG 3'), a *LacZ* (MB) forward primer (5' CTG GGA CTG GGT GGA TCA GT 3') and a *LacZ* (MB) reverse primer (5' CTG GCG ATC GTT CGG CGT A 3'). The *Cre* (MB) (5'- CCG GCT CAA GCC CGC TCA TGA TCA AAC GCA GCC GG

- 3'), the *Cre* (MB) forward primer (5' - GAT CTC GAG CCA TCT GCT G - 3') and the *Cre* (MB) reverse primer (5' - GGT CGG CCG TCA GGG ACA A - 3') were used to identify the double positive offspring from 0.9E3 *Cre*ER(T) transgenic mice crossed with the *Rosa26 - eYfp* knockin mice.

#### 3.2.9. *Transcardial Perfusion for the fixation of brain tissue*

To isolate the brain of neonatal and adult animals, transcardial perfusion was performed. The ketamine/Xylazil anaesthetic (0.1ml/10gr body weight) placed the mouse into a deep coma and this was confirmed by a tail pinch test, the absence of corneal reflex and normal breathing. A peristaltic pump was used to pump fixative into the circulation of the mouse via a 25G butterfly needle inserted into the heart. The blood was first removed by a 40 second phosphate buffered saline (PBS) rinse and followed by 4% paraformaldehyde (Sigma) in PBS (pH 7.2) kept on ice, for 10 minutes in neonates and 15 minutes in adult mice. Post-fixation was done after brain dissection in 4% paraformaldehyde for 30 minutes on ice. Three 15-minute washes in PBS at room temperature followed post-fixation and then the brain tissue was cryoprotected in a 30% sucrose/PBS solution at 4°C overnight. (All mice were killed when the fixative circulated through the brain and body).

#### 3.2.10. *Ice Hypothermia and transcardial perfusion of mouse pups*

Ice hypothermia was used as an anaesthetic for the P0 mice as they were too small to undergo i.p. injection. The technique provided immobilisation and mild analgesia and was effective due to the small surface area and body size. Pups were wrapped in a tissue to avoid freeze damage to the skin. The tissue was left open at the top to allow the pup to breathe and then the pup was placed in crushed ice for 3-4 minutes. During this time the pups became very pale. After performing the tail pinch test to confirm anaesthesia, transcardial perfusion commenced on a cooling platform to provide a constant level of hypothermia. Perfusion on pups was performed as described above for the adult.

#### 3.2.11. *Frozen Blocks*

Following overnight cryoprotection in 30% sucrose solution, brain tissue was placed in cryomold biopsy specimen moulds (Tissue-Tek Cryomold Biopsy) and embedded with OCT. The moulds were frozen on liquid nitrogen and stored at -80°C for later

analysis. Frozen tissue was sectioned into 20µm serial sections and mounted onto SuperFrost Plus slides (Menzel-glaser, Germany) using a Leica cryostat (CM3050).

### 3.2.12. *LacZ histochemistry*

Slides mounted with 20µm serial brain sections were air-dried for 30 minutes prior to the commencement of the *LacZ* staining procedure. The wash buffer was made just prior to use from 5mM EGTA (pH 8), 0.01% deoxycholate, 0.02% NP-40, 2mM MgCl<sub>2</sub>, 20mM Tris (pH 7.3) and 1x PBS (pH 7.2). Slides were rinsed in the wash buffer on the orbital shaker for 3 x 20 minutes with a change of wash buffer each time. The slides were then incubated at 37°C in the staining solution overnight in a dark location. The staining solution was made up from the 5mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5mM EGTA (pH 8), 0.01% deoxycholate, 0.02% NP-40, 2mM MgCl<sub>2</sub>, 20mM Tris (pH 7.3), 1x PBS (pH 7.2) and 1mg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma) in N, N-dimethyl formamide (40mg/ml).

The following day, slides were rinsed in PBS for 5 minutes and then dehydrated through an ethanol series—two minutes each in 70%, 90%, 100% and 100% followed by two changes in Histoclear (National Diagnostics) for two minutes each. The slides were coverslipped in Permount mounting medium (ProScitech) to prevent the *LacZ* staining from dissolving. When *LacZ* histochemistry was followed by immunoperoxidase staining, dehydration was delayed and immunoperoxidase staining commenced.

### 3.2.13. *Immunohistochemistry of mouse brain tissue for antibodies raised in mice*

The Vector MOM Immunodetection kit (Perth Scientific) was used for the Pax7, NeuN and Doublecortin (Dcx) antibodies as the kit is specifically designed for antibodies that have been raised in mice. Slides of 20µm serial brain sections were initially rinsed in PBS for 15 minutes and then the endogenous peroxidases quenched with a 0.6% H<sub>2</sub>O<sub>2</sub> in PBS solution for 20 minutes. This was followed by 3 x 15 minute PBS washes. An avidin block (4 drops of Avidin stock solution in 1000µl of PBS) was performed for 15 minutes followed by a 15-minute biotin block (4 drops of Biotin stock solution in 1000µl of PBS) using the Vector Avidin/Biotin Blocking Kit, with

both blocks rinsed afterwards for 5 minutes in PBS. The sections were further incubated for one hour in the Vector MOM mouse Ig blocking reagent (1 drop in 1250µl PBS) and washed 3 x 15 minutes in PBS. The primary antibody was added to the antibody diluent at a concentration of 1:10 (Pax7), 1:500 (NeuN) and 1:100 (Dcx). The antibody diluent was made up from a ratio of 300µl of Protein Concentrate stock solution (MOM kit) to 3750µl of PBS. The slides were drained of excess fluid and the primary antibody in antibody diluent added to the sections, covered with parafilm and incubated overnight in a humidified chamber at 4°C.

The following day, the sections were rinsed 3 x 15 minutes in PBS. The biotinylated α-mouse IgG reagent secondary antibody was diluted in the antibody diluent at 1:250 and incubated at room temperature for 2 hours. This was then rinsed 3 x 15 minutes in PBS. Sections were then incubated in the VECTASTAIN Elite ABC reagent (1 drop of reagent A and 1 drop of reagent B to 1250µl PBS) for one hour at room temperature followed by 3 x 15 minute washes in PBS. The chromogen diaminobenzidine tetrahydrachloride (DAB); (DAB Substrate Kit for Peroxidase, Vector Laboratories) was made up by adding 1 drop of Buffer Stock Solution to 2500µl of distilled water and mixed well. Then 2 drops of DAB Stock Solution were added and mixed followed by one drop of Hydrogen Peroxide solution, which was added and then mixed. DAB was visualised for colour from 2-10 minutes and the reaction stopped with the addition of PBS. Slides were then given 3 x 15 minute washes in PBS, sections flattened with a brush if required and left to dry for 30 minutes to an hour. The sections were then dehydrated through an ethanol series– two minutes each in 70%, 90%, 100% and 100% followed by 2 x 2 minutes in HistoClear (Scott Scientific). Slides were then coverslipped with Permount to prevent the *LacZ* staining from dissolving.

#### 3.2.14. Immunofluorescence for Pax7

To assess co-localisation of *Pax7* and *Sc1* in tissue sections by fluorescent and confocal microscopy, the following immunofluorescent protocol was performed for Pax7. Tissue sections were initially rinsed in PBS for 15 minutes followed by an incubation of the Pax7 primary antibody diluted 1:10 in standard antibody diluent of 1% Bovine Serum Albumin (BSA) and 0.2% TritonX-100 in PBS. Sections were



covered with parafilm and incubated in a dark humidified chamber overnight at 4°C. The following day, the primary antibody was rinsed with 3 x 15 minute PBS washes and then the secondary antibody, Cy3 goat  $\alpha$ -mouse IgG (Jackson ImmunoResearch Laboratories), having been diluted in the same standard antibody diluent at 1:300, was incubated at room temperature for 2 hours. The tissue sections were then given 3 x 15 minute washes in PBS, sections were drained of excess fluid and then coverslipped using Vectashield Mounting Medium (Vector Laboratories). Nail polish was painted around the coverslip to seal in the mounting medium and slides were stored in the dark at 4°C.

### 3.2.15. Microscopic Analysis

Light and fluorescent microscopy was performed on a Leica DMLB microscope and photographs were taken with a Leica colour digital camera DC300. Confocal analysis was achieved on a Bio-Rad MRC1000 confocal microscope at the Centre for Microscopy and Microanalysis at the University of Western Australia. The aim of this project was to look at the overlapping expression regions of *Scf* and *Pax7* from embryonic neurogenesis through to adulthood to assess co-expression between the two factors. The results reported in this study were not semi-quantitated by cell counting or density analyses.

### 3.2.16. Research Design

#### *Scf* & *Pax7* Co-localisation

The seven different timepoints were selected to follow the expression patterns of *Scf* and *Pax7* during the formation of the brain and in particular the development and subsequent innervation and lamination of the superior colliculus. Five to six timed pregnancies of *Scf-LacZ* knockin female mice were set up at each timepoint with an average of two embryonic/early postnatal litters produced. This was consistent with the 40% pregnancy rate previously achieved in the Cancer Biology Lab at the Telethon Institute for Child Health Research. The resulting litters were fixed with 4% paraformaldehyde at the timepoints of embryonic day (E) 12.5, E15.5 and E18.5. Perfusion was performed on two litters of mice at postnatal day (P) 0, P5, at weaning (P21) and in three adult mice (Fig 3.3). After brain fixation with 4% paraformaldehyde and overnight cryoprotection in 30% sucrose, the brains were

cryofrozen in liquid nitrogen and stored at -80°C. Once genotyping had been performed to detect the presence of the *LacZ* gene, mouse brains were sectioned at 20µm and mounted on SuperFrost Plus slides. Analysis of β-galactosidase and Pax7 immunohistochemistry were performed to indicate the expression patterns of *Pax7* and *LacZ*. Sections were also stained with the neuronal cell markers NeuN (Mullen et al., 1992) and Doublecortin (Rao & Shetty, 2004) to indicate neuronal subtypes within regions of co-localisation. Analysis was initially performed using light microscopy and confirmed by confocal microscopy.

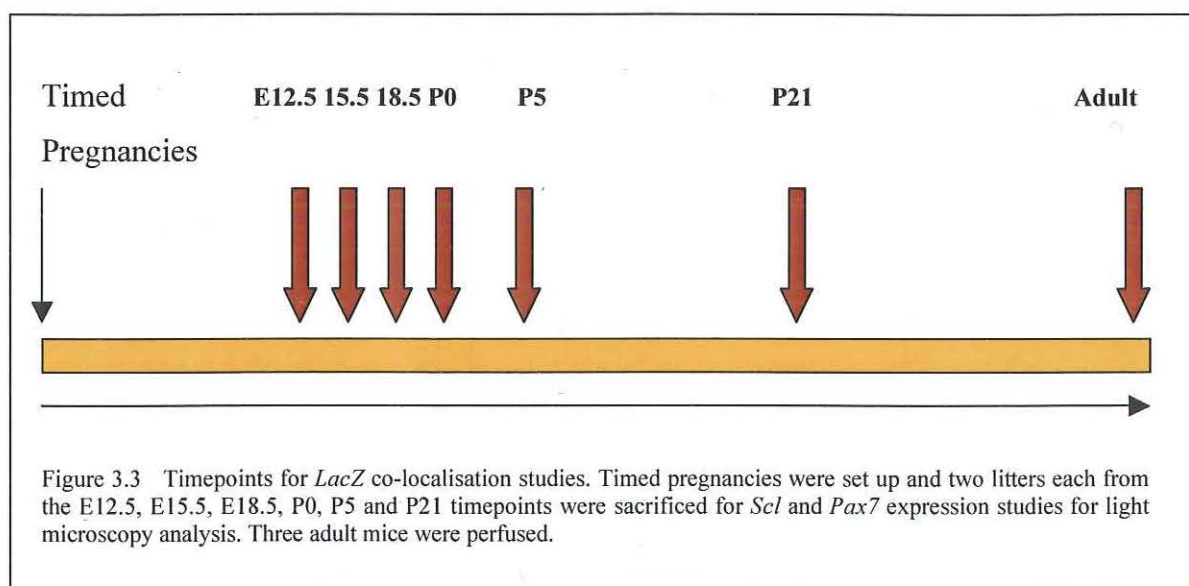
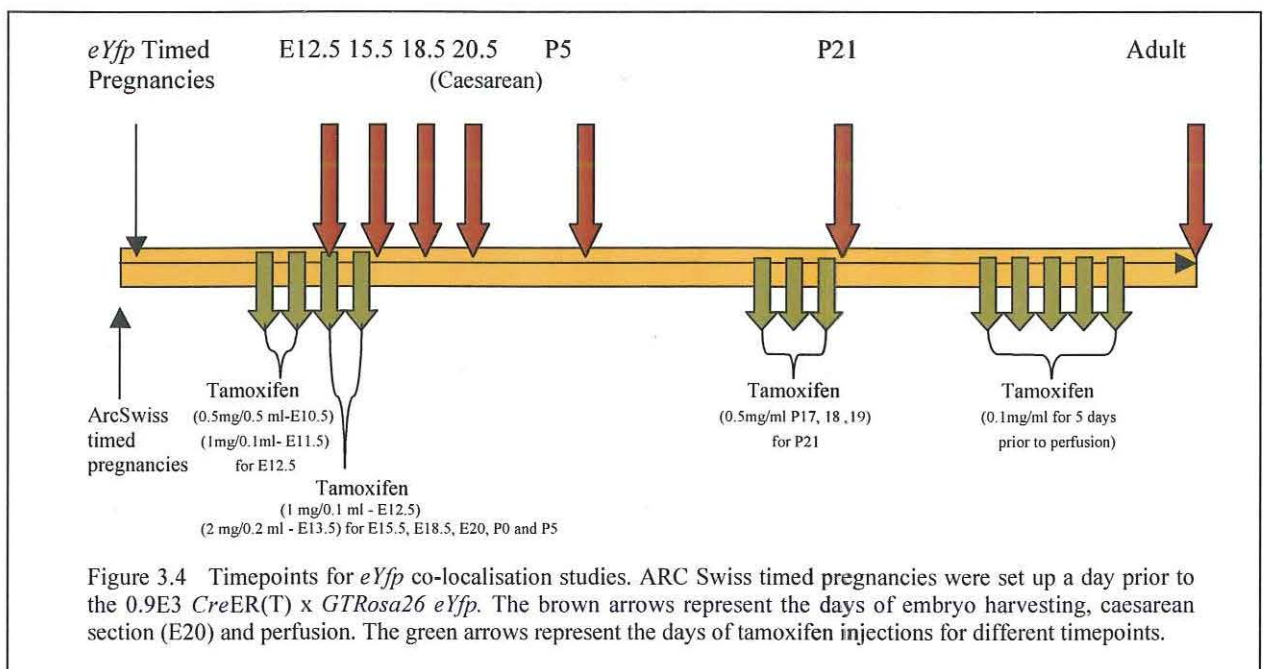


Figure 3.3 Timepoints for *LacZ* co-localisation studies. Timed pregnancies were set up and two litters each from the E12.5, E15.5, E18.5, P0, P5 and P21 timepoints were sacrificed for *Scl* and *Pax7* expression studies for light microscopy analysis. Three adult mice were perfused.

For confirmation of co-localisation using confocal microscopy, the fluorescent transgenic mouse model was used to display the pattern of *Scl* expressing cells in the CNS. Inducible 0.9E3 *CreER*(T) mice were crossed with R26R *eYfp* mice so that fluorescence could be visualised in *Scl* expressing neurons. Five to six timed pregnancies were set up with an average of two embryonic/postnatal litters produced per timepoint. To induce the *eYfp* expression in the brain, pregnant mice were given tamoxifen at 0.5mg/0.5 ml at E10.5 and 1mg/0.1ml at E11.5 for the E12.5 timepoint and 1 mg/0.1 ml was given on E12.5 and 2 mg/0.2 ml was given on E13.5 for the E15.5, E18.5, E20.5 and P5 timepoints. For the P21 timepoint, mice were given 0.5mg/ml in corn oil on P17, P18 and P19. Adult mice were given 5 daily injections of 1mg tamoxifen/ 0.1 ml in corn oil one week prior to perfusion (Fig. 3.4).

For the P0 timepoint, mothers were sacrificed and given a caesarean at E20 as it had been previously noted that some mothers had difficulty giving birth after tamoxifen injections. The E20 litters were not perfused but were fixed in 4% paraformaldehyde as with the earlier embryonic timepoints. For the P5 timepoints, mothers were sacrificed at E20 and after caesarean the pups were cross-fostered to ARC-Swiss mothers whose time pregnancies had been set up a day earlier. Once these pups reached P5 they were perfused, the brains fixed in 4% paraformaldehyde and then cryoprotected and cryofrozen. Genotyping was performed by RTPCR of DNA isolated from tail tips to determine the required genotype, 0.9E3 *CreER(T)*<sup>+</sup>, R26R *eYfp*<sup>+/w</sup>. The brains of positively identified animals were then cryosectioned and the immunofluorescent antibody staining method was performed to detect *Pax7* expression. Microscopic analysis was done using fluorescent microscopy with *eYfp* expression detected as yellow autofluorescence and *Pax7* expression as red fluorescence. The co-localisation of *Pax7* and *eYfp* expression was assessed with confocal microscopy. Cells that fluoresced orange were interpreted as cells in which yellow and red fluorescence had merged therefore co-expressing *eYfp* and *Pax7*.



## **4. RESULTS**

To assess the expression patterns of *Scf* and *Pax7* from embryogenesis into adulthood, 20µm brain sections were stained and analysed at seven different timepoints from embryonic day (E)12.5, E15.5, E18.5, P0, P5, and P21 through to the adult brain.

#### 4.1 LACZ STAINING

The expression pattern of *Scf* was visualised by light microscopy after the  $\beta$ -galactosidase enzyme assay (LacZ staining) had been performed on sections. The resulting blue staining was observed as small blue dots in the cytoplasm, possibly held within the Golgi apparatus as a non-functional protein. The overall *Scf* expression pattern was similar to that previously described (van Eekelen et al., 2003). Embryonic LacZ staining was often found to be weaker than staining in postnatal and adult brain regions. As embryonic tissue was not perfused, the 4% paraformaldehyde did not enter the circulation and so the difference in brain fixation may have possibly affected the quality of LacZ staining.

LacZ staining could be combined with *Pax7* immunoperoxidase staining for light microscopy analysis. However, background produced by the peroxidase reaction used to detect *Pax7* was too high if the combined staining procedure was performed on embryonic tissue. Moreover, in postnatal tissue where *Pax7* expression was most intense in dorsal superior colliculus, LacZ staining seemed compromised when combined with *Pax7* immunohistochemistry. This occurred at the P0 and P5 timepoints with the LacZ staining found paler when compared to controls. Strong blue staining was observed expressed in other brain regions on the same section but high magnification was needed to detect LacZ within dorsal superior colliculus. As the LacZ staining was not affected in tissue regions where *Pax7* expression was not found then the paler staining is unlikely to be a technical problem (personal observation).

#### 4.2 PAX7 STAINING

*Pax7* immunoperoxidase staining was observed as specific nuclear staining at all timepoints. The increased background observed at embryonic timepoints may be explained by differences in tissue, perhaps from increased amounts of peroxidases found within embryonic tissue. The observed expression pattern of *Pax7* has been

partially detailed in a previous report (Stoykova and Gruss, 1994). Experiments performed here have greatly extended knowledge of the *Pax7* expression profile throughout diencephalon, mesencephalon and metencephalon at several timepoints during development and postnatally.

### 4.3 FLUORESCENT AND CONFOCAL MICROSCOPY

With *Pax7* expression observed as nuclear staining and *Scf* expression as small blue dots in the cytoplasm, co-expression could not be confirmed by light microscopy. Although within a large population of the cells co-expression appeared likely, the adjacent blue staining may have come from an adjoining cell or one sitting directly above the *Pax7* expressing cell. Confirmation of co-localisation was therefore assessed by confocal microscopy that analysed sections at different focal depths. The series of virtual sections along the Z-axis were then overlayed for 3D visualisation using the Comos software. eYfp fluorescence reflecting the *Scf* expression pattern was seen under a fluorescent microscope as green nuclear staining while *Pax7* expression was seen as red nuclear staining. When detecting the cell co-expression of eYfp and *Pax7* under the confocal microscope, orange cells were observed from the merging of cellular expression whereas adjacent cells expressing eYfp and *Pax7* remained green and red. Unfortunately at most embryonic timepoints, the eYfp fluorescent signal did not reach the threshold levels necessary to visualise specific nuclear staining and so co-expression could not be confirmed at some of the earlier timepoints. The lower than threshold fluorescence was probably a result of the reduced timeframe in which the embryo had to generate fluorescence after tamoxifen administration. The tamoxifen was also given in reduced concentrations so as not to be detrimental to the embryos. The best fluorescent results for the eYfp protein were seen in the adult brain after five days of tamoxifen administration and a week was allowed for the accumulation of the fluorescence protein. However, in most cases the expression patterns of *Scf* and *Pax7* during embryonic neurogenesis were not found in the same regions and co-localisation was not suggested.

### 4.4 NEURONAL MARKERS

Some sets of sections having undergone *LacZ* histochemistry were also stained for the neuronal markers Doublecortin (Dcx) and Neuronal Nuclear Protein (NeuN) in order to identify the neuronal cell phenotype. Within the brain, NeuN is neuronal

cell-type specific and its expression initially appears at a time between the neuron progenitor cell leaving the proliferative phase of neurogenesis and the commencement of terminal differentiation. The expression continues in the mature fully differentiated neuron. In most neurons, NeuN immunoreactivity stains the nucleus however staining has been observed in the cytoplasm and even the processes of some neurons (Mullen et al., 1992). The neuronal marker Doublecortin (Dcx) is a microtubule-associated phosphoprotein (MAP) that is used as a marker to identify newly born neurons (Rao, 2004). The expression of *Dcx* is found in cells that are tangentially migrating with the strongest signal seen in both the soma and the leading processes of neurons (Francis et al., 1999). *Dcx* expression continues in the neuron during differentiation including the early growth period where dendritic and axonal growth is taking place. Some degree of overlap has been observed between *Dcx* and the mature neuron marker *NeuN* owing to the extended period of expression of *Dcx*. (Rao, 2004). *Dcx* expression normally ceases within 21 days after the birth of the neuron (Francis et al., 1999).

In this study, at the P21 timepoint, possible co-localisation of *Scf* with NeuN was seen in the superior colliculus but not in all cells as observed in the adult brain. Possible *Scf* co-localisation with Double-cortin (Dcx) in the dorsal superior colliculus, rostral inferior colliculus, the lateral interpeduncular nucleus and the posterior hypothalamic area was also observed whereas the control slide showed no specific staining. This would suggest that not all *Scf* expressing cells had fully differentiated into mature neurons at this time and some were still in an immature neuron state as indicated by their possible co-localisation with *Dcx*. However, neurogenesis is thought to be complete a few days prior to birth and the *Dcx* marker is meant to cease expression within 21 days after the birth of the cell. Our observation may indicate that *Dcx* was expressed for longer than 21 days after the birth of the cell which contradicts the current literature (Rao, 2004). On the other hand it may suggest that neurogenesis still occurs at a later stage than originally thought for which we do not have any further evidence. Maybe the most likely explanation would be that the staining observed was not indicative of *Dcx* expression for some technical reason. Unfortunately we were unable to co-stain NeuN and Dcx with Pax7 as the primary antibodies are all monoclonal and raised in mouse and therefore cannot be differentiated from each other with the  $\alpha$ -mouse secondary antibody in the



## Neuronal Markers



Figure 4.1 Expression patterns of *Scl* (blue) and *NeuN* (brown) at the P21 (A and B) and the P5 timepoints (C). Photomicrograph A illustrates *Scl* and *NeuN* staining in the superior colliculus and shows evidence of co-localisation in the vast majority of cells but not all. Photomicrograph B is a lower magnification of A.

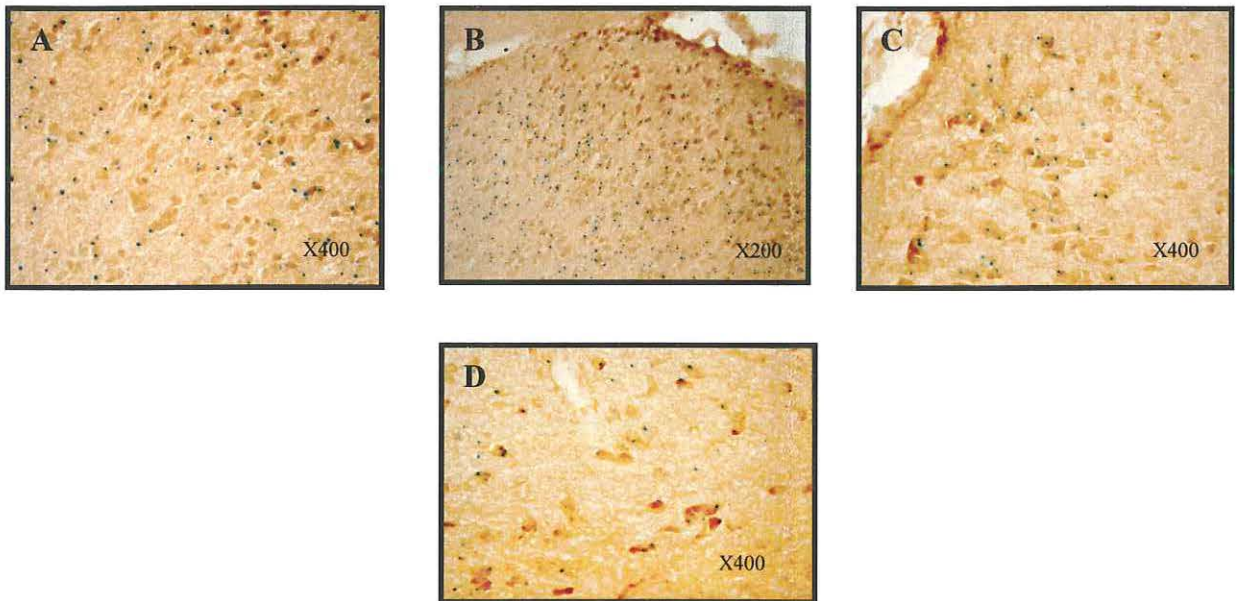


Figure 4.2 Expression patterns of *Scl* (blue) and the immature neuronal marker *Dcx* (brown). Photomicrograph A implies co-localisation between *Scl* and *Dcx* in the superior colliculus in nearly every *Scl* expressing cell at the P21 timepoint. Photomicrograph B is a lower magnification of A. Photomicrograph C illustrates possible co-localisation between *Scl* and *Dcx* in the interpeduncular region



immunohistochemical staining procedure. Immunohistochemistry using mouse monoclonal antibodies in a sequential manner has previously been performed but the results have displayed ambiguity. *Dcx* staining at the embryonic timepoints resulted in a great deal of background that did not allow for specific analysis whereas the postnatal staining was more effective and was found to co-localise with *Sc1*. The neuronal marker co-staining was not pursued as the results were inconsistent, they could not be performed in combination with *Pax7* and therefore they did not add to this research.

#### 4.5 MAPPING OF EXPRESSION PATTERNS IN THE BRAIN

The expression patterns were determined in the adult and P21 brains using the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001). For the earlier timepoint, mapping was established with the aid of prenatal mouse brain atlas (Schambra et al., 1992). The P21 and adult brain expression patterns were found to be the same and so will be described and discussed as one timepoint. Unfortunately, there is no brain map available for the early postnatal period. Despite the fact that the anatomy of the P5 brain has not fully matured at this age, the adult mouse atlas was used in the absence of a better alternative. For analysis of the P0 timepoint, the embryonic day 18 map was utilised. All analysis was done in the sagittal plane and postnatal expression patterns have been described firstly at the midsagittal level close to midline. The description then extends laterally to the midpoint of one hemisphere followed by the most lateral expression domains of *Sc1* and *Pax7*. As the embryonic brain is smaller, the description of expression patterns is all encompassing.

As the *Sc1* expression pattern is more extensive and described in great detail by van Eekelen et al (2003), the description in these results will only depict regions of expression where *Sc1* and *Pax7* are both found and where there is expression variation between different timepoints. During brain development, four main brain regions will be discussed where both *Sc1* and *Pax7* are expressed.

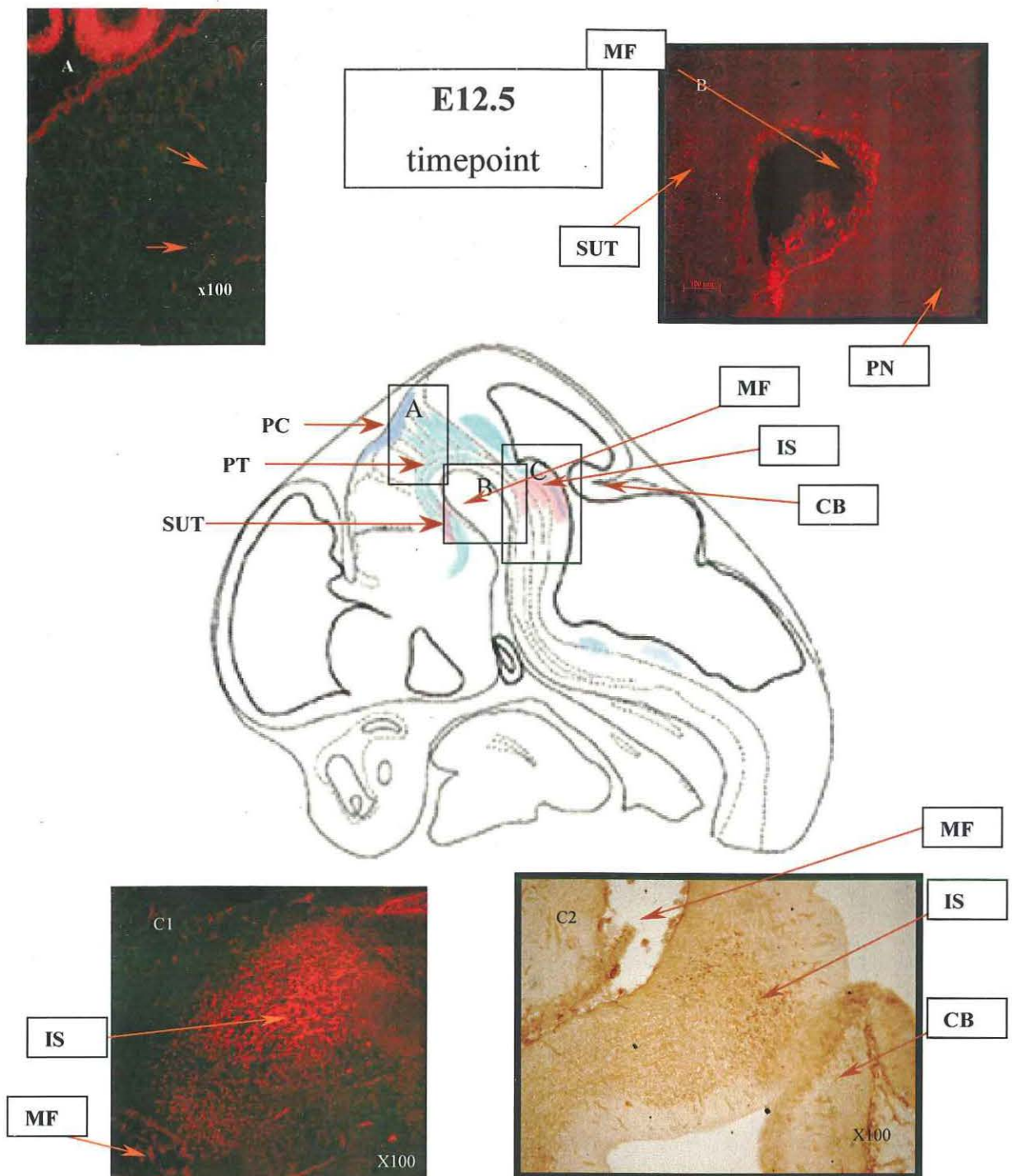


Figure 4.1 Expression patterns of *Scl* and *Pax7* at E12.5. The schematic drawing in the centre represents *Scl* (blue) and *Pax7* (pink). Indicated frames labelled A-C refer to the location of photomicrographs included in this figure. Photomicrograph A illustrates *Scl* expression in the posterior commissure (PC) and developing pretectum (PT) as analysed by confocal microscopy (indicated by yellow/green staining in the cell cytoplasm). Note some early migratory *Pax7* positive cells indicated by red staining in cell nucleus (arrows). Bright red staining of pia is considered non-specific. No co-localisation of *Pax7* and *Scl* based on the absence of red and green staining merged to orange is evident in this region. Photomicrograph B highlights *Pax7* expression in the subthalamus (SUT) and pons (PN) region around the mesencephalic flexure (MF) as analysed by confocal microscopy. Photomicrograph C1 and C2 illustrate *Pax7* expression in the isthmus (IS) as analysed by confocal microscopy (C1) and light microscopy (C2), with brown nuclear immunoreactivity.

As several different maps have been utilised to describe expression patterns, often these regions have structures that are different by name according to their level of development. For ease of identification, especially in the embryonic brain they will be described as the tectum (developing superior colliculus), the developing pretectal area and the developing tegmentum and rostral hindbrain.

#### 4.6 E12.5 TIMEPOINT

Peak cell generation occurs between E12 to E13 for the superficial layers of the superior colliculus during embryonic neurogenesis (Edwards et al., 1986b). *Pax7* was not found in the developing tectum (superior colliculus) at this timepoint but was seen in a small region of the subthalamus and in a broad expression region of high intensity within the isthmus (Fig. 4.1B). Dorsally, *Scf* expression was found in the developing tectum at the most dorsal layer, the posterior commissure, pretectum (Fig. 4.1A) and the tegmentum. The overlap of *Scf* and *Pax7* was seen in the region of the isthmus but with no indication of co-localisation after confocal analysis. These results were confirmed in three separate mice by performing both (immuno)histochemical and immunofluorescent staining for light microscopy and confocal microscopy. 139 sections underwent (immuno)histochemical staining for the mapping analysis at the E12.5 timepoint.

#### 4.7 E15.5 TIMEPOINT

At E15.5, retinal ganglion cell projections are beginning to innervate the superficial layers of the superior colliculus (Edwards et al., 1986b). At this timepoint, *Pax7* showed high expression in the dorsal ventricular zone with *Pax7* expressed in nearly every cell. From this region, scattered *Pax7* expressing cells were seen to migrate dorsally into the tectum from the ventricular zone (Fig. 4.2B). The scattered migrating cells were found throughout the superior colliculus and inferior colliculus. *Pax7* expression was also found in the rostral hindbrain in the region ventral to the dorsal raphe between the mesencephalic flexure and the aqueduct (Fig. 4.2C). More rostral, a small region of *Pax7* expression was found in the caudal thalamus.



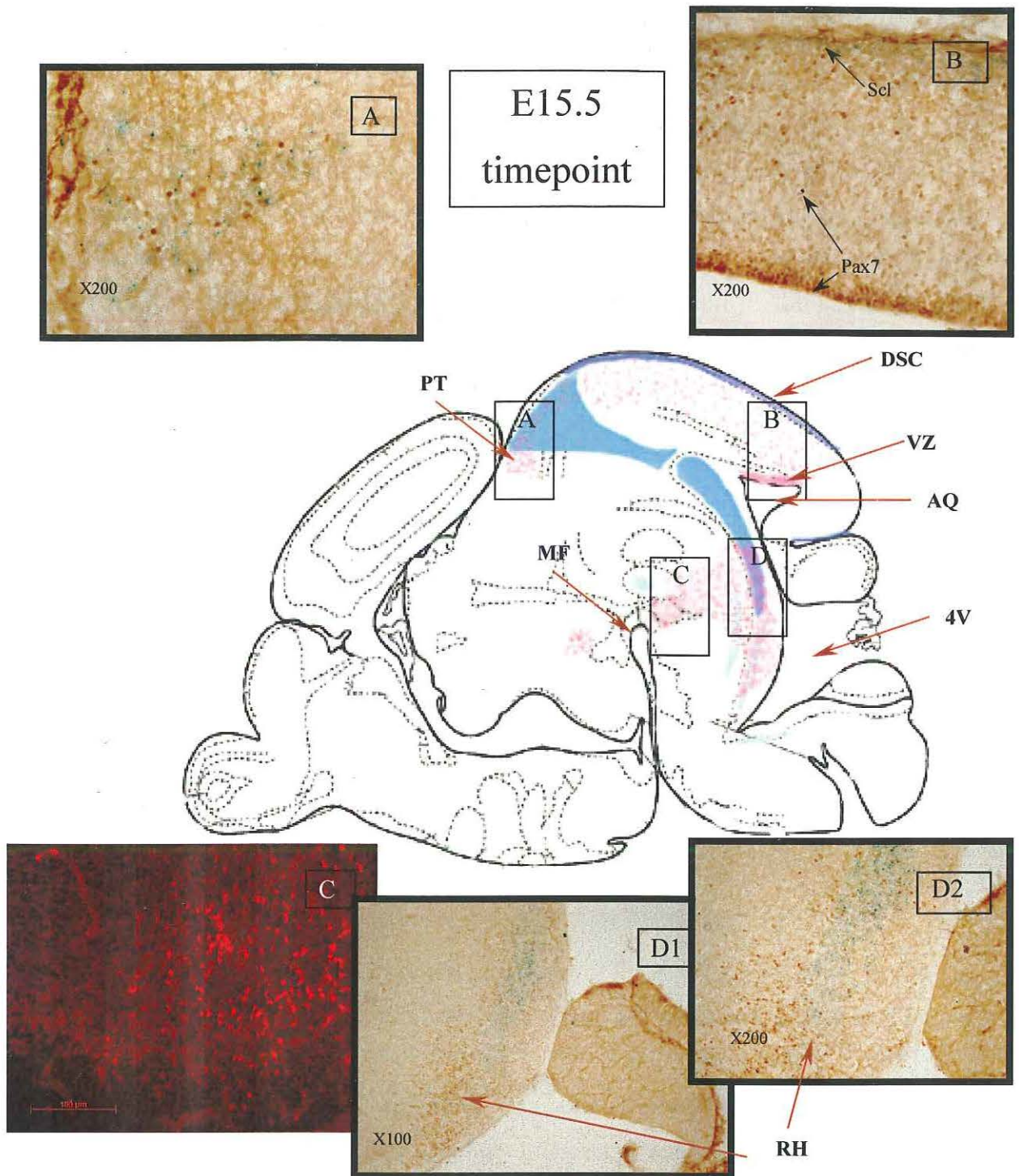


Figure 4.2 Expression patterns of *Scl* and *Pax7* at E15.5. The schematic drawing in the centre represents *Scl* (blue) and *Pax7* (pink). Indicated frames labelled A-D refer to the location of photomicrographs included in this figure. Photomicrograph A illustrates possible co-localisation of *Scl* and *Pax7* within the developing preteectum (PT). Co-localisation could not be confirmed in this region with confocal microscopic analysis, as the *eYfp* threshold was not reached. Photomicrograph B illustrates a high level of *Scl* expression in the most dorsal layer of the superior colliculus (DSC) while *Pax7* is expressed in nearly every cell in the dorsal ventricular zone. Scattered *Pax7* cells are observed migrating dorsally. Photomicrograph C illustrates fluorescent *Pax7* staining between the mesencephalic flexure (MF) and the aqueduct (AQ). Photomicrograph D1 finds *Pax7* and *Scl* expression overlapping in the tegmental region with *Scl* in caudal tegmentum and rostral hindbrain (RH) lining the fourth ventricle (4V) and *Pax7* extending into the developing rostral hindbrain. Photomicrograph D2 is the higher magnification of D1 showing the overlap but not co-localisation of *Scl* and *Pax7*.

*Scl* expression displayed a high level of expression in the most dorsal layer of the superior colliculus and was also found expressed in the inferior colliculus. *Scl* expression was seen in the subventricular zone of the dorsal tegmentum. No co-localisation between *Scl* and *Pax7* was indicated in the superior colliculus at this timepoint. Similarly, Expression of both *Scl* and *Pax7* showed possible co-localisation within the developing pretectum in the posterior commissure (Fig. 4.2A) but with no co-expression in the developing tegmental region (Fig. 4.2D1, D2). These results were confirmed after performing (immuno)histochemical and immunofluorescent staining on three separate mice. 261 brain sections were (immuno)histochemically stained for the E15.5 mapping analysis.

#### 4.8 E18.5 TIMEPOINT

In the superior colliculus at E18.5, a large population of retinal ganglion cell axons have penetrated all the superficial layers, and cell migration from the ventricular zone to the superior colliculus is almost complete (Edwards et al., 1986b; Edwards et al., 1986a). In similar patterning to that at E15.5, *Pax7* expression was seen scattered throughout the developing superior colliculus (Fig. 4.3B) extending to the inferior colliculus. *Pax7* expression also extended from the interpeduncular region (Fig. 4.3D2) to the rostral hindbrain region along the fourth ventricle crossing the presumptive median raphe region in the tegmentum. *Pax7* was again found expressed in the caudal thalamus (Fig. 4.3C).

*Scl* expression was seen in the dorsal most layer of the superior colliculus close to the midline (Fig. 4.3B). At the level of the dorsal raphe, *Scl* was found in the dorsal region while *Pax7* was seen in the ventral area. The rostral hindbrain region displayed overlapping expression but with no co-expression. For expression patterns at E18.5 three mouse brains were utilised for light microscopy analysis and two were utilised for fluorescent and confocal microscopy. A total of 132 brain sections were immunostained at the E18.5 timepoint.

#### 4.9 P0 TIMEPOINT

The P0 timepoint is a time of great activity within the superior colliculus as previously described in the literature. Innervation of the retinal axons is near completion and the superior colliculus undergoes extensive radial growth.



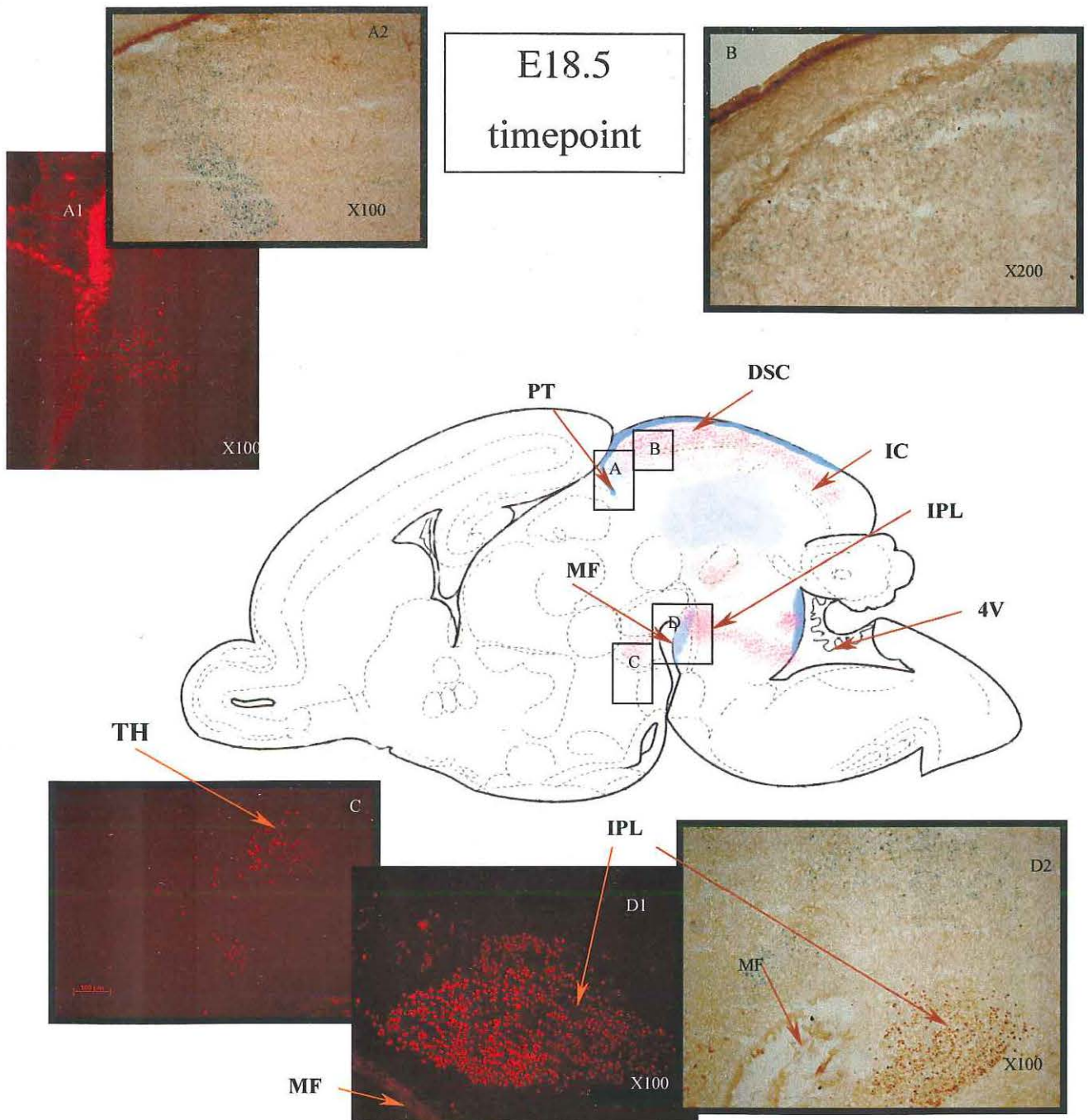


Figure 4.3 Expression patterns of *Scl* and *Pax7* at E18.5. The schematic drawing in the centre represents *Scl* (blue) and *Pax7* (pink). Indicated frames labelled A-D refer to the location of photomicrographs included in this figure. Photomicrograph A1 displays *Pax7* fluorescent staining in the developing pretectum (PT) and A2 illustrates *Scl LacZ* staining in the same region using light microscopy. Photomicrograph B illustrates high density *Scl* expression in the dorsal superior colliculus (DSC) and evenly scattered *Pax7* staining throughout the superior colliculus extending to the inferior colliculus (IC). Photomicrograph C illustrates the small expression area of *Pax7* in rostral thalamus with fluorescent staining. Photomicrograph D1 displays *Pax7* fluorescent staining in the interpeduncular (IPL) region extending toward the fourth ventricle and D2 illustrates *Pax7* and *Scl* expression surrounding the mesencephalic flexure (MF) with *Pax7* expression in caudal thalamus (TH) and the interpeduncular region. There is no co-localisation suggested between the two expression patterns at this timepoint.

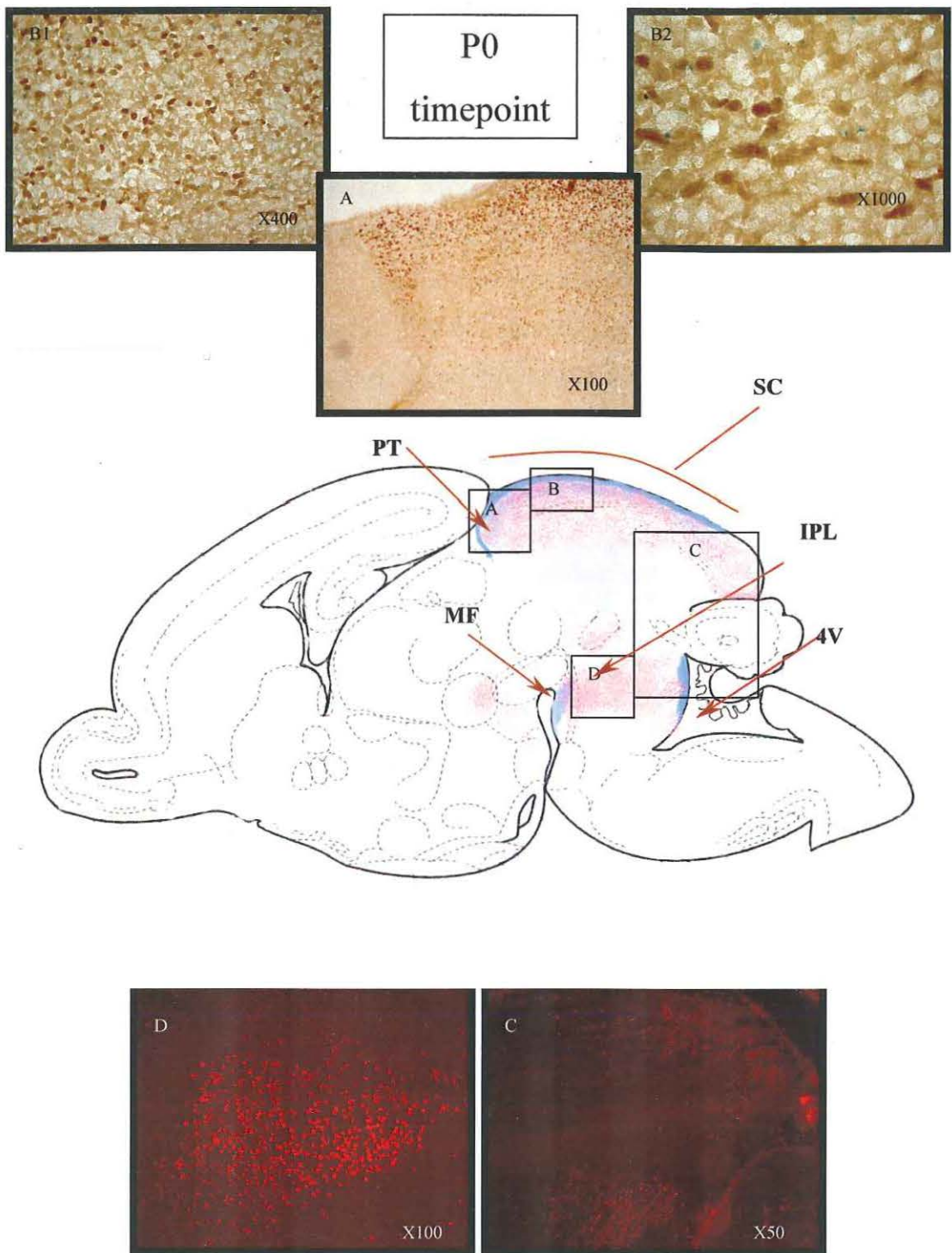


Figure 4.4 Expression patterns of *Scl* at E20.5 and *Pax7* at P0. The schematic drawing in the centre represents *Scl* (blue) and *Pax7* (pink). Indicated frames labelled A-D refer to the location of photomicrographs included in this figure. Photomicrograph A illustrates a strong band of *Pax7* expression in the preteectum (PT). Photomicrograph B1 shows *Pax7* and *Scl* in the dorsal superior colliculus (SC). *Pax7* expression is in high density throughout the dorsal superior colliculus at this timepoint and extensively throughout all the superior colliculus layers. Photomicrograph B2 is a higher magnification of B1. Note the pale *LacZ* staining amongst the dense *Pax7* staining. Photomicrograph C illustrates an overview of *Pax7* fluorescent staining in caudal superior colliculus and in rostral hindbrain showing expression along the almost mature mid/hindbrain boundary. Photomicrograph D shows the high density fluorescent *Pax7* staining between the interpeduncular (IPL) nucleus and the fourth ventricle (4V).

At this stage dense fibre bundles are present throughout all layers of the superior colliculus (Edwards et al., 1986b). Similar expression patterns were seen for *Pax7* at P0 as in the earlier timepoints but with extensive *Pax7* expression found throughout the superior colliculus and high density staining found within the dorsal layers (Fig. 4.4B1, B2). In rostral hindbrain, *Pax7* expression was found in the rostral part of the dorsal tegmental nucleus and caudal dorsal raphe (Fig. 4.4C). *Pax7* expression was also found ventral and within the interpeduncular nucleus (Fig. 4.4D). *Pax7* expression continued to be seen in a line of expressing cells from the mesencephalic flexure to the fourth ventricle along the almost mature mid/hindbrain boundary. The confined circle of *Pax7* expression consistently seen from the E12.5 timepoint was again found in the ventral thalamic region.

At P0, *Scl* expression in the superior colliculus increased into the deeper layers but not to the same extent as that observed for *Pax7* expression in this region. *Scl* was found more restricted to the most dorsal layers extending to the pretectum at a close to mid-sagittal level. *Scl* was also found in the dorsal tegmental nucleus and the dorsal raphe where expression overlapped that of *Pax7* but did not co-localise. A line of expressing cells for both factors was seen rostral to the dorsal raphe with a degree of overlap near the third ventricle. At sections more lateral from midline, *Scl* and *Pax7* expression became denser in the pretectum (Fig. 4.4A). In the most lateral sections displaying both factors, *Scl* was again limited to the most dorsal layer of the superior colliculus while *Pax7* expression remained extensive throughout the superior colliculus with a high density seen in the dorsal layers. For analysis at the P0 timepoint, three mouse brains were assessed using light microscopy and two mouse brains at E20.5 were assessed with fluorescent and confocal microscopy. In all, 217 brain sections were (immuno)histochemically stained for the mapping at the P0 timepoint.

#### 4.10 P5 TIMEPOINT

During the early postnatal period there is considerable radial growth in the superior colliculus and for the first 6 postnatal days fibre bundles are removed from the superficial grey layer while those in the optic layer remain. At the same time,



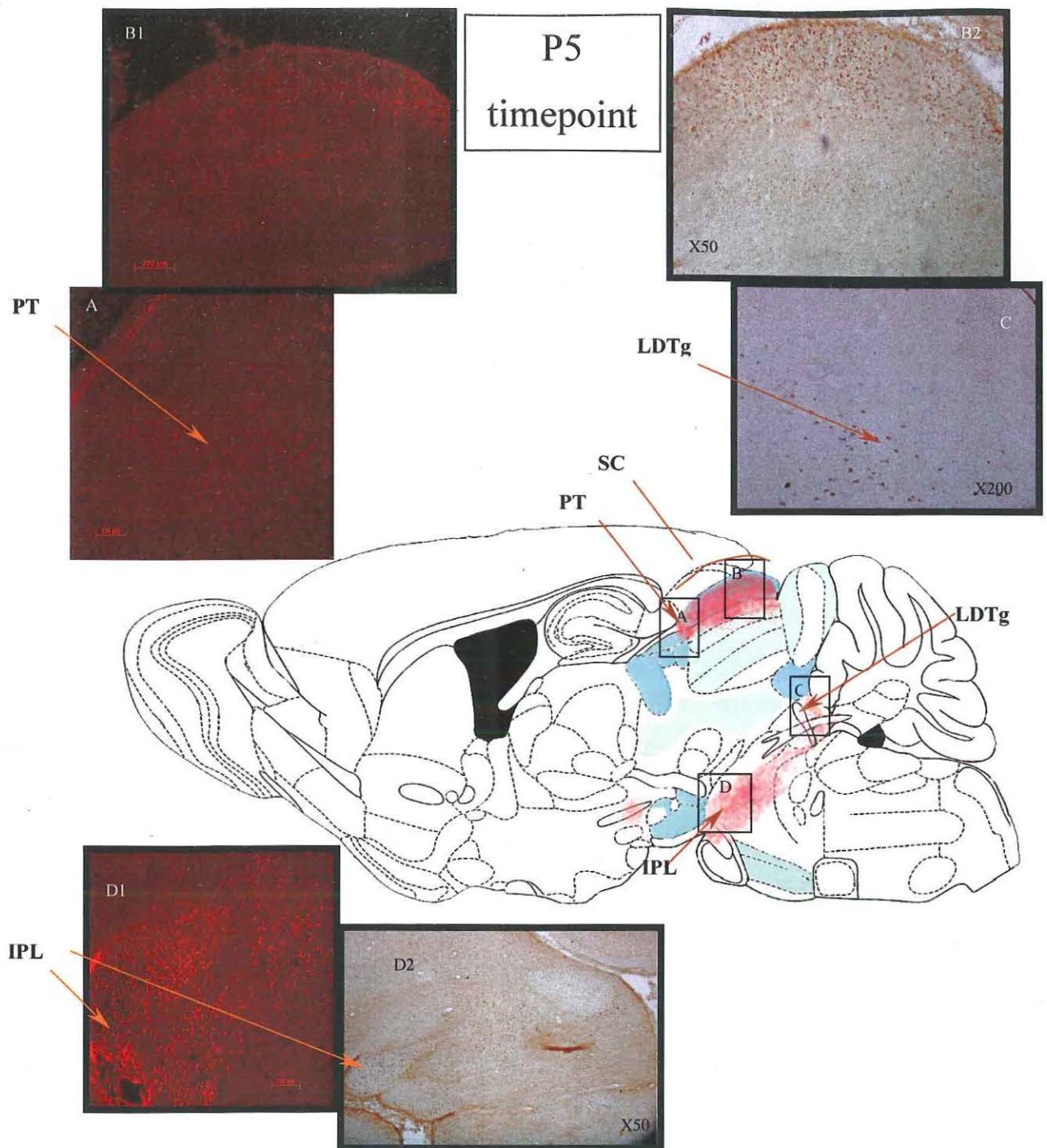


Figure 4.5 Expression patterns of *Scl* and *Pax7* at P5. The schematic drawing in the centre represents *Scl* (blue) and *Pax7* (pink). Indicated frames labelled A-D refer to location of photomicrographs included in this figure. Photomicrograph A illustrates fluorescent *Pax7* staining in the pretectum. Photomicrograph B1 displays *Pax7* fluorescent staining with extensive expression throughout the superior colliculus (SC). Note the rostro-caudal bands of *Pax7* expression seen within the layers of the superior colliculus in fluorescent and B2 in light microscopy of *Pax7* and *Scl* staining in the superior colliculus. Photomicrograph C illustrates the *Pax7* and *Scl* overlapping expression in the region of the laterodorsal tegmental nucleus without co-expression. Photomicrograph D1 shows fluorescent *Pax7* staining at the interpeduncular nucleus (IPL) and D2, light microscopy of *Pax7* expression seen from the interpeduncular nucleus to the fourth ventricle.

connections from axons in the optic layer extend and arborise the superficial grey layer (Edwards et al., 1986b).

Strong *Pax7* expression was found in the superficial layers (zonal, superficial grey and optic layer) of the superior colliculus and a medium density expression was found throughout the intermediate and deeper layers (Fig. 4.5B1, B2). Rostro-caudal bands of *Pax7* expression were seen within the layers of the superior colliculus. This is probably an indication that lamination has occurred. Unfortunately without a specific map for the P5 timepoint, it was difficult to ascertain the precise layers of the superior colliculus where the rostro-caudal bands were found expressed. The caudal high, rostral low gradient across the dorsal layers of the superior colliculus and the dorsal high, ventral low gradient across the superior colliculus (Fig. 4.6A4) was seen as described by Thomas et al, 2004. A strong pretectal expression (Fig. 4.5A) was also observed and a stream of stained cells seen expressed from the interpeduncular nucleus (Fig. 4.5D1) to the fourth ventricle (Fig. 4.5D2) although much narrower than seen at earlier timepoints. The similar circle of *Pax7* expression was again found in the caudal thalamic area of the posterior hypothalamus.

*Scf* expression was found more widespread within the superior colliculus at this timepoint (Fig. 4.5B2) and *Scf* was also found expressed in the cortex of the inferior colliculus. A strong pretectal expression was observed with possible co-localisation between *Scf* and *Pax7*. Scattered *Scf* cells were found dorsal to the line of *Pax7* expression from the interpeduncular nucleus to the fourth ventricle crossing median raphe but with no evidence of co-localisation. Both factors were expressed in the interpeduncular nucleus but again with no co-localisation confirmed. Overlapping expressions for *Scf* and *Pax7* was also found in the laterodorsal tegmental nucleus (Fig. 4.5C) with *Pax7* expressing ventrally and *Scf* dorsally with no indication of co-localisation. For analysis at the P5 timepoint, three mouse brains were assessed for light microscopy and three for fluorescent and confocal microscopy. 175 brain sections were (immuno)histochemically stained for light microscopy mapping analysis for the P5 timepoint.



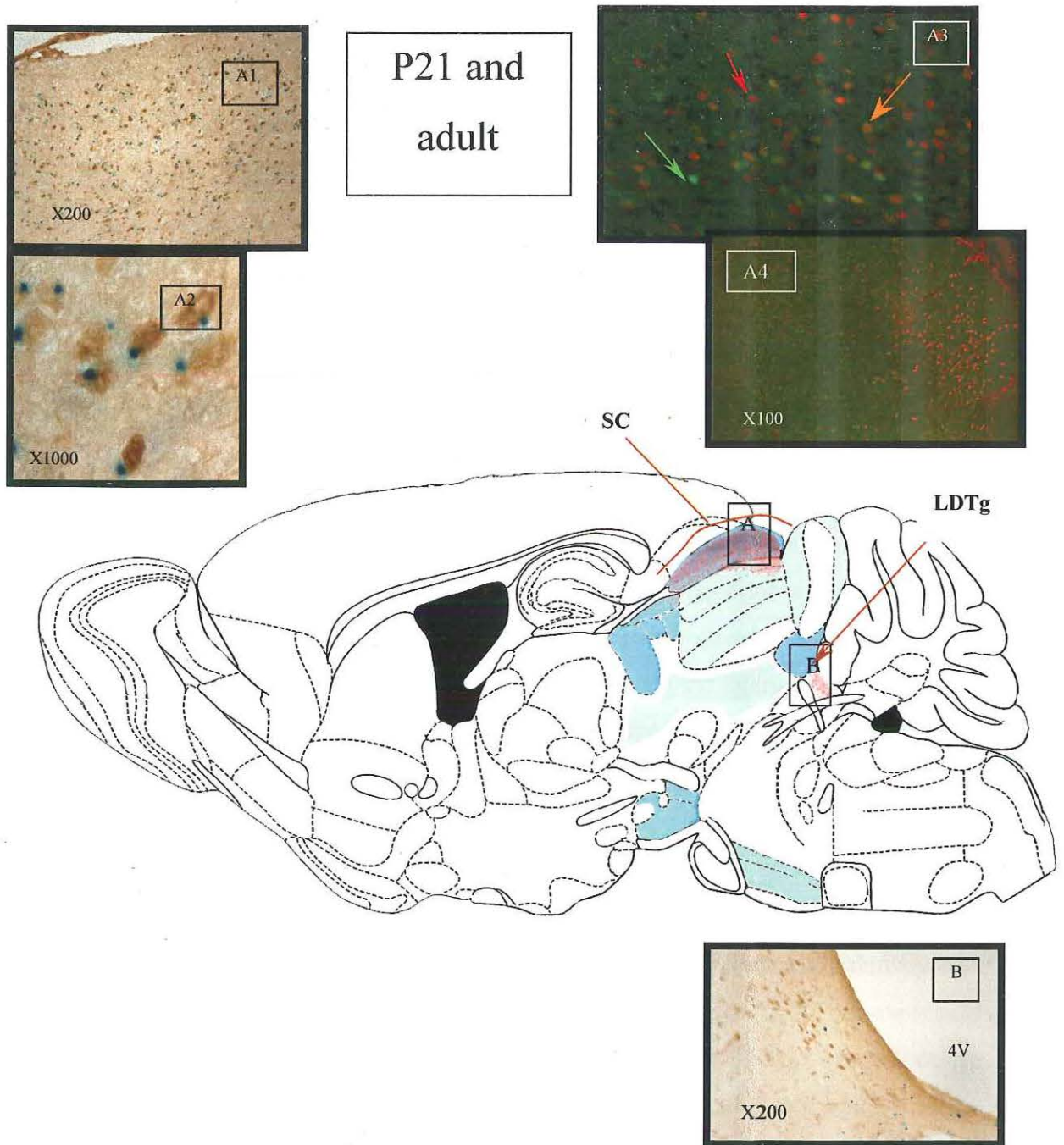


Figure 4.6 Expression patterns of *Scl* and *Pax7* at P21 and in the adult brain. Both timepoints are taken together as the expression patterns and morphology at P21 and in adult brain was found alike. The schematic drawing in the centre represents *Scl* (blue) and *Pax7* (pink). Indicated frames labelled A-B refer to the location of photomicrographs included in this figure. *Pax7* expression is restricted to the dorsal midbrain and a small region of expression in rostral hindbrain at these timepoints. Photomicrograph A1 illustrates *Scl* and *Pax7* expression in the dorsal layers of the superior colliculus indicating possible co-localisation. Photomicrograph A2 is higher magnification of A1. Photomicrograph A3 displays P21 confocal analysis of a high population of cells within the dorsal superior colliculus co-localising *Scl* and *Pax7*. (*Scl* - green, *Pax7* - red, co-localisation - orange) Photomicrograph A4 showing a strong *Pax7* fluorescent signal seen in a caudal high, rostral low gradient in the dorsal layers of the superior colliculus. Photomicrograph B illustrates the overlapping but not co-localising *Scl* and *Pax7* expression at the laterodorsal tegmentum (LDTg). This expression region in the rostral hindbrain is reduced from that at earlier timepoints.

#### 4.11 P21 AND ADULT TIMEPOINT

Remarkably, the expression patterns at postnatal day 21 and in the adult brain were found to be the same in every region for both *Scf* and *Pax7*. *Pax7* expression was found in a more restricted expression domain than that of *Scf* in the mature mouse brain.

*Pax7* was also reduced when compared to the *Pax7* embryonic expression pattern although with continued high expression in the dorsal superior colliculus. Expression was found within the superior colliculus, a few *Pax7* cells were found expressed in the inferior colliculus, the medial pretectal nucleus, the tegmentum and interpeduncular nucleus. *Pax7* was no longer found expressed in caudal thalamus or in a line of expression from mesencephalic flexure to the fourth ventricle. The caudal high, rostral low gradient across the dorsal layers of the superior colliculus and the dorsal high, ventral low gradient across the superior colliculus continued in the mature brain. *Pax7* expression was also found ventral to the laterodorsal tegmentum (Fig. 4.6B) and in the rostral part of the lateral interpeduncular nucleus. In sections further from the midsagittal cut, *Pax7* expression became limited to the superior colliculus.

*Scf* was found evenly expressed across the superficial grey layers of the superior colliculus in a high density (Fig. 4.6A1, A2). A gradient of decreasing density was observed from dorsal to ventral in the superior colliculus. *Scf* was expressed throughout cortical regions of the inferior colliculus. Outside the superior colliculus, *Scf* expression extended in more lateral sections than that of *Pax7*, mainly in the inferior colliculus and ventral midbrain region. In pretectum, *Scf* expression extended to the precommissural nucleus and parafascicular thalamic nucleus. In rostral hindbrain, *Scf* was also found expressed in the laterodorsal tegmentum (Fig. 4.6B). Co-localisation was indicated by light microscopy in the dorsal superior colliculus with a high density of co-expressing neurons found in the superficial grey layer (Fig. 4.6A1, A2). Confocal analysis confirmed that a high population of cells within the dorsal superior colliculus did co-express *Scf* and *Pax7* (Fig. 4.6A3). Although it would be useful to quantify the degree of co-expression in this area, confocal analysis does not allow for this as the laser bleaches the fluorescent signal and the light microscopy model cannot accurately enough determine those cells that co-express the

two factors. Both factors were also found to be expressed in the lateral interpeduncular nucleus. But here co-expression was excluded as *Pax7* expression was found in the rostral part of the lateral interpeduncular nucleus and *Scl* found in the ventrocaudal part of the lateral interpeduncular nucleus. For analysis at both the P21 and adult timepoints, two P21 and two adult mouse brains were utilised for light microscopy while one for each timepoint was analysed for fluorescent and confocal microscopy. For the P21 timepoint, 180 brain sections were (immuno)histochemically stained and 234 brain sections were stained and analysed under light microscopy for the adult brain analysis.

In summary, the expression pattern of both *Pax7* and *Scl* each remained relatively consistent during the process of development from E12.5 until adulthood. Comparative analysis of the two patterns over time reveals the partial overlap particularly in the developing pretectum, tectum and specific rostral hindbrain regions. However, the most striking result was that co-expression was only confirmed in the mature superior colliculus, specifically in the dorsal visual layers of the midbrain structure.

## **5. DISCUSSION**

## 5.1 NEW FINDINGS IN THIS STUDY

The major result from this study was that *Pax7* and *Scl* were found to co-localise within a subpopulation of postmitotic cells in the mature superior colliculus but with no evidence for this during embryogenesis, although co-expression in the pretectum before birth cannot be excluded. This contrasting outcome was striking because based on the preliminary studies in adult tissue, co-localisation between *Scl* and *Pax7* during brain development had been anticipated.

## 5.2 CHANGE OF FUNCTION OVER TIME FOR PAX7 AND SCL

In contrast to a range of neurogenic transcription factors which cease to be expressed after neurogenesis, like *tal2* (Bucher et al., 2000) and *mash1* (Lo et al., 1991) among others, *Scl* and *Pax7* continue to be expressed in the adult brain. This unperturbed expression over time suggests a possible change in function from playing a crucial role in neuronal differentiation before birth (Kawakami et al., 1997) to one of maintenance of a fully differentiated cellular phenotype within the dorsal layers of the superior colliculus in the adult brain.

My observation that *Scl* and *Pax7* were not found in the same cells during embryonic neurogenesis, whereas they were found co-expressed in the same cells of the fully developed brain is likely to support this proposed change in function of *Pax7* and *Scl* over time. A described molecular mechanism which would allow a change of function of transcription factor *Scl* over time and/or in different cells based on the finding that *Scl* acts as a gene regulatory protein within a multiprotein complex. Depending on the nuclear protein partners within such a complex, *Scl* is known to influence the expression of different downstream genes in different cell types (Begley and Green, 1999; Lecuyer and Hoang, 2004). Although less is known about the molecular mechanism of action of *Pax7* at the level of the DNA, a similar principle cannot be excluded. Thus, combinations of specifically co-expressed transcription proteins form multifactorial complexes that transcriptionally regulate other proteins. Expression changes of these factors may alter the target or the function of a complex and the loss of one factor may prevent the complex from forming (Lecuyer and Hoang, 2004). Such a disruption of a gene regulatory event during brain development may have consequences for the cascade of these events, which underlie proper

development of the central nervous system (Anderson, 1997). In this study, it may be that in the cascade of events defining embryonic neurogenesis in the tectum, *Scf* is downstream of *Pax7*, as *Pax7* expression is evident in the majority of proliferating neural stem cells in the ventricular zone of the aqueduct, where no *Scf* has been detected so far (this study; van Eekelen et al., 2003). If so, the absence of postmitotic embryonic tectal cells expressing both *Scf* and *Pax7* may then imply that *Scf* expression is permitted only if the *Pax7* expression is downregulated after proliferation. Alternatively, those cells that remain positive for *Pax7* and have been observed migrating from the ventricular zone to the dorsal tectal layers do not allow the expression of *Scf*. On the other hand, expression of different isoforms or splicing variants of *Pax7* and *Scf* would allow different functioning in different cells and tissues. A series of isoforms for both *Pax7* (Ziman et al., 2001a) and *Scf* (Calkhoven et al., 2002) have been reported but it is not known which isoform is present at what timepoint or in what region. Finally, the presence of a specific DNA binding sequence for *Scf*, the E-box in the *Pax7* gene suggests the theoretical feasibility that *Scf* could regulate the expression of *Pax7* (Nielsen, 1996; Begley and Green, 1999; Lecuyer and Hoang, 2004). Although this study does not provide the evidence for any of these mechanisms of action to happen, the possibility of a change in function over time is strongly implied by my observed alteration from expression of *Pax7* and *Scf* in different embryonic neurons to co-expression after maturation of the brain.

In recent times it has been suggested that neurogenesis is taking place within the adult brain. This is generally accepted to be occurring in the dentate gyrus and the subventricular zone (Arvidsson et al., 2002; Fabel et al., 2003; Jin et al., 2003; Montaron et al., 2003; Perez-Martin et al., 2003; Yoshimura et al., 2003). Other studies have explored alternate regions for evidence of adult neurogenesis (Lie et al., 2002; Zhao et al., 2003) with the latter finding progenitor cells in the substantia nigra suggesting that a basal replacement of neurons is taking place. Although the role of *Pax7* during embryonic neurogenesis involves the proliferation and early specification of neural stem cells, it seems unlikely that in the adult brain with *Pax7* expression observed in such a large number of scattered cells in the superior colliculus, these cells would indeed be neural stem cells undergoing continual regeneration. With the close proximity of the dentate gyrus to the superior colliculus, in histochemically analysed sections, studies based on the analysis of BrdU



incorporation into dividing cells being either neural stem cells or progenitor cells in the CNS (Arvidsson et al., 2002; Hellston, 2002; Fabel et al., 2003; Jin et al., 2003; Yoshimura et al., 2003) should have identified a high population of cells turning over within the superior colliculus if neurogenesis were occurring to such an extent.

Co-localisation of neural marker NeuN in every *Scl* expressing cell in adult brain indicates that *Scl* is expressed in neurons (van Eekelen et al., 2003). With confirmation of the co-localisation of *Scl* and *Pax7* in the dorsal superior colliculus, this would indicate indirect but convincing evidence that in co-localised cells, *Pax7* is also expressed in neurons. This has not previously been determined as both *Pax7* and NeuN antibodies are raised in the same species (mouse) and cannot be differentiated by the anti-mouse secondary antibody used in immunohistochemical analysis. If we are to believe that NeuN is indeed a mature neuronal marker, then the expression of *Pax7* in mature neurons is also further indication that it is unlikely that *Pax7* expressing cells in mature superior colliculus contribute to adult neurogenesis.

### **5.3 DYNAMIC EMBRYONIC EXPRESSION PATTERNS OF PAX7 AND SCL**

Information derived from knockout studies (Mansouri et al., 1996; Bradley et al., to be submitted) has shown that both *Pax7* and *Scl* are important neurogenic factors. Surprisingly in the embryonic tectum and developing superior colliculus there is no co-localisation found between the two factors. From E15.5, *Pax7* is expressed in the dorsal ventricular zone in nearly every cell. Here stained cells are seen migrating to the developing tectum. *Pax7* expression may be involved in the proliferation and early specification of newborn cells and in the persistence of the ventricular zone at this stage. At P0 and P5, extensive *Pax7* expression is seen across the superior colliculus with high density staining in the dorsal layers coinciding with a time of considerable radial growth and the removal of fibre bundles from the superficial grey layer of the superior colliculus. *Scl* expression on the other hand is found in the intermediate and marginal zone of the tectum and tegmentum at E12.5 neighbouring the ventricular zone. By E15.5 and E18.5, the *Scl* expression pattern is confined to the most dorsal layer of the superior colliculus. This highly specific and non-overlapping expression pattern of *Pax7* and *Scl* may suggest that *Scl* and *Pax7* have similar roles in neuronal subtype specification in the presumptive superior colliculus/tectum.

However, they do not seem to exert this similar function in the same cells. It is likely that *Sc1* and *Pax7* are involved in the neuronal differentiation of different subpopulations of neurons during tectal development.

*Pax7* has been described as a determinant in the mid/forebrain boundary formation in the embryonic brain (Kawakami et al., 1997; Nomura et al., 1998) where it is involved in a mutually repressive role with *Pax6*. The region of overlap between *Pax6* and *Pax7* (Stoykova and Gruss, 1994) is found in the posterior commissure. *Sc1* is also found in the posterior commissure possibly co-expressing with *Pax7* although confirmation was technically not possible (discussed later in this discussion). If there is co-localisation between *Pax7* and *Sc1* then this may imply that both factors are involved in boundary formation between the thalamus and midbrain.

Similar overlap of *Sc1* and *Pax7* expression was seen in the isthmus or wider mid/hindbrain region stretching from the mesencephalic flexure to tegmental and rostral hindbrain regions bordering the fourth ventricle. Interestingly, in this organising region *Pax7* is found expressed in rostral hindbrain and not midbrain. This is surprising as *Pax7* is described as a factor involved in the delineation, polarisation and regionalisation of the midbrain (Jostes et al., 1990; Stoykova and Gruss, 1994; Kawakami et al., 1997). With the lack of a detailed *Pax7* map this might have raised expectations in the past that *Pax7* would be found expressed on the midbrain side of the boundary.

#### 5.4 GENERATION OF AN EXPRESSION MAP FOR PAX7

To investigate the potential co-expression of *Pax7* and *Sc1*, a detailed mouse brain map was required and generated for *Pax7* in the absence of an established expression map at different embryonic timepoints and in the postnatal mouse brain. Previous *Pax7* expression patterns have been described at the E13.5 timepoint and in adult brain (Stoykova and Gruss, 1994) but the unavailability of a map at other crucial timepoints during brain development hampered accurate expression description of *Pax7*. The maps at E12.5, 15.5, 18.5, P0, P5 and P21 that were generated in this project allow the *Pax7* expression pattern to be followed during brain development and the early postnatal brain. Generally, the *Pax7* positive regions stayed the same throughout brain development although with varying densities of *Pax7* expressing

cells and/or varying intensities of *Pax7* at the different timepoints mentioned. The mapping at the P21 timepoint revealed the adolescent brain to be comparable to that of the adult brain both in terms of *Pax7* expression pattern and anatomical features.

## 5.5 APPLICATION OF GENETICALLY MANIPULATED REPORTER MICE FOR SCL EXPRESSION

In the past monoclonal as well as polyclonal antiserum against Scl have been raised in the group of Prof. Begley. Unfortunately, the use of these antisera proved limited in immunohistochemistry. Only Smith et al, 2002 has reported successful immunodetection of Scl in the embryonic spinal cord. It is thought that expression of different Scl isoforms in different tissue or brain regions may not recognise or be accessible to the antisera, explaining the failure of these antibodies in immunohistochemistry. To alleviate this problem of Scl detection in brain sections, van Eekelen et al. ('03) have previously used the *Scl LacZ* knockin reporter mouse. Newly generated transgenic mice expressing *CreER(T)* under the 0.9E3 neural enhancer of *Scl* crossed to R26R reporter mice can also be used and this has been described in detail in the introduction of this thesis. Unexpectedly, the 0.9E3 *CreER(T)* x R26R-eYfp model presented some difficulties in this study. At some embryonic timepoints the eYfp signal did not reach the required detection threshold to be able to confirm co-localisation with *Pax7* fluorescent staining using confocal microscopy. This may be because the time allowed for eYfp production after the tamoxifen injections was not long enough to generate sufficient eYfp protein to autofluoresce a strong enough signal. Alternatively, some interference while crossing the placenta may have reduced the amounts of available tamoxifen to the embryo to reduce the gene recombination of the R26R locus to express eYfp. Further, as the eYfp autofluoresces and no antibody to eYfp is available, it is not possible to amplify the signal as can be done when an immunofluorescent detection method is applied.

In summary, I have conducted a comparative expression analysis of two neurogenic factors from different transcription factor families, of which expression of both is sustained after birth in overlapping brain regions. These factors co-localise in the same neurons of the superior colliculus but only after maturation of the brain. During the process of brain development these two factors could have similar functions in

neural differentiation but in segregated cascades of gene regulatory events for different postmitotic neural progenitor populations.

## 5.6 FUTURE RESEARCH

Proposed short-term experimental research,

- To add complexity to this study, mapping the coronal sections of the mouse brain at each timepoint will allow a thorough three-dimensional analysis of the co-expression patterns of *Pax7* and *Scl*.
- To differentiate the layers of the superior colliculus particularly at those ages where no specific map is available, gold myelin and acetyl cholinesterase stainings could be carried out in adjacent sections. This is ideal to delineate the layers of the superior colliculus and will allow better interpretation of the results on a cellular expression basis.
- To ascertain whether the high expression of *Pax7* in the dorsal superior colliculus at P0 and P5 is from migrating cells into the superior colliculus, which enhance the total number of *Pax7* expressing cells or an increased or newly induced expression within existing cells of the dorsal layers, cell counting could be performed at E18.5 and P0. Conversely, cell counting could determine whether the decreased density of staining in the adult brain resulted from a downregulation of *Pax7* or an expansion of the superior colliculus. This could be achieved by co-staining with Nissl and expression of *Pax7*<sup>+</sup> cells as a percentage of total cell counts.
- To determine overlapping and abutting expression patterns of *Pax7* and other described neurogenic factors contributing to the patterning of the embryonic brain to study their involvement in the developmental cascades at the mid/forebrain and mid/hindbrain boundaries. Co-staining experiments based on the use of different antibodies against these factors involved at the boundary regions during embryonic neurogenesis could be performed.

Proposed longer term experimental research,

- To identify and quantify alterations of the expression patterns of *Scl* and *Pax7* in the adult brain, lesion studies could be investigated. The studies based on the destruction or deafferentation of specific brain regions may well verify whether *Pax7* plays a role in the repair and maintenance of the midbrain after lesioning the retino-

tectal connection thereby causing injury in need of a repair or regeneration process in the superior colliculus itself.

- To determine if different *Scl* isoforms exist within the brain, biochemical analysis using Western blots could be performed.

- Finally, to investigate whether *Scl* and *Pax7* associate with each other or with other factors in the same multiprotein complexes regulating gene expression immunoprecipitation experiments could be conducted. To study whether *Pax7* and *Scl* regulate downstream genes or each other's expression can be addressed by microarray analysis of cells in which both *Pax7* and *Scl* are expressed.

## **6. REFERENCES**

- Anderson DJ (1997) Cellular and molecular biology of neural crest cell lineage determination. *Trends Genet* 13:276-280.
- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 8:963-970.
- Bahr M, Wizenmann A (1996) Retinal ganglion cell axons recognize specific guidance cues present in the deafferented adult rat superior colliculus. *J Neurosci* 16:5106-5116.
- Begley CG, Green AR (1999) The SCL gene: from case report to critical hematopoietic regulator. *Blood* 93:2760-2770.
- Bopp D, Burri M, Baumgartner S, Frigerio G, Noll M (1986) Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of *Drosophila*. *Cell* 47:1033-1040.
- Bouchard M, Pfeffer P, Busslinger M (2000) Functional equivalence of the transcription factors Pax2 and Pax5 in mouse development. *Development* 127:3703-3713.
- Bradley CK, Takano E, Hall MA, Goethert J, Harvey AR, Begley CG, van Eekelen JA (to be submitted) The essential hematopoietic transcription factor SCL is also critical for neuronal development. *Mol Cell Biol*.
- Brocard J, Warot X, Wendling O, Messaddeq N, Vonesch JL, Chambon P, Metzger D (1997) Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc Natl Acad Sci U S A* 94:14559-14563.
- Broccoli V, Boncinelli E, Wurst W (1999) The caudal limit of Otx2 expression positions the isthmus organizer. *Nature* 401:164-168.
- Brown M, Keynes R, Lumsden A (2001) *The Developing Brain*. New York: Oxford University Press.
- Bucher K, Sofroniew M, Pannell R, Impey H, Smith A, Torres E, Dunnett S, Jin Y, Baer R, Rabbitts T (2000) The T cell oncogene tal2 is necessary for normal development of the mouse brain. *Dev Biol* 227:533-544.
- Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25:169-193.
- Calkhoven C, Muller C, Martin R, Kros G, Hoang T, Leutz A (2002) Translational control of SCL-isoform expression in hematopoietic lineage choice. *Genes and Development* 17:959-964.
- Chi N, Epstein JA (2002) Getting your Pax straight: Pax proteins in development and disease. *Trends in Genetics* 18:41-47.

- Cohen-Kaminsky S, Maouche-Chretien L, Vitelli L, Vinit MA, Blanchard I, Yamamoto M, Peschle C, Romeo PH (1998) Chromatin immunoselection defines a TAL-1 target gene. *Embo J* 17:5151-5160.
- Edwards MA, Schneider GE, Caviness VS, Jr. (1986a) Development of the crossed retinocollicular projection in the mouse. *J Comp Neurol* 248:410-421.
- Edwards MA, Caviness VS, Jr., Schneider GE (1986b) Development of cell and fiber lamination in the mouse superior colliculus. *J Comp Neurol* 248:395-409.
- Elefanty AG, Robb L, Birner R, Begley CG (1997) Hematopoietic-specific genes are not induced during in vitro differentiation of scl-null embryonic stem cells. *Blood* 90:1435-1447.
- Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci* 18:2803-2812.
- Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J (1999) Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron* 23:247-256.
- Garcia EL, Mills AA (2002) Getting around lethality with inducible Cre-mediated excision. *Semin Cell Dev Biol* 13:151-158.
- Gottgens B, Barton LM, Chapman MA, Sinclair AM, Knudsen B, Crafham D, Gilbert JG, Rogers J, Bentley DR, Green AR (2002) Transcriptional regulation of the stem cell leukemia gene (SCL)--comparative analysis of five vertebrate SCL loci. *Genome Res* 12:749-759.
- Green AR, DeLuca E, Begley CG (1991) Antisense SCL suppresses self-renewal and enhances spontaneous erythroid differentiation of the human leukaemic cell line K562. *Embo J* 10:4153-4158.
- Gruss P, Walther C (1992) Pax in development. *Cell* 69:719-722.
- Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* 75:463-476.
- Hall MA, Curtis DJ, Metcalf D, Elefanty AG, Sourris K, Robb L, Gothert JR, Jane SM, Begley CG (2003) The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. *Proc Natl Acad Sci U S A* 100:992-997.
- Hatton JD, Lin L (1992) Demonstration of specific neuronal cell groups in rat brain by beta-galactosidase enzyme histochemistry. *J Neurosci Methods* 45:147-153.



- Hellston J, Wennstrom, M, Mohapel, P, Ekdahlo, C, Bengzon, J & Tingstrom, A (2002) Electroconvulsive seizures increase hippocampal neurogenesis after chronic corticosterone treatment. *Eur J Neurosci* 16:p283-290.
- Hsu HL, Cheng JT, Chen Q, Baer R (1991) Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. *Mol Cell Biol* 11:3037-3042.
- Jin K, Sun Y, Xie L, Batteur S, Mao XO, Smelick C, Logvinova A, Greenberg DA (2003) Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice. *Aging Cell* 2:175-183.
- Jostes B, Walther C, Gruss P (1990) The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. *Mech Dev* 33:27-37.
- Joyner AL (1996) *Engrailed*, *Wnt* and *Pax* genes regulate midbrain-hindbrain development. *TIG* 12:15-20.
- Joyner AL, Liu A, Millet S (2000) Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. *Curr Opin Cell Biol* 12:736-741.
- Kallianpur A, Jordan, J. & Brandt, S. (1994) The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. *Blood* 83:1200-1208.
- Kawakami A, Kimura-Kawakami M, Nomura T, Fujisawa H (1997) Distributions of PAX6 and PAX7 proteins suggest their involvement in both early and late phases of chick brain development. *Mech Dev* 66:119-130.
- Knoll B, Isenmann S, Kilic E, Walkenhorst J, Engel S, Wehinger J, Bahr M, Drescher U (2001) Graded expression patterns of ephrin-As in the superior colliculus after lesion of the adult mouse optic nerve. *Mech Dev* 106:119-127.
- Lang D, Brown CB, Milewski R, Jiang YQ, Lu MM, Epstein JA (2003) Distinct enhancers regulate neural expression of Pax7. *Genomics* 82:553-560.
- Lecuyer E, Hoang T (2004) SCL: From the origin of hematopoiesis to stem cells and leukemia. *Experimental Hematology* 32:11-24.
- Lie DC, Dziejczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH (2002) The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci* 22:6639-6649.
- Liu A, Joyner AL (2001) EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. *Development* 128:181-191.
- Lo LC, Johnson JE, Wuenschell C, Saito T, Anderson DJ (1991) Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes and Development* 5.

- Mansouri A, Stoykova A, Torres M, Gruss P (1996) Dysgenesis of cephalic neural crest derivatives in Pax7<sup>-/-</sup> mutant mice. *Development* 122:831-838.
- Matsunaga E, Araki, I & Nakamura, H (2001) Role of Pax3/7 in the tectum regionalisation. *Development* 128:4069-4077.
- Montaron MF, Piazza PV, Aurousseau C, Urani A, Le Moal M, Abrous DN (2003) Implication of corticosteroid receptors in the regulation of hippocampal structural plasticity. *Eur J Neurosci* 18:3105-3111.
- Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116:201-211.
- Nakamura H (2001) Regionalisation and acquisition of polarity in the optic tectum. *Prog Neurobiol* 65:473-488.
- Nielsen A, Norby, P., Pedersen, F. & Jorgensen, P. (1996) E-box sequence and context-dependent TAL1/SCL modulation of basic helix-loop-helix protein-mediated transcriptional activation. *The Journal of Biological Chemistry* 271:31463-31469.
- Nomura T, Kawakami A, Fujisawa H (1998) Correlation between tectum formation and expression of two PAX family genes, PAX7 and PAX6, in avian brains. *Dev Growth Differ* 40:485-495.
- Paxinos G, Franklin K (2001) *The mouse brain in stereotaxic coordinates*, 2nd Edition. San Diego, CA: Academic Press.
- Perez-Martin M, Azcoitia I, Trejo JL, Sierra A, Garcia-Segura LM (2003) An antagonist of estrogen receptors blocks the induction of adult neurogenesis by insulin-like growth factor-I in the dentate gyrus of adult female rat. *Eur J Neurosci* 18:923-930.
- Rao MS, A. (2004) Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *European Journal of Neuroscience* Jan 19(2):p234-246.
- Robb L, Lyons I, Li R, Hartley L, Kontgen F, Harvey RP, Metcalf D, Begley CG (1995) Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. *Proc Natl Acad Sci U S A* 92:7075-7079.
- Schambra U, Lauder J, Siver J (1992) *Atlas of the prenatal mouse brain*. San Diego, California: Academic Press.
- Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA (2000) Pax7 is required for the specification of myogenic satellite cells. *Cell* 102:777-786.
- Shamim H, Mahmood R, Logan C, Doherty P, Lumsden A, Mason I (1999) Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain. *Development* 126:945-959.

- Sinclair AM, Gottgens B, Barton LM, Stanley ML, Pardanaud L, Klaine M, Gering M, Bahr S, Sanchez M, Bench AJ, Fordham JL, Bockamp E, Green AR (1999) Distinct 5' SCL enhancers direct transcription to developing brain, spinal cord, and endothelium: neural expression is mediated by GATA factor binding sites. *Dev Biol* 209:128-142.
- Srinivas S, Watanabe T, Lin CS, Williams CM, Tanabe Y, Jessell TM, Costantini F (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 1:4.
- Sternberg N, Hamilton D (1981) Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol* 150:467-486.
- Stoykova A, Gruss P (1994) Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J Neurosci* 14:1395-1412.
- Suda Y, Hossain ZM, Kobayashi C, Hatano O, Yoshida M, Matsuo I, Aizawa S (2001) Emx2 directs the development of diencephalon in cooperation with Otx2. *Development* 128:2433-2450.
- Thomas M, Lazic S, Beazley L, Ziman M (2004) Expression profiles suggest a role for Pax7 in the establishment of tectal polarity and map refinement. *Exp Brain Res* 156:263-273.
- Trokovic R, Trokovic N, Hernesniemi S, Pirvola U, Vogt Weisenhorn DM, Rossant J, McMahon AP, Wurst W, Partanen J (2003) FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmus signals. *Embo J* 22:1811-1823.
- Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23:99-103.
- van Eekelen JA, Bradley CK, Gothert JR, Robb L, Elefanti AG, Begley CG, Harvey AR (2003) Expression pattern of the stem cell leukaemia gene in the CNS of the embryonic and adult mouse. *Neuroscience* 122:421-436.
- Yoshimura S, Teramoto T, Whalen MJ, Irizarry MC, Takagi Y, Qiu J, Harada J, Waechter C, Breakefield XO, Moskowitz MA (2003) FGF-2 regulates neurogenesis and degeneration in the dentate gyrus after traumatic brain injury in mice. *J Clin Invest* 112:1202-1210.
- Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, Brismar H, Shupliakov O, Frisen J, Janson AM (2003) Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A* 100:7925-7930.
- Ziman MR, Pelham JT, Mastaglia FL, Kay PH (2000) Characterization of the alternate allelic forms of human PAX7. *Mamm Genome* 11:332-337.
- Ziman MR, Thomas M, Jacobsen P, Beazley L (2001a) A key role for Pax7 transcripts in determination of muscle and nerve cells. *Exp Cell Res* 268:220-229.

Ziman MR, Rodger J, Chen P, Papadimitriou JM, Dunlop SA, Beazley LD (2001b)  
Pax genes in development and maturation of the vertebrate visual system:  
implications for optic nerve regeneration. *Histol Histopathol* 16:239-249.