
Role of *Tbr2* in Intermediate Progenitors During Cortical Neurogenesis

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ABSTRACT

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During embryonic development neurons of the cerebral cortex are generated from various progenitor cells that have progressively restricted fate. Understanding the multiple regulatory pathways that regulate the cell cycle kinetics and the identity of neurons is crucial to comprehend the etiology of severe developmental defects such as microcephaly and polymicrogyria and also the evolutionary expansion of the mammalian cerebral cortex.

Intermediate progenitors (IPCs) express the transcription factor *Tbr2* (a T-box gene) and deletion of this gene causes a decrease in brain size and cortical thickness. However, little is known about the molecular mechanisms regulating behavior of IPCs.

In this thesis, I studied the molecular mechanisms regulating cell division and cell fate choices in IPCs using an overexpression system. I show that *Tbr2* controls the expression of key genes such as *Cdk4*, *Aspm* and *Wnt5a* by directly binding to upstream regulatory sequences. These downstream targets could explain the role played by *Tbr2* in cell cycle, spindle assembly and Wnt signaling in intermediate progenitors. The interaction with *Aspm* also suggests a possible mechanism of self-renewal of IPCs leading to an expanded generation of cortical neurons and ultimately an increased cortical size.

While the role of IPCs in cortical neurogenesis is undisputed, it is widely believed that they contribute only towards supragranular layers. Using a knock-in transgenic mouse line (*Tbr2^{Cre}*), I show that IPCs provide glutamatergic neurons (but not GABAergic neurons or GFAP+ astrocytes) towards all cortical layers in a significant proportion (20-40%). I also show that clonally generated neurons disperse within tangential dimension across the cortex significantly closer ($142.1 \pm 76.8 \mu\text{m}$) than unrelated ones ($294.9 \pm 105.4 \mu\text{m}$) though within the confines of a cortical column (300-600 μm).

Finally, I describe the similarity in the germinal zones of a large-brained gyrencephalic rodent, agouti and a lissencephalic primate, marmoset. Both these species show similar germinal zone cytoarchitecture and distribution of various progenitors. Further, the number of IPCs is grossly expanded thus demonstrating the conserved role of IPCs in cortical expansion regardless of the folding status of the cortex in these two species.

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STATEMENT OF AUTHORSHIP

All work shown in this thesis is original unless otherwise stated. No work or part thereof has been submitted for consideration for another degree.

Chapter 2 is based on work done by Dr. Jamin DeProto towards his D.Phil thesis titled “**Elucidating transcription factor driven molecular cascades involved in embryonic neurodevelopment**” submitted in 2012.

Figure 2.3A has been modified from his thesis. E14.5 brains of *Sox1^{Cre} x Tbr2^{flox/flox}* were provided by Dr. Matthias Groszer (INSERM, Paris).

Chapter 3 has been submitted as a research article titled “**Tbr2 expressing intermediate progenitors contribute to all cortical layers in the developing brain**” and is currently under review.

I undertook the work shown and discussed in this chapter including setting up of mating pairs, in-utero electroporation, perfusion of brains, sectioning of the brain tissue, immunohistochemistry for different antibodies, imaging of the tissues, cell counting and analysis. The manuscript was written by me and edited in close discussion with all authors.

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LIST OF ABBREVIATIONS

AP2gamma: Activating protein 2 gamma
Aspm: Abnormal spindle-like microcephaly-associated protein
bHLH: Basic helix-loop-helix
BLbp: Brain lipid binding protein
bp: Base-pair
BrdU: Bromo deoxy-uridylate
C: Celsius
CAG: Cytomegalovirus actin beta-globin promoter
Cdk4: Cyclin dependent kinase gene 4
cDNA: Copy deoxy-ribose nucleic acid
cHBSS: Combined Hank's balanced saline solution
ChIP: Chromatin Immunoprecipitation assay
cKO: Conditional knockout
CMV: Cytomegalovirus enhancer element
CNVs: Copy number variations
CP: Cortical plate
Cre: Cre recombinase
cRNA: Copy ribosenucleic acid
Ctip2: COUP-TF interacting protein 2
Ctnnb1: Beta-catenin gene (also β -catenin)
Cux2: Cut-like homeobox 2 gene
Cxcl12: Chemokine (CXC motif) ligand 12
DAPI: 4',6'-diamidino-2-phenylindole
DIG: Digoxigenin
DNA: Deoxy-ribose nucleic acid
DTT: Dithiothreitol
Dvl: Dishevelled
E: Embryonic day
E.coli: Escherichia coli
EDTA: Ethylene diamine tetra-acetic acid
EGFP: Enhanced green fluorescent protein
EYFP: Enhanced yellow fluorescent protein

FACS: Fluorescence activated cell sorting
FCS: Fetal calf serum
Fgfr1: Fibroblast growth factor receptor 1
GABA: Gamma amino butyric acid
GAD: Glutamate dehydrogenase
GFAP: Glial fibrillary acidic protein
GFP: Green fluorescent protein
GLAST: Glutamate aspartate transporter
GSK3 β : Glycogen synthase kinase 3 beta
GI: Gyrification index
HCl: Hydrochloric acid
Hes1: Hair cell enhancer of split 1 gene
IFL: Inner fibre layer
IgG: Immunoglobulin
INM: Interkinetic nuclear migration
IPC: Intermediate progenitor cell
IRES: Internal ribosome entry sites
ISVZ: Inner subventricular zone
IZ: Intermediate zone
Ki67: Monoclonal antibody against antigen KI 67
LGE: Lateral ganglionic eminence
LINES: Long interspersed nucleotide sequences
M-phase: Mitotic phase
M: Molar
mg: Milligram
MGE: Medial ganglionic eminence
ml: Millilitre
mT-Sapphire: Membrane tethered Sapphire fluorescent protein
NaCl: Sodium Chloride
Nes: Nestin gene
Neurod6: Neuronal differentiation 6 gene
Ngn2: Neurogenin 2
NPC: Neural precursor cell
oRG: Outer radial glial cell

OSVZ: Outer subventricular zone
Pax6: Paired box 6 gene
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
pH3: Phospho-histone H3
pVim: Phosphorylated vimentin
qPCR: Quantitative polymerase chain reaction
RC2: Intermediate filament-associated protein 2
RGC: Radial glial cell
RIPA: Radio-Immunoprecipitation Assay buffer
RNA: Ribose nucleic acid
RNase: Ribonuclease
Ror2: Receptor tyrosine kinase like orphan receptor 2
rpm: Revolutions per minute
RT: Real time
S-phase: Synthetic phase
Satb2: Special AT-rich sequence binding protein 2
Sfrp1: Secreted frizzled receptor protein 1
siRNA: Short interfering ribose nucleic acid
Sox2: SRY-box containing gene 2
Svet1: Subventricular expressed transcript 1
SVZ: Subventricular zone
Tbr1: T-box brain related gene 1
Tbr2: T-box brain related gene 2
tdTomato: Tandem dimer Tomato fluorescent protein
TE: Tris-EDTA
T α 1: alpha tubulin 1 gene
U: Units
VZ: Ventricular zone
Wnt5a: Wingless integrated gene 5 isoform a
ZO-1: Zona occludens 1 gene
 μ g: Microgram
 μ l: Microlitre

Chapter 1

General Introduction

1.1 GENERAL INTRODUCTION

The human brain contains on an average 86 billion neurons and an equal number of non-neuronal cells. The cerebral cortex in turn makes up for roughly 80% of the brain mass while only housing a fifth of the total neurons in the brain (Azevedo et al., 2009). This mass of neurons however controls important aspects such as speech, thought, movement and consciousness.

The mature cerebral cortex is arranged as a hexalaminar sheet comprising of neurons (including glutamatergic and GABAergic neurons), astrocytes, oligodendrocytes and microglial cells (Azevedo et al., 2009). The glutamatergic neurons are the predominant cell type found in this hexalaminar sheet varying in connectivity, identity and functionally (Mountcastle, 1997; Shipp, 2007). Starting from the top, the layer 1 (also called molecular layer) contains small Cajal-Retzius neurons that are amongst the earliest born cell populations and influence the proper laminar development of the cortex (reviewed in Marín-Padilla, 1998). Layers 2 and 3 form an external layer containing majorly granule cells and pyramidal cells respectively with apical dendrites remaining within the external layers and with axons connected to nearby areas or to the contralateral cortex in an associative role. The layer 4 cells (also Internal granule layer) receive inputs from the thalamic neurons and possess tangentially oriented fibres. The visual cortical layer 4 contains spiny stellate cells that lack an apical dendrite and are involved in local processing. Somatosensory spiny stellate cells similarly do not connect to cells outside of the same barrel. The Internal pyramidal layer (layer 5) contains the large pyramidal cells that form the corticospinal (and corticopontine) tract. These cells are thickly myelinated and have very long axons to reach target areas via the internal capsule. The lowest situated layer (layer 6 or multiform layer)

is further divided into an inner and outer layer on the basis of cell densities. The outer layer contains during early development the subplate zone (sometimes classified as layer 6b or layer 7) that receives transient thalamocortical innervation. Inner layer 6 cells both receive and project to the thalamus thus functioning as a relay in the circuit. Some cells in this layer project specifically to layer 4 while others project beyond them (Figure 1.1) (Bear et al., 2006; reviewed in Shipp, 2007).

Figure 1.1: Canonical circuitry of the various neuronal cell types in a typical cerebral cortical area.

In general layer 2/3 cells receive input from lower layers and relay it back within the layer or to lower layers. Layer 4 cells receive thalamic input and layers 5 and 6 receive input from upper layers and thalamus and send outputs to subcortical and thalamic nuclei respectively. Reproduced from (Shipp, 2007).

1.2 EMBRYONIC DEVELOPMENT OF THE CEREBRAL CORTEX

1.2.1 Neurulation and neural tube formation

The nervous system (including central and peripheral nervous system) arises from a sheet of ectodermal cells located at the dorsal midline of the gastrula called the neural plate. In order to form a closed structure that is later specified into the various parts of the nervous system (forebrain, hindbrain, spinal cord etc.), the neural plate undergoes several changes leading to its folding into a tubular structure. This process is called neurulation and begins with the folding of the neural plate to form a neural groove. The ends of the groove then come together

to fuse and form a tubular structure called the neural tube (reviewed in Smith and Schoenwolf, 1997). Simultaneously, the neural plate also lengthens anteriorly (Figure 1.1). Neural tube closure in mammals and humans occurs at multiple sites unlike that in chick and amphibian embryos (Golden and Chernoff, 1993; Van Allen et al., 1993). This precise activity is orchestrated beginning with expression of dissimilar cell adhesion molecules that allow or prevent fusion of adjacent cells (Detrick et al., 1990; Fujimori et al.).

Figure 1.2: Neurulation and neural tube formation

The neural plate at the dorsal midline (purple) invaginates, folds and pinches off from the non-neural ectoderm (blue) at the dorsal side to form a cylindrical neural tube that elongates antero-posteriorly to become the rudiments of the central nervous system. Reproduced from Gammill and Bronner-Fraser, 2003.

1.2.2 Development of the CNS

Upon formation of the neural tube and its elongation, differential changes set in at the anterior-posterior axis. The anterior pole undergoes rapid cell division to elongate and form a 3-vesicle structure (prosencephalon, mesencephalon, rhombencephalon) that will form the brain.

The prosencephalon further undergoes division to give rise to the telencephalon (cerebrum) and diencephalon (thalamus, hypothalamus, epithalamus and subthalamus). Similarly the rhombencephalon subdivides to form the metencephalon (pons and cerebellum) and myelencephalon (medulla). During this transformation, the brain also undergoes several flexures. However, while the cervical and pontine flexures straighten out, the cephalic flexure remains. This

causes the forebrain to lie in a different axis than that of the spinal cord (Figure 1.2). These five subdivisions together with the posterior part of the neural tube that subsequently develops into the spinal cord form the central nervous system.

Figure 1.3: Lateral view of development of the brain from the neural tube

Elongation and differentiation of the anterior neural tube leads to the initial formation of a 3-vesicle structure that contains the forebrain, midbrain and hindbrain. Over time, the forebrain further develops unequally leading to a large cerebral cortex in comparison to the smaller hindbrain and spinal cord. Reproduced from BrainFacts (www.brainfacts.org)

1.2.3 Development of the neocortex

Shortly after the formation of the telencephalon, signalling from the anterior neural ridge (ANR), the roof plate and the cortical hem (collectively called as 'organisers') specifies dorso-ventral and medio-lateral identity (Figure 1.3A). This also divides the former single telencephalon into left and right halves. Together with secreted factors from these organising centres, transcription factor expression further specifies the cortical ventricular zone leading to a regional specification of the neural tissue (Monuki and Walsh, 2001). One of the earliest genes to be expressed is the forkhead transcription factor gene 1 (*Foxg1*) by induction from *Fgf8* released from the ANR (Shimamura and Rubenstein; Tao and Lai, 1992). The telencephalic induction from *Foxg1* is then reinforced into cortical VZ identity by expression of neurogenenins and *Gli3* as well as by the cortical selector gene *Lhx2* (Mangale et al., 2008; Monuki et al., 2001). Thereon, unequal rates of division coupled with gradients of transcription factor expression (*Emx2*, *Pax6*), leads to an expansion of the VZ and initiation of the cortical neurogenesis program (Mallamaci and Stoykova, 2006; reviewed in Wilson and Rubenstein, 2000) (Figure 1.3B).

Figure 1.4: Specification and patterning of the telencephalon

A combination of secreted factors from the organising regions (A) and transcription factor expression (B) serves to specify the telencephalon and to demarcate the neocortex, hippocampus and the ganglionic eminences (Hébert and Fishell, 2008(B); reproduced from Marín and Rubenstein, 2001(A))

1.2.4 Neurogenesis in the neocortex

Neocortical neurogenesis in mammals serves to give rise to cortical projection neurons. These neurons are derived from the progenitors lining the dorsal surface of the lateral ventricles in the ventricular (VZ) and subventricular zones (SVZ). In addition, neurogenesis in the dorsal cortex proceeds in an ‘inside out’ manner i.e. earliest born cells occupy infragranular positions while later born cells are positioned superficial to the ones already generated (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1974).

Transplantation experiments where tritiated (H^3 incorporated) embryonic progenitors were grafted into neonatal ferret brains destined to produce layer 2/3 neurons showed that labelled cells that had completed the S-phase of the cell cycle migrated in keeping with the birthdate of the donor tissue. However when cells were grafted prior to completion of the S-phase, they adopted the laminar positions of the environment i.e. the host tissue (Frantz and McConnell, 1996; McConnell and Kaznowski, 1991). This shows that specification and laminar positions are determined both during and after neurogenesis. Identification of laminar specified progenitor has added to this debate (Franco et al., 2012). It is

hypothesised that the environment of the germinal niche might permit survival of a certain group of cells and not others (Franco and Müller, 2013).

In addition to the 'birthdate' and cell cycle, neuronal identity is also enforced by expression of a host of transcription factors (reviewed in Molyneaux et al., 2007). Additionally, choices between projection fates such as that seen in subcortical and callosal projection neurons are decided by mutual repression between genes such as *Ctip2* and *Satb2* (reviewed in Leone et al., 2008). *Satb2* is expressed by callosally projecting neurons while *Ctip2* marks subcortical projection neurons. Genetic mutants for either gene show aberrant phenotype of axon fibres (Alcamo et al., 2008; Arlotta et al., 2005; Britanova et al., 2008). Relaxation of the repression caused by *Ctip2* (by ectopic overexpression of *Satb2*) leads to reduced subcortically projecting cells and prevents axonal projections from crossing the cerebral peduncle (Britanova et al., 2008). Other genes in the upper layers (*Cux2*, *Brn2*, *Rorb* etc) perform similar functions.

Figure 1.5: Temporal and genetic specification of neuronal identity

Neurogenesis in the mice brain follows a temporal sequence (A) and cells born are arranged in an 'inside out' manner. (B) Laminar identity is also further enforced by a number of genes that are expressed in various patterns (reproduced from Molyneaux et al., 2007).

Neuronal migration in the developing cortex occurs in two main ways. Glutamatergic neurons generated in the dorsal cortex migrate radially outwards whereas GABAergic (gamma amino-butyric acid) neurons generated in the LGE and MGE migrate tangentially into the dorsal cortex (Anderson, 1997; De Carlos et al., 1996; Tamamaki et al., 1997).

Radial migration of neurons was proposed as a general trend for the movement of newborn neurons by Santiago Ramón y Cajal (Cajal, 1911). Apart from glial guided migration (Rakic, 1972), postmitotic neurons also undertake somal translocation. Cells that migrate using glial support display a saltatory movement with the help of a leading process twined around the basal process of the glia (Nadarajah et al., 2001). In contrast, neurons also display somal translocation where they migrate owing to a basal process attached to the pia. Such cells thicken and shorten their basal process during migration before terminating at the laminar position (Nadarajah et al., 2001). A third kind of migration is that of multipolar cells that do not possess a basal process or use glia but migrate by growth of their processes radially and occasionally tangentially (Nadarajah et al. 2001; Tabata and Nakajima, 2003).

Tangentially migrating GABAergic cells in comparison employ a wider diversity of migratory routes. Early born interneurons from the MGE follow a superficial route near to the piriform cortex and migrate into the marginal zone (MZ) or subplate (SP) before scattering into the cortex. Subsequently born cells from the MGE and LGE undertake a deeper route closer to the subpallial SVZ and migrate into the dorsal cortical SVZ in addition to the SP and MZ (Hernández Miranda et al., 2010; reviewed in Marín and Rubenstein, 2001).

Once into the cerebral cortex, interneurons then reach laminar positions by multipolar migration within the cortex (Nadarajah et al. 2001; Tabata and Nakajima, 2003).

Figure 1.6: Radial and tangential migration in the developing neocortex

(A) Radial migration occurs majorly by either somal translocation that involved thickening and shortening of the basal process of neurons (purple) or by glial guided movement where neurons display a saltatory movement (yellow cells) using a prominent leading process wrapped around glial processes (black lines). (B) Tangential migration is most prominently observed with interneurons that migrate towards the dorsal cortex. The upper panel shows the major routes taken while the lower panel explains the sites at which interneurons enter the developing cortex (modified from Gupta et al., 2002; Hernández Miranda et al., 2010).

1.3 PROGENITOR CELLS IN THE DEVELOPING NEOCORTEX

The developing mammalian neocortex shows considerable morphological diversity of progenitor types leading to generation of a diverse cell population consisting of glutamatergic neurons, astrocytes and oligodendrocytes. It is composed of largely multipotent progenitor cells that are able to generate a diverse population of cells differing in laminar positions, gene expression, morphology and function (Franco and Müller, 2013; Kriegstein and Alvarez-Buylla, 2009; (Betizeau et al., 2013)).

The progenitor cells can be differentiated by their morphology, cell biology as well as by expression of key genes that influences their behaviour (Kriegstein and Alvarez-Buylla, 2009).

In addition to differences in progenitor cells, there are also differences in the germinal zones between rodent and primate brains (Bayatti et al., 2008; Smart et al., 2002). Primate brains contain an extra germinal zone termed as the outer subventricular zone (OSVZ) that houses a large number of progenitors (Smart et al., 2002). In recent times, other species have also been shown to possess this germinal zone indicating its role in the evolution of larger cortices (Fietz et al.,

2010; García-Moreno et al., 2012; Karsten B Nielsen, 2010; Kelava et al. 2012). Despite these differences, common to all mammalian cortices are the following progenitor cells:

1.3.1 Radial glial cells (RGCs)

During neural tube closure, the neuroepithelial cells surrounding the ventricles lose expression of *occludin* but not of *ZO-1* (zona occludens protein 1) (Aaku-Saraste, 1996). This sets into motion, events leading to remodelling of the neuroepithelial cells to RGCs that then form the major progenitor cell population of the early neocortex (Malatesta et al., 2000; Noctor et al., 2002).

RGCs divide by asymmetric division during early neurogenesis to self-renew and also produce neurons (Miyata et al., 2001; Noctor et al., 2001) while at later stages of embryonic development, RGCs switch towards producing macroglial cells such as astrocytes and oligodendrocytes (Malatesta et al., 2000; Williams and Price, 1995). During neurogenesis, RGCs are characterised by their typical morphology i.e. having a nucleus in the ventricular zone that undergoes INM (interkinetic nuclear migration); a feature common to pseudostratified epithelial tissue (Misson et al., 1988; Miyata et al., 2001; Noctor et al., 2001; 2002; Sauer, 1935). They are also distinguished by the presence of a slender process that elongates towards the pial surface and acts as a scaffold for the migration of neurons born in the germinal zones (Miyata et al., 2001; Noctor et al., 2001; Rakic, 1972). Immunoreactivity against glial markers such as GFAP (glial fibrillary acidic protein), GLAST (glutamate and aspartate transporter), RC2 (intermediate filament-associated protein), Sox2 (SRY-box containing gene 2) and Pax6 (paired box

gene 6) can also be used to label radial glial progenitors (Gotz et al., 1998; Hutton and Pevny, 2011; Malatesta et al., 2000) (Figure 1.6).

1.3.2 Outer radial glia (oRG)

The outer radial glial progenitors were initially identified in human and ferret brains (Fietz et al., 2010; Hansen et al., 2010) and hence thought to be a hallmark of gyrencephalic brains (Fietz and Huttner, 2011). However, they were also subsequently identified in mouse (Shitamukai et al., 2011; Wang et al., 2011) and in lissencephalic primate brains (García-Moreno et al., 2012; Kelava et al. 2012) thus questioning their role in promoting gyrencephaly in mammals.

As suggested by the name, oRG cells share similarities with RGCs. They express similar genes and also undergo somal translocation (Fietz and Huttner, 2011; Hansen et al., 2010). However they differ crucially in not being attached to the ventricular surface and in being located at the margin of the intermediate zone and cortical plate in rodents (Shitamukai et al., 2011; Wang et al., 2011) and in the outer subventricular zone in primates and other gyrencephalic species (Fietz et al., 2010; Smart et al., 2002) (Figure 1.6). In addition, primate oRG cells have been shown to be more heterogeneous in terms of morphology than previously thought (Betizeau et al., 2013). Some oRG cells retain both an apical and basal process while others might have only one of them. Further, it is now believed that oRG cells are also capable of directly generating neurons in addition to generating IPCs (Betizeau et al., 2013). Thus, careful comparative assessment of progenitors might shed light on crucial evolutionary differences leading to an increase in neuronal diversity and differences in characteristics.

Figure 1.7: Comparative description of progenitor cells in the mammalian cortex

(A) Developing rodent cortices have a simpler germinal zone architecture comprising of only the VZ and SVZ. A small proportion of oRG cells (red cells) are present at the border of SVZ and IZ. (B) The human cortex displays an expanded germinal zone with a subdivided inner and outer SVZ and an amplification in IPC (green with red nucleus) and oRG cell numbers (reproduced from Lui et al., 2011)

1.3.3 Intermediate progenitor cell (IPC)

Intermediate progenitor cells (IPCs) derive from asymmetric divisions of RGCs and usually undergo terminal differentiation to produce a pair of neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). In this way, they are thought to amplify the output of neurons during cortical development (Cheung et al., 2007; 2010; Martinez-Cerdeno, 2006). Morphologically, IPCs do not possess a basal process as seen in RGCs and oRGs and are multipolar (Miyata et al., 2004; Noctor et al., 2004). They are present basal to RGCs in a zone termed as subventricular zone (SVZ) (Angevine et al., 1970; Hatai, 1902) where they undergo usually symmetric terminal mitosis before generating neurons (Haubensak et al., 2004; Noctor et al., 2004).

In terms of gene expression, IPCs have been characterised by the expression of *Tbr2* (Englund et al., 2005), *Cux2* (Cubelos et al., 2008; Nieto et al., 2004), *Svet1* (Tarabykin et al., 2001) and *Neurod6* (Goebbels et al., 2006; Wu et al., 2005).

Gene knockout studies reveal that mutants for *Tbr2* or *Cux2* show changes in mitotic division in the SVZ and also in cortical cell numbers (Arnold et al., 2008; Cubelos et al., 2008; Sessa et al., 2008). However there are differences in the observations from separate groups with Arnold et al. reporting a 25% decrease in the non-VZ pH3+ cells while Sessa et al. report a 75% decrease. In keeping with

their individual observations, Sessa report only a reduction in the upper layer density while Sessa et al. describe a 29% reduction in thickness of lower layers and a ~50% decrease in the thickness of upper layers. However there are important caveats to both studies. Both studies report pH3+ cell reduction only at stages of supragranular layer genesis while reports show that *Tbr2* is expressed at earlier stages corresponding to infragranular layer genesis as well (Kowalczyk et al., 2009). In addition, Sessa et al. report a stronger phenotype possibly due to their use of the *Foxg1^{Cre}* mouse line that is known to be haploinsufficient and to produce 35% fewer IPCs (Siegenthaler et al., 2008). Thus while both studies indicate a role for Tbr2+ IPCs in corticogenesis, their conclusions varies grossly due to differences in strategies.

1.3.4 Progenitor cells in evolutionary development

Despite being a metabolically expensive organ, the mammalian brain has nevertheless increased in size during evolution (Pilbeam and Gould, 1974). Further, the increase in neocortical size has particularly been higher in comparison to other structures of the brain (Finlay and Darlington, 1995). The obvious and hence easy explanation for the increase can be made based on the increase in radial units stemming from an increase in neural stem cell pool (Rakic, 2009). However this maybe an oversimplification of the evolutionary events that have lead to an expansion of the cerebral cortex.

IPCs in the SVZ present an alternative neurogenic niche that has been argued to be involved in giving rise to the supragranular layers of the cerebral cortex (Tarabykin et al., 2001, Lukaszewicz et al., 2005). Indeed the temporal pattern of

pH3+ expression in the SVZ and the effect of knocking out essential IPC genes shows that these cells do contribute heavily towards the generation of layers 2-4 (Wu et al., 2005; Martinez-Cerdeno, 2006). However, enough evidence is not available to limit them to the generation of these layers.

The rudimentary 3-layered reptilian cortices lack a SVZ despite the presence of a small population of scattered abventricular mitoses (Cheung et al., 2007; Martinez-Cerdeno, 2006). Avian brains on the other hand, do have a substantial abventricular mitotic zone in the subpallium that might be called a subventricular zone (Figure 1.7). The presence of a SVZ in the dorsal pallium of birds however is debatable (Striedter and Keefer, 2000; Cheung et al., 2010).

Prior to discussing the presence of a subventricular zone in avian cortices, it might be prudent to understand the definition of an SVZ proposed by the Boulder Committee Report (Angevine et al., 1970). The SVZ is defined as:

“...located at the junction of the ventricular and intermediate zones...the initial cellular occupants come into position soon after the intermediate zone has begun to form. Subventricular cells are small and round or oval. They are distinguished from the young neurons of the intermediate zone by their proliferative activity and, unlike ventricular cells; they remain stable in position, without a to-and-fro-nuclear displacement, during the mitotic cycle.” (Angevine et al., 1970, pp 259)

Based on this definition, it can be argued that many species of birds (such as chicken and quails) do not possess a well-defined SVZ not due to absence of abventricular mitoses (which are regularly observed in the hyper, meso and nido-pallium) but due to absence of a ‘zone’ like clustering of these mitoses (Cheung et al., 2007). Some species such as parakeets and zebra-finches do show

aggregation of abventricular mitoses into a thin band above the VZ (Striedter and Charvet, 2009). However, the proportion of abventricular mitoses in the hyperpallium (considered homologous to the dorsal pallium of mammals) is negligible compared to the proportions observed in the meso and nido-pallium (Cheung et al., 2007) (Figure 1.7). Despite this difference in clustering of abventricular mitoses, it has been shown that developing chick brains do express *Tbr2*; a marker for basal progenitors in mammalian cortices (Englund et al., 2005; Suzuki et al., 2012).

Figure 1.8: Distribution of mitotic cells in the developing cortex in different species

Mitotic cells stained with an antibody against phosphohistone H3 (pH3) are distributed unequally in the developing cortex of turtle (A), chick (B), marsupial (C) and mouse (D). While the mouse cortex has a well-organised abventricular compartment in the SVZ, the other species lack the same. The chick has a large number of pH3+ cells in the medial pallium (MP) but not the hyperpallium, which is considered equivalent to the dorsal cortex (dCtx) of the mouse. Scale bars = 400 μ m in A–C, 100 μ m in D. *Abbreviations*: DVR: dorsal ventricular ridge; dCtx: dorsal cortex; GE: ganglionic eminence; Hyp: hyperpallium; LGE: lateral ganglionic eminence; MGE: medial ganglionic eminence; MP: mesopallium; NP: nidopallium; Sep: septum; Str: striatum. (Reproduced from Abdel-Mannan et al., 2008).

A similar problem also exists in identifying a SVZ in metatherian mammals such as the grey short-tailed opossum (*Monodelphis domestica*). *M.domestica* like all marsupials displays altricial development where young ones are born immature and cling to the teats (reviewed in Keyte and Smith 2008). Marsupial cortices though display a hexalaminar arrangement similar to that seen in rodents and humans (Gray, 1924) leading towards the debate whether the presence of a SVZ pre-dates the eutherian-metatherian split. Few studies exist that have studied

neurogenesis in *Monodelphis* cortex in detail and they differ in outcomes of experiments.

Puzzolo and Malamacci (Puzzolo and Mallamaci, 2010) argue against the presence of a basal progenitor compartment based on pH3, Tbr2 and BrdU stainings. In doing so, they differ from the conclusions made by Cheung et al. (Cheung et al., 2010) who used pH3 immunostaining to show that an aggregation of abventricular mitoses exists at P16. A closer look at the methods used by the two papers reveals that Puzzolo et al. used 10 µm sections for their staining while Cheung et al. used 40 µm sections. This however doesn't answer the presence of a significant population of Tbr2+ cells basal to the VZ during the period of neurogenesis (P1-P20) that fails to co-localise with pH3 immunoreactivity.

The absence of co-labelling of pH3 and Tbr2 in the marsupial germinal zone might suggest that Tbr2 (or other IPC markers') immunoreactivity is not a sine-qua-non for the SVZ. It is possible that the abventricular mitosis observed in them might instead be due to the recently discovered oRG cells (Hansen et al., 2010; Fietz et al., 2010) and thus has a separate identity from that of rodents and higher mammals. Alternatively, IPCs in marsupials might be Tbr2 negative and this population of cells has been lost or has coalesced with IPC marker identity during the evolution of the neocortex. A comparative gene expression study shall serve well to outline the differences between the abventricular divisions in these species versus those that show a well-defined abventricular mitotic compartment. Despite these alternate explanations, the role of Tbr2 expression in SVZ located cells is an interesting observation. It is known that the SVZ (and lower intermediate zone) serves as a route for migration of GABAergic interneurons in the neocortex of rodents (Del Rio et al., 1992). It has also been shown that Tbr2 regulates

interneuron migration into the SVZ by regulating the expression of *Cxcl12* (Sessa et al., 2010). In keeping with these observations, Puzzolo and Mallamaci show that GABA⁺ and GAD⁺ cells migrate into the SVZ in *M.domestica*. Hence, the presence of Tbr2 expressing cells in the brains of developing marsupials might have an alternative function as a mediator of interneuron migration rather than as a progenitor cell.

At the other end of the mammalian spectrum, species such as ferrets, marmoset, macaques and even gyrencephalic rodents such as the agouti (*Dasyprocta agouti*) and *Capybara* show that a further increase in the neurogenic potential can be attained by an elaboration of the subventricular zone (García-Moreno et al., 2012; Kelava et al. 2012; Reillo et al., 2010). These animals show a compartmentalisation of the SVZ roughly around mid-gestation into an inner and outer compartment termed as the ISVZ and OSVZ bisected by the inner fibre layer (IFL) of unknown composition and origin (Smart et al., 2002) (Figure 1.8). The ISVZ consists majorly of IPCs generated from the underlying radial glia (RGCs) and maintains its thickness during cortical neurogenesis (Fietz and Huttner, 2011; García-Moreno et al., 2012; Hansen et al., 2010; Reillo et al., 2010). In contrast, the OSVZ consists of a mixture of IPCs and a recently described progenitor; the outer radial glia (oRG) that is similar in gene expression and function to the RGCs found in the VZ (Fietz and Huttner, 2011; Hansen et al., 2010; Reillo et al., 2010).

Figure 1.9: Germinal zone cytoarchitecture in mouse and monkey cerebral cortex

(A) While the mouse cerebral cortex has only two germinal compartments (VZ and SVZ) that produce neurons during development, the monkey cortex (B) has an additional germinal zone called the OSVZ that houses oRG cells and IPCs. The large increase in progenitor population leads to an increase in cortical thickness and cortical surface. The monkey ISVZ resembles the mouse SVZ in being largely populated by IPCs. However a fibre layer (IFL) bifurcates the ISVZ from OSVZ (reproduced from Smart et al., 2002).

The IPCs contained in the OSVZ might originate both from the VZ as well as within the OSVZ through the asymmetric division of oRG progenitors. This might indicate either a dominant role of IPCs towards generation of neurons in species with OSVZ or OSVZ like compartments or the functioning of IPCs as a conserved conduit through which a large number of neurons are generated regardless of their origin and cell-fate.

While the expansion of the neocortex provides an increased substratum for development of newer connections and computational schema, additional mechanisms are required for generation of diversity at the cellular level. Such diversity is usually at the level of genes and their transcripts and serves to create differences even in cells born at similar times and positioned in the same layer.

An example of heterogeneity in progenitor cells was described by Franco et al. (Franco et al., 2012) where *Cux2* expressing radial glia give rise to upper layer neurons even during periods where infragranular neurons are being generated. Fate mapping of *Cux2* expressing cells by crossing *Cux2^{Cre}* with the *Ai9-tdTomato* reporter mice shows a salt and pepper labelling of progenitors at the ventricular zone. This might indicate a mottled arrangement of fate limited progenitor cells. A similar fate-mapping experiment performed using a combination of *Tα-1* (alpha tubulin 1), *GLAST* and *BLbp* (brain lipid binding protein) promoter driven Cre with fluorescent reporter plasmids also leads to a segregation of progenies into different classes (Tyler and Haydar 2013). Other genes have been shown to regulate progenitor fate in a spatial manner (Pinto et al., 2009). Taken together, it

indicates that a seemingly homogeneous pool of progenitors can be fate restricted or induced to give rise to a heterogeneous progeny by genetic means. Single cell transcriptomic profiling of neural progenitor cells indicates that cells 'marked' by the same gene can differ at the level of the global transcriptome (Kawaguchi et al., 2008; Narayanan et al., 2012). One can appreciate that even a handful of genes can lead to a high level of diversity just by permutation and combination of mRNA levels of those genes.

Another way by which variety in the progenitor cells can be achieved is through chromosomal manipulation. Copy-number variations (CNVs) can increase or decrease the gene dosage leading to severe brain malformations (Kariminejad et al., 2011). Alternatively, retrotransposition of long interspersed elements (LINEs) can also lead to a change of genetic status (Coufal et al., 2009). Such variations in the neural progenitor cells can lead to differences in functioning in a clonal manner (Singer et al., 2010). Coupled with the inherent genetic stochasticity, it can generate a high amount of diversity that can even be individual specific.

1.4 ROLE OF *Tbr2* IN INTERMEDIATE PROGENITORS DURING CORTICAL NEUROGENESIS

The T-box genes belong to a family of 19 genes that share a common 'T-box' DNA binding domain and have arisen through tandem duplication of previous genes (Agulnik et al., 1996; Naiche et al., 2005) (Figure 1.9). The first member of this gene family; *Brachyury* (*T*) was identified by a short-tailed phenotype in mice

(Dobrovolskaia-Zavadskaia, 1927; 1933). T-box genes have been shown to regulate development of multiple embryonic tissues and mutations in them have been linked with several disease conditions in humans (reviewed in Naiche et al., 2005).

Expression of *Tbr2* (also called *Eomesodermin*) was first identified in the developing mouse brain by probing with a probe generated from E14.5 cortex (Kimura et al., 1999). On comparing the expression with chick *Tbr2*, the two were found to be conserved in expression in the telencephalon. In contrast, expression of *Tbr2* differs from that of *Tbr1* with the pattern being complementary and in germinal regions (Bulfone et al., 1999).

Figure 1.10: Conservation in the T-box gene family

The members of the T-box gene family show a high degree of similarity in the T-box binding element region and can be arranged in subfamilies on this basis. *Tbr2* belongs to the *Tbr1* subfamily consisting of *Tbx21* and *Tbr1* apart from itself. (Reproduced from Naiche et al., 2005)

Subsequently, expression of *Tbr2* was reported to be localised to the IPCs in the SVZ (Englund et al., 2005). This is further supported by expression of GFP (green fluorescent protein) in different transgenic strains of *Eomes::GFP* (Arnold et al., 2009; Kwon and Hadjantonakis, 2007). Expression of *Tbr2* has also been reported in the Cajal-Retzius cells and during adult neurogenesis in the dentate gyrus in addition to IPCs (Arnold et al., 2008; Hodge et al., 2008; 2013). The expression pattern of *Tbr2* suggests that it is involved in a conserved genetic program leading to the generation of glutamatergic neurons (Hevner et al., 2006). As *Tbr2* is required for the development of the trophoblast, genetic ablation leads to arrest of embryos at the blastocyst stage (Russ et al., 2000). Conditional knockouts have

thus been used to study the role of *Tbr2* in cortical neurogenesis (Arnold et al., 2008; Sessa et al., 2008).

Such mutants show a direct relation between cell division in the SVZ and also cortical thickness. Further, the effect on upper layer neuron generation is more severe than that on infragranular layers (Arnold et al., 2008; Sessa et al., 2008). Forced expression of *Tbr2* leads to an increase in mitosis in the VZ (Sessa et al., 2008) and an overproduction of IPCs. In humans, mutation at the TBR2 locus has been linked with microcephaly and polymicrogyria (Baala et al., 2007) (Figure 1.10) thus indicating that the role of this gene is conserved across mammalian species.

Figure 1.11: Loss of TBR2 in human causes microcephaly and polymicrogyria

(A) Saggital MRI scan image of individual with mutation in the TBR2 locus leading to loss of TBR2 expression. Apart from a smaller brain size, agenesis of the corpus callosum can also be seen (dotted arrow). (B) Individuals also show bilateral polymicrogyria (multiple small gyri) in addition to microcephaly. (C) TBR2 expression in 12 post-conception week (PCW) human cortex. Expression can be seen in the ISVZ and OSVZ. (Modified from Baala et al., 2007; Bayatti et al., 2008).

In addition to its role in cell division and neurogenesis, *Tbr2* is also involved in mediating expression of *Cxcl12* in the SVZ that serves as a chemoattractant for interneurons migrating from the LGE/MGE (Sessa et al., 2010).

However, the exact signalling pathway through which *Tbr2* exerts its role as well as how the ratio of RGC to IPC is maintained in the developing cortex remains unknown. Further, the identity of cells generated by *Tbr2*⁺ IPCs and their laminar positions is also unknown.

1.5 AIMS OF THIS THESIS

Over the last decade, the interest in cortical progenitors has steadily increased. IPCs, due to their neurogenic potential, offer a means to generate specific population of cortical neurons. Thus they can potentially be used in regenerative therapies such as ALS and stroke. However, prior to that, there is a need to understand the basic mechanisms that influence generation of specific neuron types. Additionally, mechanisms controlling IPC division are key to understanding the evolutionary expansion of the cerebral cortex in humans.

This thesis hence attempts to understand various aspects of the role of *Tbr2* (and indirectly IPCs) in cortical neurogenesis.

In Chapter 2, I attempt to understand the molecular function of *Tbr2*. I will describe analysis of genes up/down regulated after forced expression of *Tbr2* in embryonic mouse cortex. I will also validate some effectors of *Tbr2* function by using a variety of molecular biology methods (quantitative PCR and chromatin immunoprecipitation). I will also detail some experiments comparing gene expression of downstream genes in wildtype and *Tbr2* cKO cortices and discuss some probable mechanisms that might play a crucial role in the evolution of cortices.

In Chapter 3, I describe the fate of cells born from *Tbr2*⁺ IPCs using transgenic mouse lines (*Tbr2*^{Cre} and *Ai9-tdTomato*). I will show additional regions (and migratory patterns) of *Tbr2* expression in the cortex and outside that have previously not been described. Lastly, I will also discuss the clonal generation of

neurons from IPCs using a novel fluorescence based clonal analysis method that labels all cells born from a cell in the same manner. Results from this method will be used to describe the relation between clonally generated neurons. I will discuss the results in light of the upper layer hypothesis attributed to IPCs.

In Chapter 4, I link the expansion in IPC population and simultaneous changes in germinal zone architecture in *Callithrix jacchus* (marmoset) and *Dasyprocta agouti* (agouti) brains. The marmoset is a lissencephalic primate and the agouti is a gyrencephalic rodent. It has been suggested previously that gyrencephaly might be caused by presence of oRG progenitors and absence of these might lead to a smooth brain. These two species are outliers to the general trend seen in their respective phylogenetic subclass. Hence any similarity between gyrencephalic (or lissencephalic) species should apply to both the brains studied here. With this idea, I shall compare if there are differences with regard to the architecture of the germinal zones or progenitor cell types in these species. I will also discuss other mechanisms that might affect folding of the brain.

Each of these chapters contains the relevant experimental details and discussions pertinent to the findings described.

Finally, in Chapter 5, I will conclude the major findings from all chapters and discuss them in the broader context of understanding the role of genetic mechanisms in development, disease and evolution.

Chapter 2

Molecular Role of *Tbr2* during Cortical Neurogenesis

2.1 INTRODUCTION

Intermediate progenitor cells (IPCs) are born via asymmetric divisions of radial glial cells (RGCs) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). While both RGCs and IPCs produce neurons, only the RGCs also generate astrocytes in the latter stages of embryonic development (Culican et al., 1990; Voigt, 1989). These cells differ morphologically and in their transcriptomes (Cameron et al., 2012; Englund et al., 2005; Kawaguchi et al., 2008) in that IPCs are multipolar cells while RGCs are connected to the ventricular surface through endfeet and to the pia via a basal process thus making them bipolar in shape (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2002). In terms of gene expression, IPCs express genes such as *Tbr2*, *Cux2* and *Svet1* (Englund et al., 2005; Zimmer et al., 2004; Tarabykin et al., 2001) while RGCs do not express such molecules and instead express *Pax6* and *Sox2* (Gotz et al., 1998; Hutton and Pevny, 2011). Thus despite their similarity in function (i.e. generation of neurons) they differ in form and expression.

In order to elucidate the molecular mechanisms underlying intermediate progenitor biology, studies have looked at the effect of knocking out IPC specific genes and also at transcriptomic analysis (Arnold et al., 2008; Cameron et al., 2012; Cubelos et al., 2008; Sessa et al., 2008). Gene knockout studies reveal that conditional deletion of *Tbr2* leads to a decrease in mitotic division in the sub-ventricular zone (SVZ) and also a decrease in cortical cell numbers (Arnold et al., 2008; Sessa et al., 2008). Transcriptome analysis from

Tbr2::GFP mouse line has shown that *Tbr2*⁺ cells express basic helix-loop-helix (bHLH) genes, mature neuronal markers and also genes involved in axonal growth (Cameron et al., 2012). Studies have also shown that expression of *Cxcl12*; a chemo-attractant molecule is dependent on *Tbr2* expression and is involved in directional migration of interneurons through the SVZ (Sessa et al., 2010).

Further, overexpression of *Tbr2* leads to induction of an IPC fate (Sessa et al., 2008). Hence, induction or repression of *Tbr2* by genes functioning upstream can regulate the generation of IPCs (Pilaz et al., 2009). This is useful in titering neurogenesis during development and might also be evolutionarily important as an increase in IPCs is linked with an increase in cortical thickness (Cheung et al., 2010; García-Moreno et al., 2012; Kelava et al., 2012; Martinez-Cerdeno, 2006; Nonaka-Kinoshita et al. 2013).

The role of several genes expressed upstream of *Tbr2* such as *Sox2*, *Pax6*, *Hes1*, *Ngn2*, *AP2gamma* etc. has been described (Farkas et al., 2008; Ochiai et al., 2009; Pinto et al., 2009; Quinn et al., 2007). Recently, the role of a microRNA; miRNA-92b has also been noted in regulating the number of IPCs generated from RGCs (Nowakowski et al. 2013). Thus direct transcriptional control is an important means of regulating IPC genesis and therefore neuronal numbers in the cortex.

However, a carefully regulated system is also required to maintain a balance between generation of IPCs (and hence neurons) and maintenance of RGCs. A

tilt of this system toward any side would result in fewer or greater number of neurons than is necessary and might compromise on proper cortical development (Baala et al., 2007; Way et al. 2009). In this regard, the Wnt signaling pathway has an important role in maintaining this balance between the different progenitors and generation of neurons in the developing brain.

The canonical Wnt signaling pathway consists of transcriptional control of several genes by a molecule: beta-catenin that is rapidly degraded in the “OFF” state and stabilized in the “ON” state (reviewed in MacDonald et al., 2009; van Amerongen and Nusse, 2009).

Figure 2.1: Mechanism of action of the canonical Wnt signaling

(A) In the absence of Wnt ligands, β -catenin is phosphorylated by the action of GSK3 β that leads to its degradation through the proteasome pathway. (B) When the ligand binds to Frizzled receptors on the cell membrane, it leads to activation of secondary messengers such as Dvl that inhibits action of GSK3 β leading to stabilization of β -catenin and its translocation to the nucleus where it initiates transcription of genes. (Reproduced from Luo et al., 2007).

During cortical development it has been shown using the *Axin2*^{d2EGFP} and *BAT-GAL* mice (*Axin2* is a direct target of canonical Wnt signaling) that Wnt signaling is downregulated in intermediate progenitors (Machon et al., 2007; Mutch et al., 2010). Further, a conditional knockout of beta-catenin (*Nes*^{Cre}:: *Ctnnb1*^{fl/fl}) causes an increase in the IPC population while stabilization of beta-catenin by deletion of its N-terminal region (*Nes*^{Cre}:: *Ctnnb1*^{fl (ex3)}) leads to a decrease in IPC numbers (Mutch et al., 2010). Conditional knockout of a Wnt downstream gene *N-myc* on the other hand causes a decrease in IPCs and its overexpression leads to an increase in IPCs (Kuwahara et al., 2010).

On the other hand, *Wnt5a* is generally considered to trigger the non-canonical Wnt signaling (Oishi et al., 2003; Yuzugullu et al., 2009) though it can also activate β -catenin in certain cases (van Amerongen et al., 2012) and has a role to play in maintaining the balance between RGCs and IPCs. Treating neural precursor cell (NPC) cultures with siRNAs against *Wnt5a* and *Ror2* leads to a decrease in *Tbr2*⁺ cells (Endo et al., 2012).

Figure 2.2: Canonical and Non-canonical Wnt pathways

(A) As described earlier, the canonical Wnt pathway operates via the stabilization of β -catenin upon activation by Wnt ligands such as Wnt3a, Wnt7a, Wnt1 etc. (B) On the other hand, non-canonical Wnt ligands such as Wnt5a initiate a separate pathway that is β -catenin independent and effects cell function through the calcium pathway. (Reproduced from Olivares-Navarrete et al., 2011).

However, despite this knowledge, a functional understanding of the role of IPCs has been evasive. It is unknown whether cell-signaling pathways are involved in regulating the division versus differentiation choice in IPCs. More specifically, it is not known if IPCs and *Tbr2* play any role in maintaining signaling cascades that serve a role in mediating cell fate determination.

In order to study the role played by *Tbr2* in IPC homeostasis and neuron production, I used an overexpression microarray based study that looked at changes in gene expression 24 hours post *Tbr2* overexpression in E13.5 mouse germinal zone. The genes obtained were analysed for pattern of expression in the VZ/SVZ and also studied for their mRNA levels in the conditional knockout. Genes showing a correlation with both the overexpression and cKO were then analysed for being direct targets of *Tbr2* action by use of chromatin immunoprecipitation protocols.

This chapter hence attempts to find molecular mechanisms that influence IPC genesis, maintenance and division.

2.2 MATERIALS AND METHODS

2.2.1 Slice culture

In order to over-express *Tbr2* in radial glial progenitors, an ex-vivo approach was followed (Polleux and Ghosh, 2002) followed by slice culture.

For this, time-mated C57BL6/J mice were euthanised at embryonic day (E) 13.5 by cervical dislocation and embryos removed. A mouse *Tbr2*-IRES-GFP plasmid and/or EYFP- expressing pCAGGS vector was injected into the lateral ventricle using a glass micropipette (Harvard Apparatus). Embryo heads were removed and paddle electrodes (Genetropes, Harvard Apparatus) placed on either side for electroporation with 4x100msec pulses of 35V with 100msec intervals (Electro Square Porator, Harvard Apparatus). Brains were dissected, embedded in 4% low melting agarose (Sigma), sectioned to a thickness of 300 μ m using a vibrating microtome (Leica VT1000S) and placed on laminin/poly-L-lysine pre-coated culture inserts over 1.8ml of culture media (20mM 1M D-glucose, 1mM L- glutamine, 100units/ml penicillin, 0.1mg/ml streptomycin, in Basal medium Eagle and complete HBSS at 3:1) in six-well plates. The cortical slices were incubated at 37°C with 5% CO₂ for 24 hours. Culture slices were checked for GFP/EYFP expression at the end of 24 hours under a dissecting microscope (Leica). Cortical regions showing moderate to high expression of the fluoroprotein were dissected using a fine micro-scalpel blade (MSP Supersharps) and fine forceps and stored in RNAlater (Ambion) at -20 °C until further use.

2.2.2 RNA extractions, cDNA synthesis and quantitative PCR

RNA was extracted from over-expression slices and *Tbr2* conditional knockout cortices (*Sox1^{Cre}* $\times$ *Tbr2^{flox/flox}*) by using RNAeasy Mini kit (Qiagen) and eluted in 12 microliters of Tris-Cl: EDTA buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA). RNA was stored at -20C ° until further use.

Extracted RNA was used to synthesise cDNA using random hexamers in a reaction containing 50 ng random hexamers, 1 µg total RNA, 500 µM dNTP mix, 50 µM DTT, 40 U RNaseOut and 200 U Superscript III reverse transcriptase (Invitrogen). The reactions were carried out by incubating the mix at 25 °C for 5 mins and 55 °C for 60 mins followed by inactivation of the enzyme at 75 °C for 15 mins. RNaseH (5 Units) was added to degrade all RNA products. For quantitative PCR reactions, this reaction was diluted 1:1 with RNase free TE buffer. Tubes were stored at -80 °C until further use.

Primer sets were designed using Universal Probe Library's assay design software (<http://www.roche-applied-science.com>) and are detailed in Appendix A.1. Reactions were carried out in 96 well optical plates using the ABI Prism 7000 Sequence Detection System thermal cycler. Reactions contained 10pmol of each primer, and 10µl of 2X Power SYBR® Green Master Mix (Applied Biosystem). Cycling conditions followed were initial denaturation at 95 °C for 5 mins followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Data was exported into MS-Excel (Microsoft) for further analysis by the $\Delta\Delta C_t$ method for relative quantification.

2.2.3 Microarray and data normalization (from DeProto, 2012)

Labeled cRNA from each sample was hybridized to a corresponding Affymetrix 430 2.0 whole mouse genome chip (Affymetrix #900496). Following hybridization, chips were then washed, stained, and scanned the following day. Chips were processed on a GeneChip Fluidics Station and Scanner. Data from the hybridized samples was then extracted using Genechip Operating System (GCOS) software (Affymetrix).

The Affymetrix .CEL files containing raw array images were normalized using GCOS software that subtracted background, analysed intensity of each probe to generate an averaged intensity for each probe set call. Robust Multi-array Average (RMA) algorithm (Irizarry et al., 2002) and G/C content Robust Multi-array Average (GCRMA) in Genespring was used to analyze the GCOS output files of each chip for background correction, normalization, and summarization.

2.2.4 In-situ hybridization

Probes were prepared by ligating Polymerase Chain Reaction (PCR) generated products from cDNA synthesized from E15.5 wild-type brain tissue into pCRII vector (Invitrogen). Primers used for this are given in Appendix A.2. Reactions were setup in 20 µl volumes containing 500 nM each of forward and reverse primers alongwith 10 µl of 2X OneTaq Master Mix (New England Biolabs).

DIG-labelled riboprobes were synthesised by using linearized plasmids as templates in a 40 µl reaction containing 80 U Protector RNase Inhibitor (Roche),

1x DIG Labelling buffer (Roche), 40 U T7 or SP6 RNA Polymerase in a 1x Transcription buffer (Roche). This reaction was incubated for 4 hours at 37°C. Probes thus synthesized were extracted by incubating with 300 µl ethanol (100%), 60 µl TE buffer and 10 µl LiCl (4M) on ice for 30 mins. 5 µl of GlycolBlue (Invitrogen) was added to allow visualization and this mixture was centrifuged at 13,000 rpm for 20 mins at 4°C. The pellet was washed with 70% ethanol, dried and dissolved in 20 µl of RNase free TE buffer. Tubes were then stored at -80°C until use.

For ISH, brains were fixed with 4% PFA (Sigma Aldrich) for 4 hours, cryoprotected with 30% sucrose overnight and frozen in OCT (TissueTek). Sections of 16µm thickness were cut on a cryostat (Jung CM3000; Leica) and stored at -80°C. On the day of use, sections air-dried, postfixed with 4% PFA for 5 min, deproteinized with 0.1 N HCl for 5 min and acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine hydrochloride). Pre-hybridization was carried out at room temperature (RT) for at least 1 h in a solution of 50% formamide, 200 µg/mL *E.coli* transfer RNA, 1x Denhardt's solution, 10% dextran sulfate, 10 mM Tris (pH 7.6), 600 mM NaCl, 0.25% sodium dodecyl sulfate, and 1 mM ethylenediaminetetra-acetic acid. Hybridizations were carried out overnight at 66-68°C with riboprobes diluted in the pre-hybridization solution. Sections were washed in 30 mM NaCl/3 mM sodium citrate at 66-68°C for an hour and blocked with 1% blocking reagent (Roche) in 100 mM Tris-Cl (pH 7.5) and 150 mM NaCl containing buffer. This was followed with overnight incubation in anti-DIG antibody (1:500, Horseradish peroxidase conjugated, Roche) at 4°C. Sections were developed using a Tyramide plus Cyanine 3 kit

(Perkin Elmer), counterstained with DAPI, covered with a coverslip and sealed with nail varnish. Sense cRNA probes were used as negative controls.

2.2.5 Imaging and Image Analysis

Images were captured using either a Leica DMR fluorescence microscope (Leica Microsystems) or a Zeiss LSM 710 confocal microscope (Carl Zeiss Microimaging). Images were analysed using ImageJ (Image Analysis in Java, NIH) or Adobe Photoshop CS3 (Adobe Systems Inc.).

2.2.6 Chromatin Immunoprecipitation

Embryonic cortices from E14.5 animals were dissected in ice-cold PBS and collected in microcentrifuge tubes. Tissue was homogenised in pre-cooled tissue douncers (Wheaton) and the resulting homogenate fixed in 1% PFA at room temperature for 15 mins followed by quenching with 0.25M Glycine (final concentration). The homogenate was washed twice in ice-cold PBS with protease inhibitors (Roche), pelleted (13000 rpm, 4 °C, 5mins) and lysed with 1 ml of RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0). Supernatant was collected in 150 µl aliquots and all further work undertaken in cold with protease inhibitors unless specified.

Lysate was sonicated at Medium power for 15 mins (Bioruptor, Diagenode) to fragment the chromatin into roughly 500 bp fragments and contents of each tube diluted ten-fold with ChIP dilution buffer (proprietary, Millipore).

The chromatin was pre-cleared with 50% Protein-A agarose slurry (Santa Cruz) and supernatant collected by gentle centrifugation (1000 rpm, 2 mins). Appropriate antibodies (anti-Tbr2, anti-RNA Pol II or IgG) were added at a concentration of 2 µg and tubes incubated on a rotor overnight at 4°C. The following day, 20 µl of MagnaChIP Protein-A magnetic beads (Millipore) was added to each tube to bind immune-chromatin complexes. Beads were collected by placing on a magnetic rack (Millipore) for 5 mins and washed with the following buffers for 5 mins each at room temperature.

Table 2.3: List of buffers used for washing Protein-A bound immune complexes prior to elution.

	Low Salt	High Salt	LiCl Immune Complex	TE
SDS	0.1 %	0.1%	-	-
Triton X-100	1 %	1 %	-	-
EDTA	2 mM	2 mM	1 mM	1 mM
Tris-HCl (8.1)	20 mM	20 mM	10 mM	10 mM
NaCl	150 mM	500 mM	-	-
LiCl	-	-	0.25 mM	-
Igepal CA360	-	-	1 %	-
Sodium doexycolate	-	-	1 %	-

Chromatin was eluted twice by washing the beads with a 1% SDS and 0.1M NaHCO₃ containing buffer at room temperature and then combined.

The eluate was incubated with 0.2M NaCl at 65°C for 4 hours to reverse formaldehyde cross-links followed by EDTA (1 mM) and Proteinase K for digesting protein complexes. DNA was precipitated using standard Phenol-Chloroform extraction and Sodium acetate/Ethanol precipitation. PCRs were performed with this DNA for chosen upstream regions. Forward and reverse primers used with annealing temperatures are listed in Appendix A.4 of appendix.

2.3 RESULTS

Previous attempts to understand the molecular mechanisms underlying IPC biology have utilized either a loss of function model of *Tbr2* or a whole transcriptome analysis of *Tbr2*^{GFP} mouse (Arnold et al., 2008; Cameron et al., 2012; Sessa et al., 2008). These studies have shown a decrease in mitotic cells in the SVZ (Arnold et al., 2008; Sessa et al., 2008) or a decrease in interneuron migration in the SVZ (Sessa et al., 2010) indicating a link between *Tbr2* and cell cycle or with *Cxcl12* (Sessa et al., 2010).

However the loss of function model does not show a complete loss of SVZ mitosis or a large decrease in cortical thickness (discussed in Chapter 2). This hence points towards either heterogeneity in the identity of IPCs or a redundancy of *Tbr2* function. However, forced over-expression of *Tbr2* in the developing cortex does lead to an increase in mitotic cells at the VZ/SVZ (Sessa et al., 2008) thus indicating that by itself *Tbr2* does control regulation of cell proliferation.

2.3.1 Overexpression of *Tbr2* identifies novel downstream genes

Thus to probe the role of *Tbr2* in cell fate determination, a complementary method of gain of function experiment was chosen. This was performed by ex-vivo electroporation of embryonic tissue and slice culture followed by a microarray analysis of the electroporated region (DeProto, 2012). A similar approach (with modifications) has been reported in multiple studies (Sansom et

al., 2009) where transcriptional regulation during neurogenesis was being studied.

Analysis of the overrepresented pathways from the microarray analysis lead to identification of several signaling pathways involved in regulation of cell proliferation, differentiation and specification (Figure 2.3).

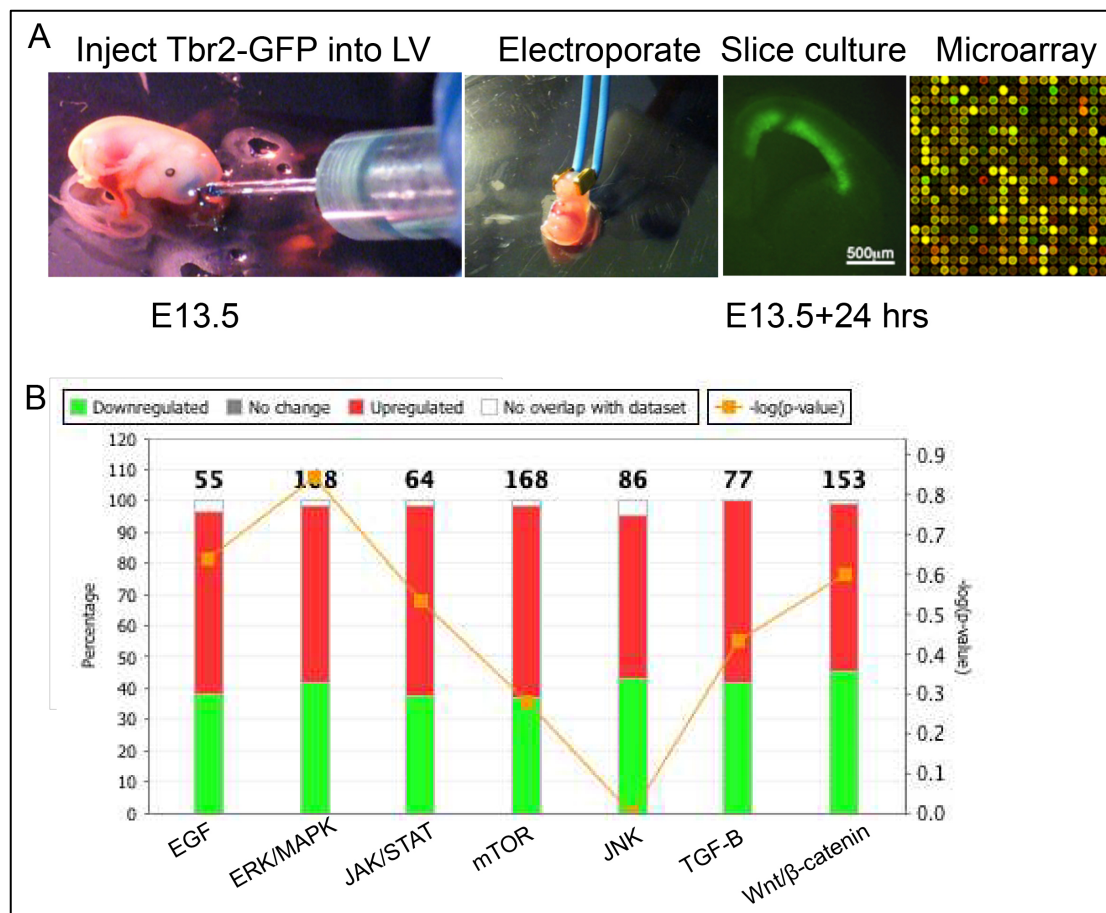


Figure 2.3: Experimental scheme used for over-expression of *Tbr2* and analysis of downstream genes

(A) Mouse *Tbr2*-IRES-GFP and/or EYFP construct was electroporated into the lateral ventricles of E13.5 embryos followed by electroporation. Brain slices (300 μ m) were cultured for 24 hours followed by microarray of overexpressed tissue. (B) Analysis of major pathways represented after over-expression. Yellow lines represent the $-\log P$ value computed by using the right-tailed Fisher exact test that compares the total number of genes in the microarray list related to a pathway to the total number of genes in the reference set of that pathway changed genes to total genes in the pathway. Numbers mentioned above the bars denote total number of genes changed. (Panel A modified from DeProto, 2012).

To validate and study the expression of upregulated genes, I chose to look at the pattern of expression of some of the highest fold change genes by using the Genepaint in-situ hybridization image database (www.genepaint.org, Visel, 2004). Only genes for which in-situ expression patterns were available were included in this analysis.

Out of 30 genes analysed from the dataset, most genes could be characterized into 3 categories in relation to their expression in the germinal zones. Genes were found to be either VZ specific, VZ+SVZ expression or SVZ specific (Figure 2.4). However, at closer inspection, most VZ specific genes showed expression at the lower borders of SVZ and hence were added to the latter group. Expression patterns were compared with those of *Pax6* and *Eomes* (*Tbr2*), genes known to be specific to progenitors in the germinal zones (Englund et al., 2005) (Figure 2.4).

Genes included in this list were then referenced for previous studies. Important genes involved in cell cycle (*Cdk4*), spindle assembly (*Aspm*) and Wnt signaling (*Wnt5a*, *Beta-catenin*, *Sfrp1*) were further chosen for analysis by qRT-PCR due to reports on their roles in cell fate determination, progenitor renewal and corticogenesis.

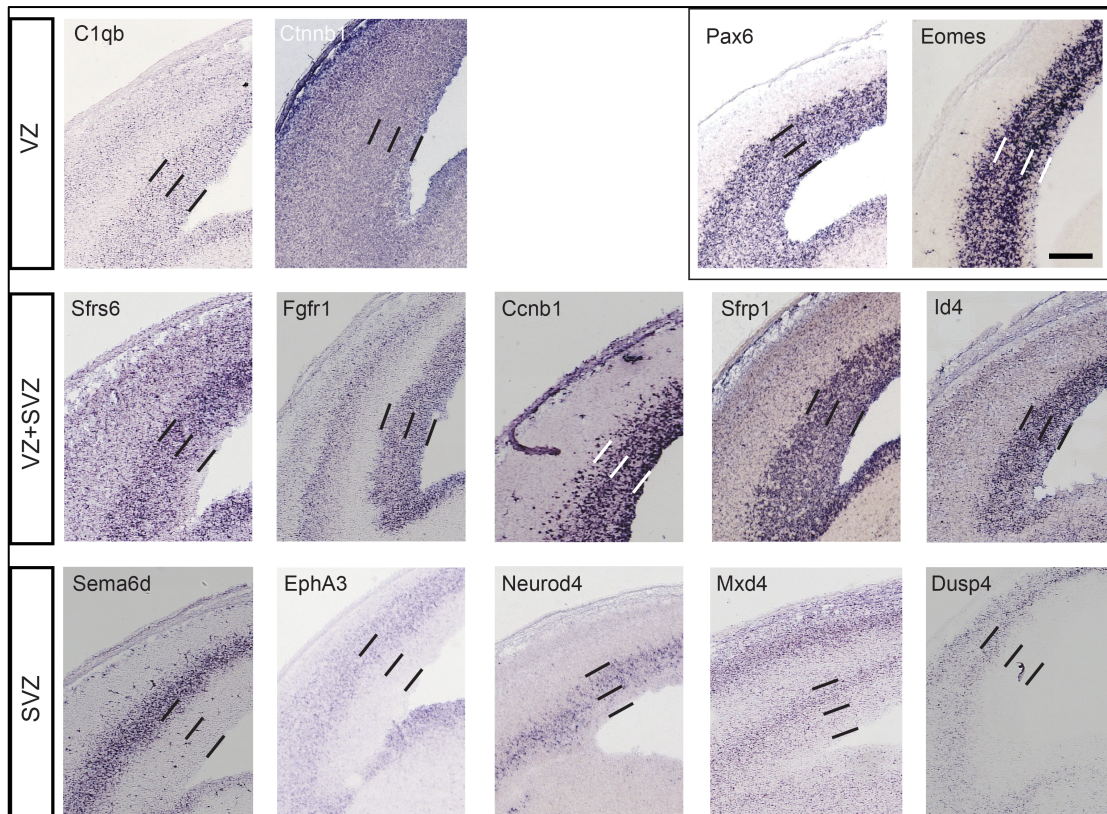


Figure 2.4 (on previous page): Expression pattern of upregulated genes by in-situ hybridization at E14.5 using the Genepaint database

In-situ hybridization patterns of genes from the over-expression gene list can be classified roughly under 3 classes; those that are predominantly in VZ (*C1qb* and *Ctnnb1*), those shared between VZ and SVZ (*Sfrs6*, *Fgfr1*, *Sfrp1* etc.) and those predominantly in the SVZ (*Sema6d*, *Neurod4*, *Mxd4* etc.). Expression of *Pax6* and *Eomes* (*Tbr2*) is shown for correlation. Black bars indicate VZ, SVZ boundaries. Scale bar 100 μ m

2.3.2 Validation of microarray by quantitative PCR

Having analysed expression of genes after over-expression of *Tbr2*, I next sought to validate changes in expression by performing quantitative real-time PCR (qRT-PCR). For this, I followed the same protocol used for over-expression of *Tbr2* prior to microarray (Figure 2.3).

Over-expression was performed in embryos from 5 separate litters and all slices obtained from a single litter were pooled to represent a replicate.

Validation by qRT-PCR showed that most genes selected showed similar fold change values as compared to the microarray values (Figure 2.5). Genes such as *Cdk4* (2.9 ± 0.49) and *Aspm* (2.71 ± 0.29) showed consistent upregulation further strengthening the idea of changes in cell cycle and centrosome proteins on *Tbr2* overexpression (Dehay and Kennedy, 2007). In comparison, the overexpression of *Tbr2* mRNA was ~1000 fold due to the use of a strong constitutive promoter (CAG). The fold change values of *Sfrp1* (3.37 ± 0.4) and *Wnt5a* (2.0 ± 0.3) indicate the possibility of a non-canonical Wnt signaling taking place upon overexpression of *Tbr2*. This fits well with observations using *Axin2*^{d2EGFP} that show a decrease in GFP expression as well as Axin2 mRNA levels in *Tbr2*⁺ cells (Mutch et al., 2010). However, *β-catenin* (2.31 ± 0.4) was also upregulated following overexpression contrary to suggestions elsewhere (Mutch et al., 2010; Sessa et al., 2008).

To address if the nature of regulation by *Tbr2* is two-way, I also chose to perform qRT-PCR analysis on RNA collected from *Tbr2* conditional knockout brains at E14.5 (Figure 2.5). At E14.5, *Tbr2* conditional knockouts show a decrease in phospho-histone H3⁺ (pH3⁺) and Ki67⁺ cells in the SVZ (Arnold et al., 2008). Further, there is also a decrease in *Cux2*⁺ and *Svet1*⁺ cells at this stage showing there is in addition to a difference in proliferation, also a change in specification of IPCs.

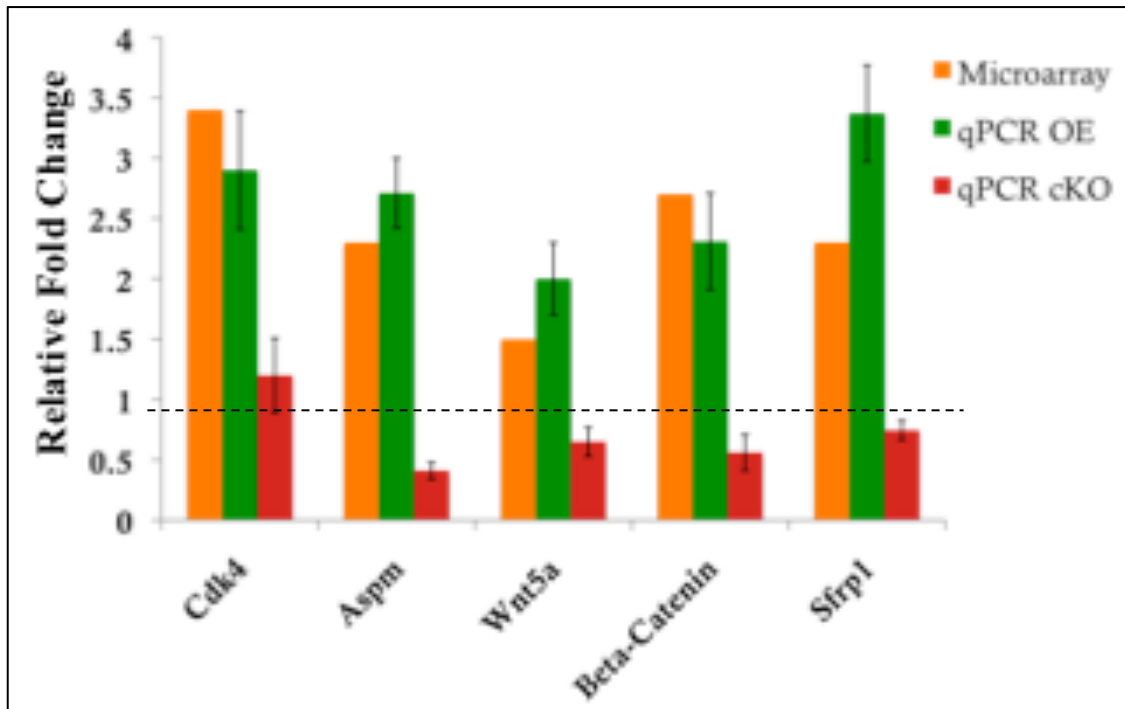


Figure 2.5: Quantitative RT-PCR analysis of genes after *Tbr2* overexpression and knockout

Validation by qPCR analysis of highest fold change genes from the *Tbr2* overexpression gene list shows that most genes showed similar fold changes (green) as compared to the microarray values (orange) with *Cdk4* and *Aspm* showing the largest changes. In contrast, qPCR analysis of the *Tbr2* conditional knockout (red) shows a decrease compared to WT. *Aspm* (0.41), *Wnt5a* (0.65), *Sfrp1* (0.75) and β -catenin (0.56) also showed appreciable decrease. Only *Cdk4* didn't show any change. N=5 for overexpression qPCR and N=3 for knockout qPCR. Genes were normalized using β -actin and further normalized with either EYFP overexpression samples (qPCR OE) or with WT samples (qPCR cKO) respectively using the DDCT method.

Analysis showed that there was indeed a decrease in the relative expression of *Aspm* (0.41 ± 0.07), *Wnt5a* (0.65 ± 0.12), *Sfrp1* (0.74 ± 0.08) and β -catenin (0.56 ± 0.15) as compared to wild-type controls. The decrease in expression for *Wnt5a*, *Sfrp1* and β -catenin was only 25-50% in magnitude and hence not very large (Figure 2.5). This could be explained as; *Sfrp1* and β -catenin are also expressed in the VZ (Figure 2.4) that could compensate the effect of the knockout leading to lower than expected values.

However, there was no change noted in the case of *Cdk4* (1.2 ± 0.31) in the knockout (Figure 2.5). It can be appreciated that expression of cell-cycle genes would be under homeostatic control as loss of function could lead to lethality or disease.

2.3.3 *Tbr2* binds to upstream regulatory regions to regulate expression

Having identified genes that changed in response to change in *Tbr2* expression, I also wished to address the mechanism of action of this regulation. For this, upstream regions of genes in Figure 2.5 were analysed by bioinformatics tools to search for a putative *Tbr2* binding motif (TCACACCT) within 1-2 kb upstream of the transcription start site (Teo et al., 2011) and only genes that showed presence of this motif were chosen. I undertook chromatin immunoprecipitation using an antibody against *Tbr2* followed by PCR for in the upstream region in E14.5 wild-type tissue (Costello et al., 2011; Teo et al., 2011).

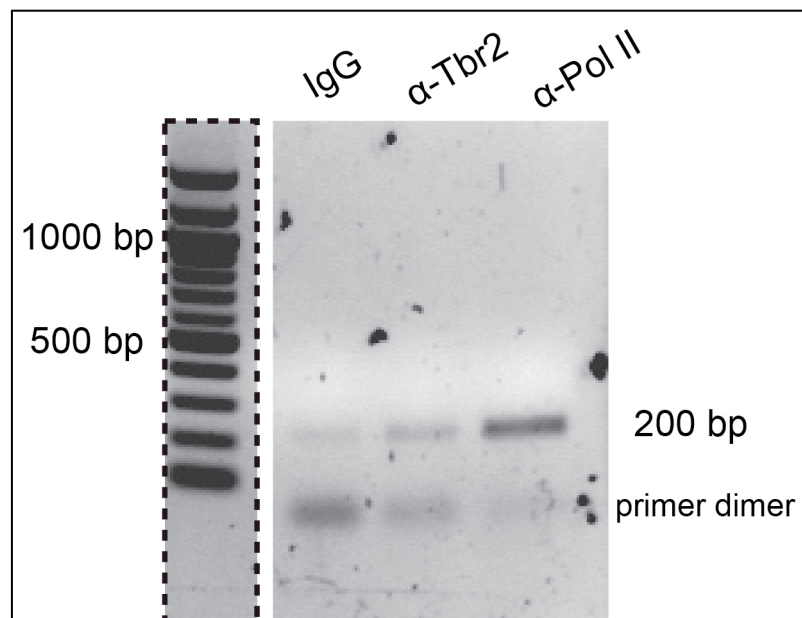


Figure 2.6: *Tbr2* regulates *Wnt5a* expression by binding to upstream regions

Chromatin immunoprecipitation (ChIP) using an antibody against *Tbr2* shows that *Tbr2* binds to sequences upstream of the *Wnt5a* gene. (L-R): 100 bp ladder, IgG pull-down, *Tbr2* pull-down and RNA Pol II pull-down. The amplified product is of 200 bp.

ChIP-PCR of an upstream sequence of *Wnt5a* showed that compared to the IgG control, the Tbr2 antibody shows preferential binding near the transcriptional start site as the same sequence is also pulled down by RNA polymerase II (Figure 2.6). This shows that the regulation of *Wnt5a* by Tbr2 is direct and by binding to upstream regulatory sequences.

I also checked the binding of Tbr2 to upstream regions for *Cdk4* and *Aspm* to ascertain if the same mechanism applies. In both cases, a preferential enrichment in the Tbr2 pulldown was noted over a no antibody as well as IgG control. In addition, regions of both genes were present close to transcriptional start sites as with *Wnt5a* (Figure 2.7 and Figure 2.8) though the amplification was much lower for RNA Pol II bound sequence than for Tbr2.

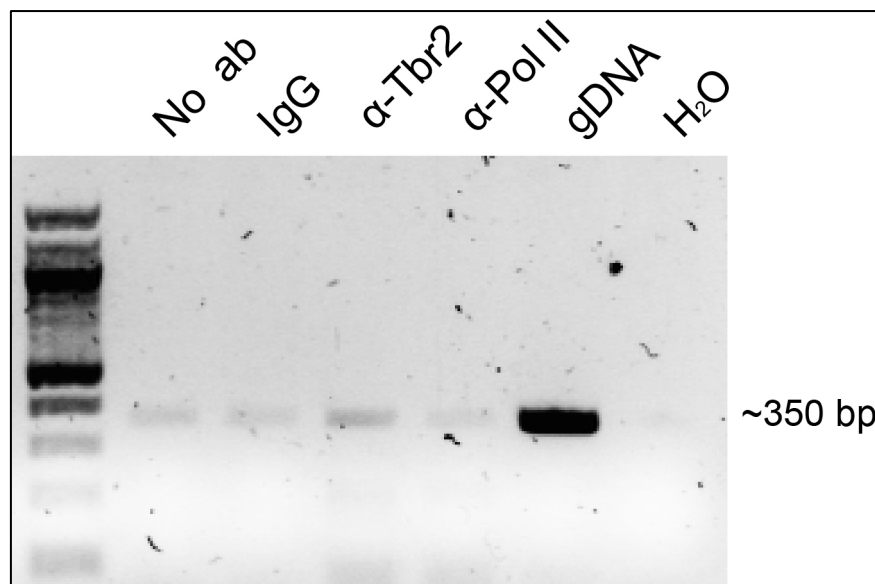


Figure 2.7: Chromatin immunoprecipitation for upstream sequence of *Cdk4*

Pull down with Tbr2 followed by PCR analysis of an upstream region (~350 bp) shows that the pull down is enriched with Tbr2 compared to both no antibody or IgG controls. Also shown are genomic DNA and H₂O as positive and negative controls for PCR. Image has been adjusted for brightness and contrast and cropped.

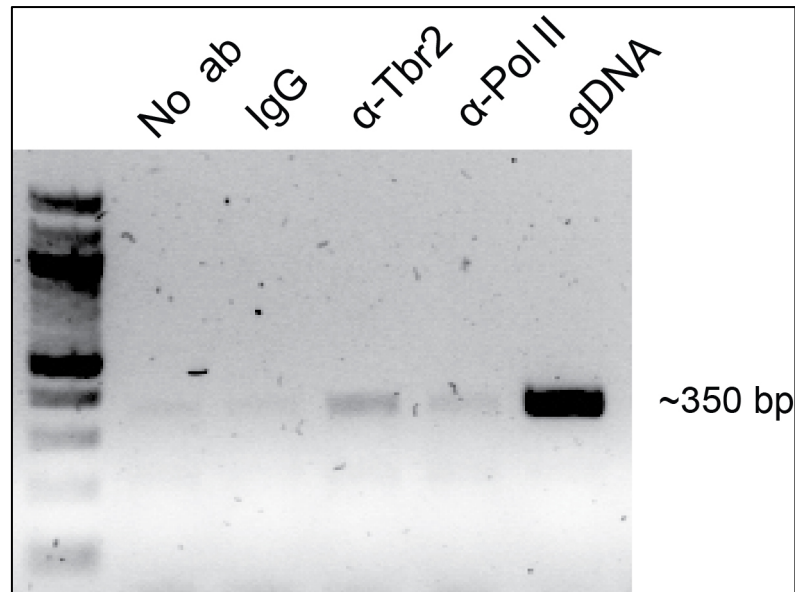


Figure 2.8: Chromatin immunoprecipitation for upstream sequence of *Aspm*

Tbr2 pull down is also enriched for *Aspm* upstream region as shown by the higher intensity of the Tbr2 lane compared to no antibody and IgG controls. Image has been adjusted for brightness and contrast.

As the enrichment shown by PCR was subtly higher when viewed qualitatively, I also performed quantitative PCR to define the fold enrichment so as to be able to compare the samples better. Samples were once again analysed by the DDCT method using the input fraction as the first step of normalization followed by using the IgG as the second normalization step. This showed that in both *Cdk4* and *Aspm*, the enrichment was ~3 fold (3.3 ± 0.03 and 3.29 ± 0.007 respectively) as compared to the IgG control (Figure 2.9).

This conclusively shows that genes such as *Cdk4*, *Aspm* and *Wnt5a* are direct downstream targets of Tbr2 and that Tbr2 regulates a variety of genes involved in different pathways.

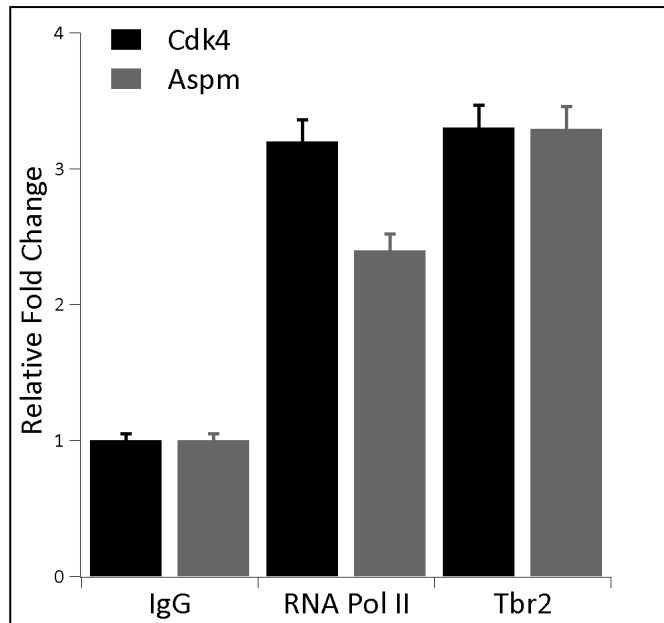


Figure 2.9: Quantitative detection of enrichment of upstream sequences after Tbr2 immunoprecipitation

qPCR analysis of pull down from IgG, RNA Pol II and Tbr2 shows a preferential enrichment in Pol II and Tbr2 samples for both *Cdk4* (black bars) and *Aspm* (grey bars). Genes were normalized using the input fraction.

2.3.4 In-situ expression of *Wnt5a* and β -catenin in the wild-type and *Tbr2* cKO

In addition to showing at a molecular level the function of Tbr2, I also wanted to determine the mechanism through which *Tbr2* influences the generation and fate of intermediate progenitors. For this, I concentrated on the interactions between β -catenin and *Wnt5a* due to their mutual negative regulation of each other. However, the changes noted for β -catenin in the overexpression and knockout conditions presents a difficult scenario for analysis. If β -catenin is required to maintain the radial glial fate then overexpression of *Tbr2* should cause a decrease and higher levels of β -catenin should be noted in the knockout. Hence, I decided to study if the change in mRNA levels is also

reflected in-situ. For this, I performed fluorescent in-situ hybridization (FISH) for *Wnt5a* and β -*catenin* in the *Tbr2* conditional knockout at E14.5 (Figure 2.10).

I analysed the pattern of mRNA expression in two areas namely the dorsal cortex and the medial located cortical hem (Figure 2.10). The cortical hem is known to secrete Wnt ligands and plays a crucial role in the development of the dorsal cortex (Grove et al., 1998). True to this, *Wnt5a* expression could be detected in the cortical hem (Figure 2.10G,H). However, expression in the dorsal cortex was very low and sparse (Figure 2.10E,F). The low expression of *Wnt5a* made it improbable to detect any qualitative changes in the dorsal cortex. However, in the cortical hem, the *Tbr2* cKO showed a restricted expression as well as decreased intensity (Figure 2.10G-H). In contrast, both the dorsal cortex and cortical hem showed robust expression of β -*catenin* in the VZ (Figure 2.10A-D). However, no qualitative difference could be detected in the knockout in either region (Figure 2.10B,D).

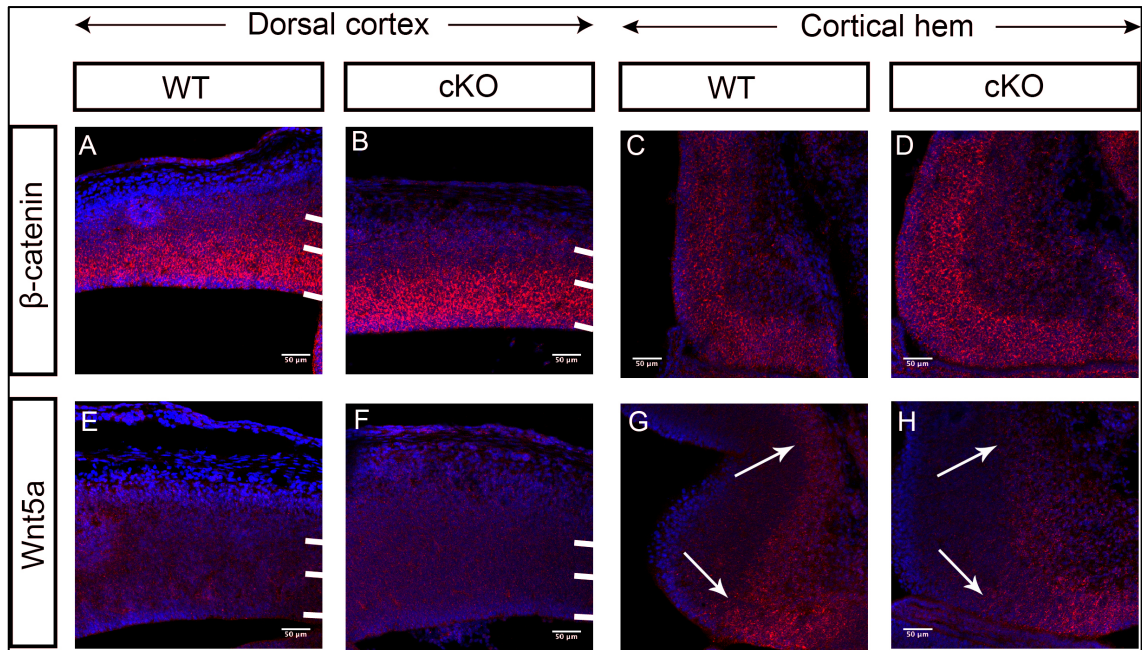


Figure 2.10: In-situ expression of *Wnt5a* and *β-catenin* in the dorsal cortex and hem of E14.5 wild-type and *Tbr2* cKO embryos

(A-D) Expression of *β-catenin* in the dorsal cortex (A-B) and cortical hem (C-D) remains unchanged in the WT (A,C) and KO brains (B,D). *Wnt5a* expression is very low in the dorsal cortex (E-F) and does not change in the KO (F). In the cortical hem (G-H), *Wnt5a* expression recedes medially in the KO (H, arrows) and also decreases in intensity. Scale bars: 50 μm. White bars demarcate approximate VZ, SVZ boundaries.

The absence of any difference between *Wnt5a* and *β-catenin* in the conditional knockout leads to questions about redundant effects of *Tbr2* in the developing cortex. These issues are discussed in the next section.

2.4 DISCUSSION

Tbr2 is an important gene for the specification of intermediate progenitors. Knockout models of *Tbr2* show that absence of this gene leads to a decreased cortical thickness and corpus callosum agenesis (Arnold et al., 2008; Sessa et al., 2008). In human, mutation in the locus containing TBR2 also causes microcephaly with polymicrogyria (Baala et al., 2007). However, the mechanism of specification and the mechanisms through which cell-cycle and neuronal fate is specified are less understood (Dehay and Kennedy, 2007; Elsen et al. 2013; Nelson et al., 2013; Visel, 2004).

This chapter attempts to look at changes in gene expression in response to overexpression of *Tbr2*. The reason for choosing an overexpression model over traditional loss of function models is two fold. Firstly, knockout models have limited use when dealing with redundant expression (Gitter et al., 2009). In the case of *Tbr2*, many other T-box genes are also expressed in the VZ/SVZ and might play an additive role in the induction or repression of downstream targets. Secondly, network stabilization by non-homologous genes would also mitigate some effects of transcription factor activity (Mutch et al., 2010; Niven, 2004; Siegal and Bergman, 2002).

In contrast, using an overexpression induces artificial conditions that push the transcriptional activity beyond tolerable differences. The forced expression of *Tbr2* itself presents an example where overexpression induces expression of genes such as *Cdk4* and *Aspm* that have not been revealed by knockout and

FACS sorting methods (Cameron et al., 2012; Elsen et al. 2013; Mutch et al., 2010; Sessa et al., 2008).

While the upregulation *Cdk4* might be explicable due to its prominence as a cell division kinase, and hence resistant to changes in expression, that of *Aspm* presents proof that overexpression models can uncover new interactions.

Aspm is a centrosomal protein that stabilizes mitotic spindle formation and promotes symmetric division in neuroepithelial cells (Arnold et al., 2008; Fish et al., 2006). Loss of function of *Aspm* leads to severe microcephaly with mental retardation (Bond et al., 2002; Costello et al., 2011; Teo et al., 2011). Thus the interaction with *Tbr2* is a curious one as intermediate progenitors do not show a plane preference during division (Attardo et al., 2008; Grove et al., 1998). However, imaging with *Tis21-GFP* shows that ~90% of basal progenitors undergo symmetric neurogenic divisions (Arnold et al., 2008; Haubensak et al., 2004; Sessa et al., 2008). Conversely, roughly 10% of basal progenitors undergo self-renewing divisions (Baala et al., 2007; Noctor et al., 2004). It is quite possible that the expression of *Aspm* occurs in that subset of *Tbr2* expressing cells and forced expression of *Tbr2* might increase the number of such progenitors at the expense of neurogenic divisions. However such a claim would need to be supported with imaging studies, as it is improbable to correlate expression of *Aspm* with kind of division for intermediate progenitors. If found true, this would also indicate that despite being multipolar cells, asymmetric inheritance can occur in IPCs.

More importantly, this also provides a possible mechanism through which

expansion of the intermediate progenitor pool could occur leading to increased neuron production and a larger cortical surface. Thus it would be interesting to study this comparatively with newer tools

This chapter also describes interactions between *Tbr2* and the Wnt signaling pathway genes such as *Wnt5a* and β -*catenin*. *Tbr2* has a direct and reciprocal effect on both genes and also binds upstream to *Wnt5a* to regulate its expression. While, in-vitro data showed that increasing or decreasing *Tbr2* levels had an impact on the two genes, this could not be corroborated with the in-situ data. This could be due to higher sensitivity of qPCR (Bao et al., 2007) or could be an artifact due to the very high expression levels achieved. However, in-situ hybridization did detect a difference in *Wnt5a* expression in the cortical hem. As Wnts are secreted ligands, this difference could manifest in the surrounding medial germinal zone and also travel as far as the dorsal cortex. The investigation of such non-autonomous changes will however for lack of tools to determine the migration of *Wnt5a* in the dorsal cortex.

2.5 APPENDIX

A.1: List of primers used for qRT-PCR on cDNA from Tbr2 overexpression

GENE	OLIGO (5'-3')
Aspm-L	GAT GGA GGC CGA GAG AGG
Aspm-R	CAG CTT CCA CTT TGG ATA AGT ATT TC
Cdk4-L	GCA GTC AGT GGT GCC AGA
Cdk4-R	GGA AGG CAG AGA TTC GCT TA
Ctnnb1-L	GCA GCA GCA GTT TGT GGA
Ctnnb1-R	TGT GGA GAG CTC CAG TAC ACC
Sfrp1-L	CAG TTG TGG CTT TTG CAT TG
Sfrp1-R	GAG GGA AGG GAG AGG GTT C
Tbr2-L	ACC GGC ACC AAA CTG AGA
Tbr2-R	AAG CTC AAG AAA GGA AAC ATG C

Wnt5a-L	TGA AGC AGG CCG TAG GAC
Wnt5a-R	AGC CAG CAC GTC TTG AGG
β -actin-L	CTA AGG CCA ACC GTG AAA AG
β -actin-R	ACC AGA GGC ATA CAG GGA CA

A.2: List of primers used for generating in-situ hybridization probes

PRIMER	OLIGO (5'-3')
Ctnnb1-F	GCA TAA TCT CCT GCT CCA TCA G
Ctnnb1-R	GGT TTC TGA GAG TCC AAA GAC AG
Wnt5a-ISH-F	TTC ATG AAT GGG CCT TTT TC
Wnt5a-ISH-R	GGC ACC ATT AAA CCA CAT CC

A.4: List of primers used to detect promoter sequences after Tbr2 chromatin immunoprecipitation

a. ChIP-PCR

PRIMER	OLIGO (5'-3')
Aspm_Prom_L	GTT CGC TCA TTG GCT CTT TT
Aspm_Prom_R	CAG AAG TGG CTG AGC AAC AG
Cdk4_Prom_L	GGC CTG TGT CTA TGG TCT GG
Cdk4_Prom_R	GAC ATC GCA CTA GGC ACA AA

b. ChIP-qPCR

PRIMER	OLIGO (5'-3')
Cdk4_ChIP_L	GGT GAG TGC AGT CTC ATC CA
Cdk4_ChIP_R	GGG CCA CCT TCC ATA ATC A
Aspm_ChIP_L	GCT GTA GCG AGG AGG TTC C
Aspm_ChIP_R	TTT TGC TCG GTT CAA ATA TCG

Chapter 3

Lineage and Fate Mapping of Tbr2+ Intermediate Progenitor Cells

3.1 INTRODUCTION

Intermediate progenitor cells (IPCs) are transient transit-amplifying progenitors present during neocortical neurogenesis that arise by asymmetric divisions of radial glial progenitors (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004) and reside in the sub-ventricular zone (SVZ) where the majority terminally divide to produce a pair of neurons (Noctor et al., 2004). Additionally, they differ from radial glial progenitors in being solely neuronogenic and largely undergoing terminal divisions (Noctor et al., 2004).

Intermediate progenitors are defined by expression of the long non-coding RNA *Svet1* (Tarabykin et al., 2001) and by transcription factors such as T-brain gene-2 (*Tbr2*; Englund et al., 2005) and *Cux2* (Zimmer et al., 2004) (Figure 3.1). Both of these transcription factors are known to play a significant role in IPC biology since mice knockouts of *Tbr2* and *Cux2* display changes in specification of IPCs and subsequent generation of neurons (Arnold et al., 2008b; Cubelos et al., 2008; Sessa et al., 2008). Conditional knockout models show a 50% decrease in supragranular and a ~30% decrease in infragranular layer thickness respectively (Sessa et al., 2008). However, the use of *Foxg1*^{Cre}

to ablate *Tbr2* expression is questionable as haploinsufficiency at the *Foxg1* locus causes a 35% decrease in *Tbr2*⁺ population and a 42% decrease in cycling cells at E13.5 (Siegenthaler et al., 2008).

Figure 3.1: IPCs contribute towards the upper layers of the cortex

(A) RGCs and other progenitors (yellow cells) in the VZ express genes different from IPCs (blue cell in SVZ). (B) Expression of mature neurons in the cortical plate indicates that upper layer neurons share expression patterns of IPCs while lower layer neurons express similar genes as the RGCs. (Modified from Dehay and Kennedy, 2007).

Evolutionarily, IPCs are thought to be instrumental in the expansion of the neocortical thickness by amplifying the neuronal output from radial glia (Cheung et al., 2010; Martinez-Cerdeno, 2006). It has been shown that non-ventricular surface divisions increase at late embryonic stages, during the formation of upper-layers (Martinez-Cerdeno, 2006), therefore relating IPC-derived neurons to supragranular layers. Previous fate-mapping by use of a *Neurod6* (*NEX*) driven cre mouse line has shown IPCs to contribute towards the formation of upper layers (Wu et al., 2005). This is further supported by the large decrease in the upper cortical layer thickness (20%) and *Cux1*⁺ cells in *Tbr2* conditionally deleted mice (Arnold et al., 2008b). However, in macaque fetal cortices, during periods of infragranular layer generation, nearly 30% of tritiated thymidine is

incorporated into non-ventricular zone progenitors (inner/outer SVZ) (Lukaszewicz et al., 2005). With recent studies showing large numbers of IPCs in both these regions (Fietz and Huttner, 2011; García-Moreno et al., 2012; Hansen et al., 2010; Reillo et al., 2010), it suggests that IPCs are also involved in formation of infragranular layers, at least in larger brains. In mice, the early expression of *Tbr2* at E10.5 in the *Tbr2::GFP* transgenic mouse line suggests that IPCs might contribute towards the generation of all cortical layers (Kowalczyk et al., 2009).

Direct evidence showing contribution from *Tbr2*⁺ IPCs towards infragranular layers is however lacking. A quantitative understanding of the neuronal output from IPCs towards cortical neurogenesis is currently absent and impeding our understanding of neuronal cell number regulation during development and evolution of the forebrain. According to the upper layer hypothesis, IPCs contribute only towards formation of upper layers (Tarabykin et al., 2001; Wu et al., 2005). However this is based solely on gene expression similarities. On the other hand the intermediate progenitor hypothesis (Kriegstein et al., 2006) suggests that an increase in cortical thickness can be achieved by production of a larger number of IPCs. This delimits the ventricular space by production of

non-epithelial progenitor cells and thus can achieve higher neuron production. This also suggests that the mechanism is not stage specific but rather applies throughout the neurogenic phase.

While it is known that most IPCs divide terminally to generate a pair of neurons in the rodent cortex (Noctor et al., 2004), it is not known whether these two neurons come to lie at the same laminar positions. Additionally, as neurons sharing functional properties lay in the same radial column (Mountcastle, 1957; 1997), it begs the question whether neurons derived from IPCs that lie in the same column are also related in function.

A reverse approach of labelling sister cells in the ontogenetic column (by using a low-titre retrovirus) followed by studying their electrophysiological properties has shown that such cells develop preferential connections in the postnatal cortex (Yu et al., 2009). Further such connections are unidirectional and mature with development reaching a plateau in the second week after birth (Yu et al., 2009). Similarly, clonally related neurons in the visual cortex of mice also were shown to respond similarly to orientation of a grating pattern (Li et al., 2012). However such approaches are based on labelling of a very small population of

progenitor cells (ideally 1-2) that are well separated and form radial columns. It is well known that RGCs undergo an amplification step during early neurogenesis (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001). This would duplicate radial columns and lead to an over-representation of neurons with the same functional characteristics. Additionally, the radial arrangement of neurons after retroviral infection is an over-simplification of neurogenesis. It has been shown before that clones labelled with retroviruses disperse sometimes >500 μm with dispersion increasing as IPCs generate majority of the neurons (Walsh and Cepko, 1993; Reid et al., 1997).

I hence investigate these issues by using the *Tbr2^{Cre}* and *Ai9-tdTomato* mouse lines (Costello et al., 2011; Madisen et al., 2009). I also make use of a novel clonal analysis method: CLoNe (Clonal Labelling of Neural progenitors) that is based on stable transfection of multiple Cre-inducible fluorophores in combination with a transposase expressing plasmid by in-utero electroporation (Garcia-Moreno et al. unpublished results) to understand clonal distribution of IPC derived cell populations.

Additionally, the chapter will also describe and address expression of *Tbr2* in regions not described before.

3.2 MATERIALS AND METHODS

All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986).

The date when vaginal plug was observed is defined as E0.5. Day of birth is defined as P0.5. Mice were maintained on a C57BL/6 genetic background in specific pathogen-free conditions on 12 hours light: dark cycle at a temperature of 22° C, 60-70% humidity, food and water access *ad libitum*. Cages were provided with cardboard tunnels for environmental enrichment.

Tbr2^{Cre} animals (Costello et al., 2011) were bred with *Ai9-tdTomato* reporter (Madisen et al., 2009) mice to label all neurons derived from the Tbr2+ IPCs. Pups born from this mating were perfused at P7 and P21 and n=3 mice were used for cell counts and immunostaining.

All cortical images were taken at the level of the S1 cortex.

3.2.1 Fluorescent In-Situ Hybridization (FISH)

In order to validate the expression of Cre, fluorescent in-situ hybridization was carried out on *Tbr2^{Cre}* embryos (n=4) at E12.5 on coronal sections containing

the developing dorsal cortex.

Probes were prepared by ligating into pGEMT (Promega), the PCR product from the following primers on a known Cre expressing plasmid (pCAG-Cre).

DIG-labelled riboprobes were synthesized using T7 and SP6 RNA polymerase according to manufacturers protocols (Roche).

Forward (5'-3'): GCCGCCACCATGGCCAATTT

Reverse (5'-3'): GCGGCCGCTATCACAGATCT

For ISH, tissue was fixed with 4% PFA (Sigma Aldrich) for 4 hours, cryoprotected with 30% sucrose overnight and frozen in OCT (TissueTek).

Sections of 16µm thickness were cut on a cryostat (Jung CM3000; Leica) and stored at -80 degrees until use. Sections were postfixated with 4% PFA for 5 mins, deproteinized with 0.1 N HCl for 5 mins and acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine hydrochloride). Pre-hybridization was carried out at room temperature (RT) for at least 1 hour in a solution of 50% formamide, 200 µg/mL E. coli transfer RNA, 1x Denhardt's solution, 10% dextran sulfate, 10 mM Tris (pH 7.6), 600 mM NaCl, 0.25% sodium dodecyl sulfate, and 1 mM ethylenediaminetetra-acetic acid (EDTA). Hybridizations were carried out overnight at 65°C with riboprobes diluted in the pre-hybridization solution. Sections were washed in 30 mM NaCl/3 mM sodium

citrate first at 65°C for an hour and then at RT for 5 mins. Sections were blocked with 1% blocking reagent (Roche) in 100 mM Tris-Cl (pH 7.5) and 150 mM NaCl. This was followed with overnight incubation in anti-DIG antibody (1:500, Horseradish peroxidase conjugated, Roche) at 4°C. Sections were developed using the TSA-plus Cyanine 3 Kit (PerkinElmer) according to the manufacturers instructions, counterstained with DAPI and mounted with PBS.

3.2.2 Plasmids

In order to label individual IPC clones, two kinds of plasmid vectors were used namely: labelling vectors (that contained a loxP-STOP-loxP insert ahead of a fluorescent protein gene) and transposase gene (that ubiquitously expressed transposase under a CMV promoter) (Figure 3.2).

To generate labelling vectors, general molecular cloning strategies were employed. As an example, a synthetic stop SV40 signal (containing SV40 polyadenine sequence) flanked by directly oriented LoxP sites was PCR amplified from the pBS302 plasmid (Sauer, 1993; kindly provided by Dr. Sauer; Addgene plasmid 11925) using primers with 5' BamHI and 3' NcoI sites. The PCR product was then subcloned through BamHI and NcoI restriction into a

pPB-UbC-EGFP vector (kindly provided by Wellcome Trust Sanger Institute; Yusa et al., 2009) between UbC promoter and EGFP sequence. The UbC promoter was subsequently replaced with the CAG promoter due to its stronger expression to generate a pPB-CAG-loxP-STOP-loxP-EGFP and was referred as pPB-STOP-EGFP. Similar vectors were constructed replacing the fluorophore with EYFP, mCherry, Sapphire etc. This gave a cytoplasmically localised expression of fluoroproteins.

To obtain separate subcellular localisation, human H2B histone (Genbank ID X00088.1; to label nucleus) and a palmitoylation sequence (to tag the cellular membrane) from CMV-Brainbow-1.1 M were amplified by PCR and cloned in frame at the 5' end of the fluorophore. The resulting vectors were referred as pPB-STOP-H2B-XFPs and pPB-STOP-Mb-XFPs respectively.

The expressing transposase vector (or mPB), kindly provided by Wellcome Trust Sanger Institute (Yusa et al., 2009), is required for insertion of the fluorophore plasmids into the genome of progenitor cells.

Scheme of use of these plasmids is diagrammatically represented in Figure 3.2.

Figure 3.2 Diagrammatic representation of plasmids and mechanism of labelling

(A) Labelling vectors are shown in the arrested form. Fluoroprotein expression is blocked (white) by the SV40 STOP signal (red hexagon). When electroporated into *Tbr2^{Cre}* mouse, recombination occurs at the loxP sites (green triangles) leading to excision of the STOP signal generating the active vector and driving expression (yellow). (B) Transposition of the active vector occurs by the action of transposase that is coded by the mPB plasmid. The transposase detects 5' and 3' terminal repeats (TR; blue with black border) and inserts intervening sequences into the genome in a variable manner generating a unique combination. Expression of XFPs is hence inherited. Modified from García-Moreno et al. (unpublished).

3.2.3 In-utero electroporation

To label individual IPCs for fate mapping and for analysis of clonal generation, I performed in-utero electroporation on *Tbr2^{Cre}* mice. Using this method, plasmids were electroporated into cells lining the ventricular surface. However, as the Cre is thought to be expressed only in Tbr2+ IPCs, the expression shall be achieved only in Cre+ cells. In-utero electroporation was performed as reported by Shimogori and Ogawa (2008) with modifications. Time-mated pregnant dams were administered Vetergesic intraperitoneally (0.08 mg/kg) for analgesia prior to anesthesia with Isoflurane. A midline laparotomy was undertaken and uterine horns exposed gently. DNA (mixed with Fast Green, Sigma) was injected into the lateral ventricles using pulled glass capillaries (Harvard Apparatus). Square pulses (at 30-35V, 5x50 ms with 950 ms resting periods) were delivered using tweezer electrodes (CUI650P3, Nepagene)

connected to a square pulse generator (Electro Square Porator, Harvard Apparatus). Warm phosphate-buffered saline (PBS) was squirted regularly on the embryo sacs to keep them at near body temperature. In addition, a warm blanket was used to keep the female warm throughout the duration of the surgery.

Following electroporation, the uterine horns were gently placed back in the abdominal cavity. Surgical incisions were sutured (Vicryl 4/0, Ethicon) and the skin held together using surgical clips. Animals were allowed to recover after removal of anaesthesia and kept on heating pads until they regained movement. Animals were monitored post-operatively to check for signs of pain.

A total of n=4 pups at P7 were analysed from electroporations at E12.5, and n=3 pups at P7 from electroporations at E15.5.

3.2.4 Immunohistochemistry

Pups born from *Tbr2^{Cre}* and *Ai9-tdTomato* breeding were used to perform immunohistochemistry. At each age (i.e. P7 and P21), n=3 brains were analysed.

Drop-fixation of embryonic brains was carried out at 4°C overnight in 4% paraformaldehyde (PFA; Sigma-Aldrich) and subsequently stored in PBS with 0.05% Sodium Azide (Sigma). For postnatal ages, animals were transcardially

perfused first with cold PBS and then with cold 4% PFA before collecting the brains. Brains were sectioned on the Leica VT1000S vibrating microtome at 40-50 μ m and collected in PBS. Sections were washed in PBS thrice for 5 mins each and permeabilised in PBS- Triton-X (0.1%; PBST) for 30 mins. Following blocking with 5% serum (in PBS-T) for 2 hours, sections were incubated overnight at 4°C in primary antibody diluted in PBS-T with 1% serum. The following day, the sections were again washed before incubation with secondary fluorescent-labelled antibodies (Molecular Probes, Invitrogen) for 2 hours at room temperature. Sections were counter-stained with DAPI (4',6-diamidino-2- phenylindole, Invitrogen) and mounted on slides with PBS. The edges were sealed with clear nail-varnish and allowed to dry.

Table 3.1: List of antibodies used

ANTIBODY	SOURCE AND CATALOGUE NO.	CONCENTRATION
	Primary antibodies	
Chick anti-EYFP	GFP-1020; Aves Lab	1/1000
Chick anti-Tbr2	AB15894; Millipore	1/250
Goat anti-Nurr1	RD Systems	1/200
Mouse anti-Calbindin	300; Swant D-28k	1/500
Mouse anti-Otx1	Giorgio Corte, Univ. of Genova	1/1000

Mouse anti-Satb2	ab51502; Abcam	1/200
Rabbit anti-Calretinin	AB149; Chemicon	1/500
Rabbit anti-ChAT	ab70219; Abcam	1/500
Rabbit anti-Cux1	sc-13024; Santa Cruz	1/200
Rabbit anti-GABA	A2052; Sigma	1/1000
Rabbit anti-GFAP	G9269; Sigma	1/500
Rabbit anti-Ki67	ab15580; Abcam	1/500
Rabbit anti-Tbr1	ab31940; Abcam	1/1000
Rat anti-Ctip2	ab18465; Abcam	1/500
Sheep anti-TH	ab113; Abcam	1/1000
ANTIBODY	SOURCE AND CATALOGUE NO.	CONCENTRATION
	Secondary antibodies	
Donkey anti-chick 488	703-546-155; Jackson Labs	1/1000
Donkey anti-goat 488	A11055; Molecular Probes	1/500
Donkey anti-rabbit 488	A21206; Molecular Probes	1/500
Donkey anti-rabbit 568	A10042; Molecular Probes	1/500
Donkey anti-rabbit biotinylated	ab7082; Abcam	1/100
Donkey anti-rat 488	A21208; Molecular Probes	1/500
Goat anti-mouse 488	A11029; Molecular Probes	1/500
Goat anti- sheep biotinylated	BA-6000; Vector Labs	1/100

3.2.5 Tbr2 cKO cell comparison

In order to obtain *Tbr2* cKO neonatal animals, *Sox1^{Cre}* (Takashima et al., 2007) animals were crossed with *Tbr2^{flox/flox}* (Arnold et al., 2008a) and the resultant Cre+ F1 generation back-crossed to *Tbr2^{flox/flox}*. Pups from this breeding were provided by Prof. Elizabeth J Robertson (Sir William Dunn School of Pathology).

Images of Nissl stained sections (40 µm thickness) were taken using a Leica light microscope (Leica DMR 500). Cells were counted in 100 µm widths using NeuroLucida (MBF Bioscience) and Adobe Photoshop CS6 (Adobe Systems Inc).

Cell counts between heterozygotes and homozygotes for the deletion were compared.

3.2.6 Imaging and Image Analysis

Images were captured using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microimaging). Similar image parameters (gain, pinhole and wavelengths) were maintained for images from all brains. Z-stacks were taken individually for each channel and then collapsed to get maximum intensity projections. Images were analysed using ImageJ (Image Analysis in Java, NIH) or Adobe Photoshop CS6 (Adobe Systems Inc.).

3.2.7 Image Segmentation

Confocal images of multi-channel fluorescently labelled cells were analysed using a histogram based multi-level thresholding algorithm (Arora et al., 2008) on MATLAB software (R2013a; Mathworks). The code is appended to this thesis in A.5.

An initial weighted mean is computed for the image using the pixel intensities (0-255) and hence leads to a single threshold value (say x). Once the first threshold is computed, the algorithm segments the histogram into two parts (i.e. values from 0 to $x-1$ and from $x+1$ to 255). The threshold for these parts is once again calculated leading to sub-thresholding. This process is iterated many times over to get the optimum number of thresholds (n) required to segment the image (7-12 usually) which is determined based on changes of the PSNR (peak signal to noise ratio) values with each iterative step.

Finally, thresholded values were compared with the figure to verify that they showed similarly labelled cells i.e cells suspected to be clonally should show similar thresholded values in all three channels.

An example of an image before and after thresholding alongwith the histogram is shown in Figure 3.3. As can be seen, post-thresholding, only three thresholded values are sufficient to reconstruct the image.

Thus by comparing a group of cells within defined columnar widths, one can verify the similarity in the colour hue of the cells.

Finally, in order to generate statistical confidence, ImageJ was used to obtain the mean intensity values of “clonally related” cells in all channels. Cells were taken to be related only if the mean intensity value of two cells was comparable in all channels within the standard deviation values.

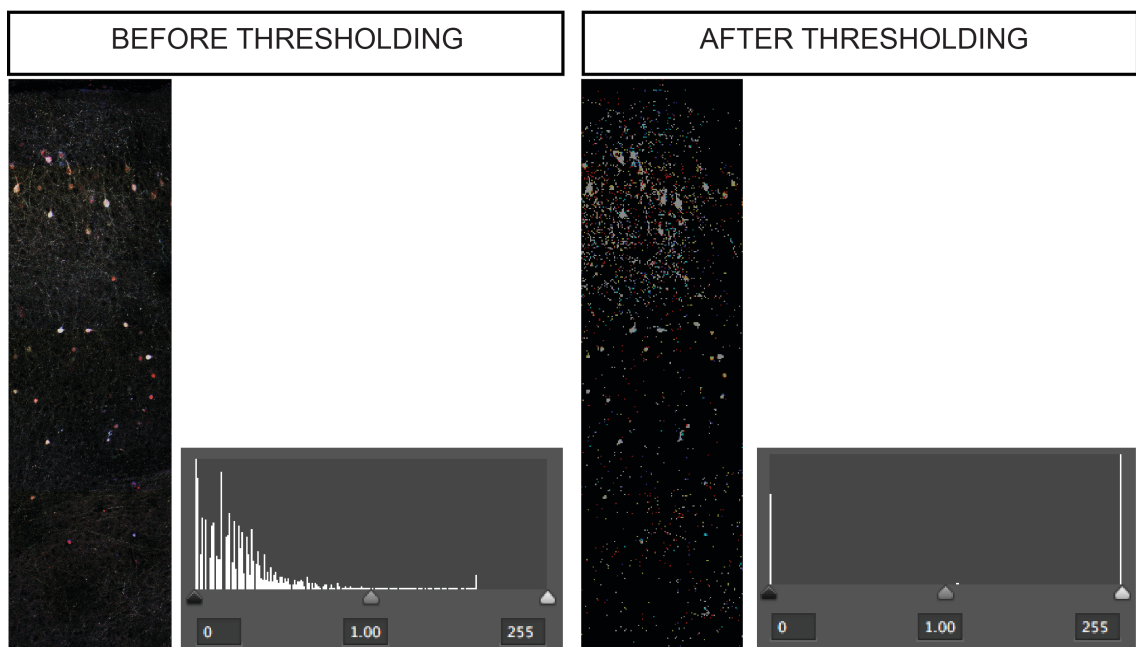


Figure 3.3: Effect of thresholding based image segmentation

The unthresholded image on the left is shown with its histogram. As can be seen, a variation of pixel intensities exists in the image from 0-255. The image on the right shows a thresholded image alongwith its histogram. As can be observed, the thresholded histogram shows only 3 peaks as against the numerous observed before. These three peaks represent the segmentation of the image based on pixel intensities.

3. 3 RESULTS

The expression of *Tbr2* has been previously described in the developing brain, and specifically in progenitors of the subventricular zone (SVZ) (Englund et al., 2005; Kimura et al., 1999). Compared to other SVZ markers, such as the *Cux2* gene, *Tbr2* is expressed only in the intermediate progenitor cells (IPCs) while *Cux2* expression can also be seen in the upper cortical layers of the cerebral cortex (Zimmer et al., 2004). Further, *Cux2* also labels a subpopulation of radial glial cells that are destined towards the upper layers (Franco et al., 2012) thereby complicating its use as an IPC marker. Thus I chose the *Tbr2^{Cre}* mouse line (Costello et al., 2011) to fate map the entire progeny of IPCs in the mouse brain.

3.3.1 Analysis of IPC derived neurons in the *Tbr2^{Cre}* mouse

Expression of Cre-recombinase was characterized by fluorescent in-situ hybridization (FISH) at E12.5. It was found that the Cre-recombinase is expressed in proliferative zones but not in the thin cortical plate at this age (Figure 3.4 A-A'). Cre expression was also compared with that of *Tbr2* protein (on an adjacent section) and it was found to be largely correlative in the SVZ

(Figure 3.4 B-B'). Cells in the *Tbr2* negative region of the VZ were also positive for Cre mRNA indicating that transcription of the transgene occurs during the transition to IPC fate.

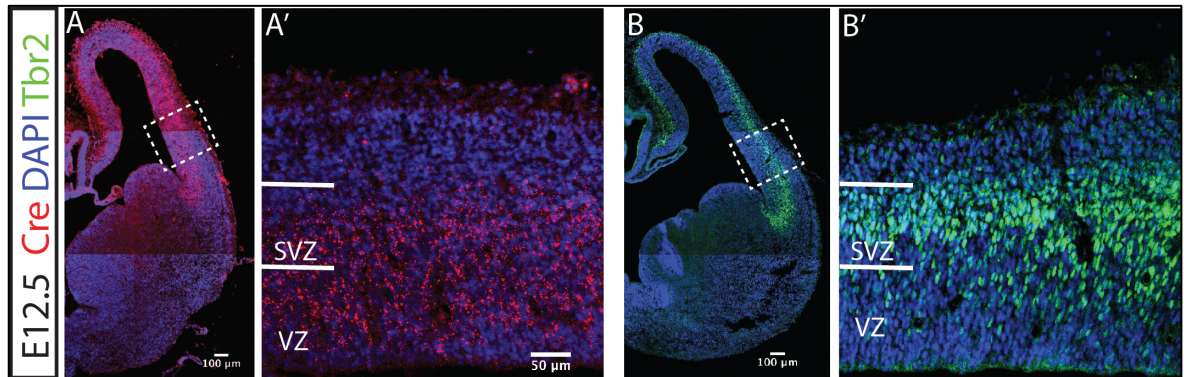


Figure 3.4: Cre-recombinase expression in *Tbr2*^{Cre} cerebral cortex at E12.5

(A-A') Expression of the Cre-recombinase in *Tbr2*^{Cre} at E12.5 by fluorescent in-situ hybridization. Cre mRNA is expressed in the dorsal pallium only and not in the subpallium in accordance with the expression of *Tbr2*. Boxed region in A is shown in A'. (B-B') Immunohistochemistry for *Tbr2* on an adjacent section to the one in A to show correlation of expression. Boxed region is shown in B'. Scale bars: A,B: 100 μm, A',B': 50 μm

In order to label the progeny of IPCs, two separate strategies were employed. Firstly, the *Tbr2*^{Cre} (Costello et al., 2011) was crossed with the *Ai9-tdTomato* reporter mice ((Madisen et al., 2009) henceforth referred as Ai9 brains) to label all cells born from a *Tbr2*⁺ lineage across the brain during development. Secondly, I performed in-utero electroporation in *Tbr2*^{Cre} pregnant mice at 12dpc (E12.5) in order to label timed lineages. A cocktail of transposase

expressing plasmid and STOP-arrested fluoroprotein expressing plasmids was used. This cocktail labels all progenies of focally electroporated progenitors that pass through a Tbr2⁺ lineage with the same combination of fluorophores thus labelling clonal populations (Figure 3.5A; (Garcia-Marques and Lopez-Mascaraque, 2013), Garcia-Moreno et al. personal communication).

Short-term labelling (E12.5-E14.5) with only CAG-STOP-EYFP labelled cells exiting the SVZ (Figure 3.5B). Importantly, no label was found in the ventricular zone that houses radial glial progenitors (Malatesta et al., 2000) displaying the specificity of the labelling. Immunostaining for Tbr2 showed only a small proportion of EYFP⁺ cells in the SVZ (Figure 3.5B) with the majority in the intermediate zone (IZ) and cortical plate (CP). Additionally, none of the labelled cells co-localised with Tbr2 possibly indicating that expression of the fluorophores occurs during exit from SVZ during or after the final cell division.

In order to test the process of combinatorial fluorescent labelling of progenies, a cocktail containing mT-Sapphire (membrane anchored), GFP (cytoplasmically targeted) and mCherry (nuclear targeted) was electroporated at E12.5 and the brains collected 2 days later. As with the single fluorophore electroporation,

most of the cells were positioned above the SVZ with some even sending axons through the IZ towards subcortical targets. Two independent pairs of clones could be observed exiting the SVZ. The first pair (Figure 3.5C; thick arrowheads) was negative for Sapphire, weakly positive for GFP and strongly expressing mCherry while the latter was positive for all three (thin arrowheads). Thus, clonal labelling of progenies of IPCs is possible using this method. In addition, the descent of axons from these cells (Figure 3.5D and 3.5E) showed that a proportion of these cells are subcortically projecting and hence infragranular in nature. To confirm this, I performed immunostaining for Ctip2 and Tbr1 separately in E14.5 Ai9 brains (Figure 3.5F-G). Both Ctip2 and Tbr1 co-localised with Ai9+ cells (Figure 3.5F-H) above the IZ showing that infragranular layer neurons do arise from Tbr2+ IPCs during early neurogenic periods.

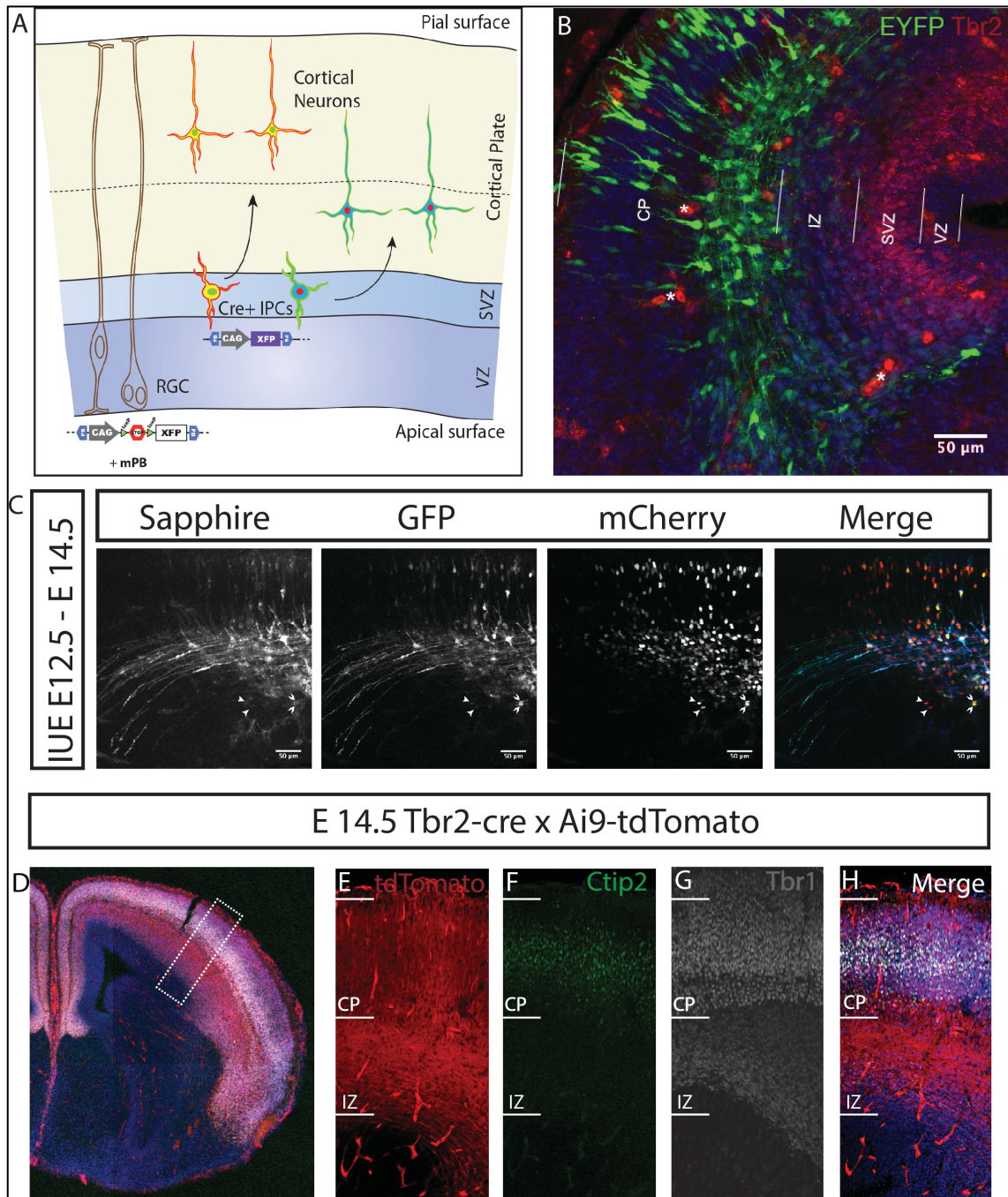


Figure 3.5: Analysis of IPC derived neurons in the *Tbr2^{Cre}* mouse with stable transfection of fluoroproteins and Ai9-tdTomato reporter mouse at E14.5

(A) Schematic representation of the lineage analysis with stable transfection of fluoroproteins using a transposase plasmid by in-utero electroporation. (C) Single fluorophore (STOP-EYFP) transfection at E12.5 (collected at E14.5) revealed labelled cells in IZ, SP and CP, but not in VZ and SVZ. Immunoreactivity for Tbr2 is shown in red. Asterisks indicates blood vessels. (D) The method outlined in B can be extended to various fluoroproteins to identify clonally derived cells. The example utilised three

fluorophores (Sapphire, GFP, mCherry). Arrowheads indicate pairs of cells expressing the same combination of fluoroproteins. (D-H) Immunostaining sections from brains derived from breeding *Tbr2^{Cre}* and *Ai9-tdTomato* mouse lines at E14.5 revealed that Ctip2 (F) and Tbr1 (G) immunoreactive neurons are derived through Tbr2 positive IPCs. Scale bars: D: 100 μ m, rest are 50 μ m

3.3.2 Tbr2 positive IPCs derived neurons contribute to all layers of the cortex

As neurons continue to migrate well into postnatal stages, the final positions of neurons generated from Tbr2+ IPCs needed to be addressed. For this, *Tbr2^{Cre}* mice were crossed with *Ai9-tdTomato* reporter mice to label all cells born from a Tbr2+ lineage throughout the brain during development. A developmental stage (P21) was chosen after the completion of neuronal migration (Figure 3.6A). Ai9+ cells were observed across all layers of the cortex though this varied across different regions. Cingulate areas and the lateral cortex had Ai9+ neurons spread across the length of the cortex while in more dorsal regions fewer Ai9+ cells were present in infragranular layers (Figure 3.6A-A''). To quantify contribution to different cortical layers, cell counts were done from the lateral cortex. Staining for known layer-specific markers such as Cux1, Otx1 and Nurr1 (Frantz et al., 1994; Hoerder-Suabedissen et al., 2009; Nieto et al., 2004) was carried out to confirm cell identities (Figures 3.6B-D).

Immunostaining for these markers revealed that Ai9⁺ neurons displayed fate identities in keeping with their positions. Majority of Ai9⁺ cells in supragranular layers also labelled for Cux1 while a smaller proportion of infragranular layers was positive for Otx1 (Figure 3.6B'-C', arrowheads). Interestingly, a subset of Nurr1⁺ subplate cells was found to be Ai9⁺ (Figures 3.6D', arrowheads). Subplate neurons are the earliest born cells in the cortex born between E11.5 and E12.5 and are involved in mediating corticothalamic and thalamocortical innervation (McConnell et al., 1989; Hoerder-Suabedissen and Molnár 2009). It has been reported that Tbr2⁺ IPCs also give rise to Cajal-Retzius cells in the developing brain (Hodge et al., 2013) and considered together with data shown here, this shows the contribution of Tbr2⁺ IPCs towards formation of early cortical neurons that play a substantial role in proper patterning and layering of the cortex. Additionally, the number of neurons derived from Tbr2⁺ IPCs in each layer was quantified in Ai9 brains at P21. Cells born from Tbr2⁺ IPCs were present in all layers of the cortex (Figure 3.6E) with varying proportions. Layers 2/3 and layer 4 had the highest proportions of Ai9⁺ cells with 40.2%±6.4 and 31.3%±5.4 respectively. In comparison, layer 6 and subplate (SP) had fewer cells with 29%±4.9 and 27.2%±6. Layer 5 contained the fewest cells born through an intermediate progenitor with 19.8%±4.2. Since Ai9⁺ cells were

counted as a percent of all cells in the cortex (thereby including glial cells and interneurons in the denominator) the percentages of neurons derived from Tbr2+ IPCs are under-represented in these observations.

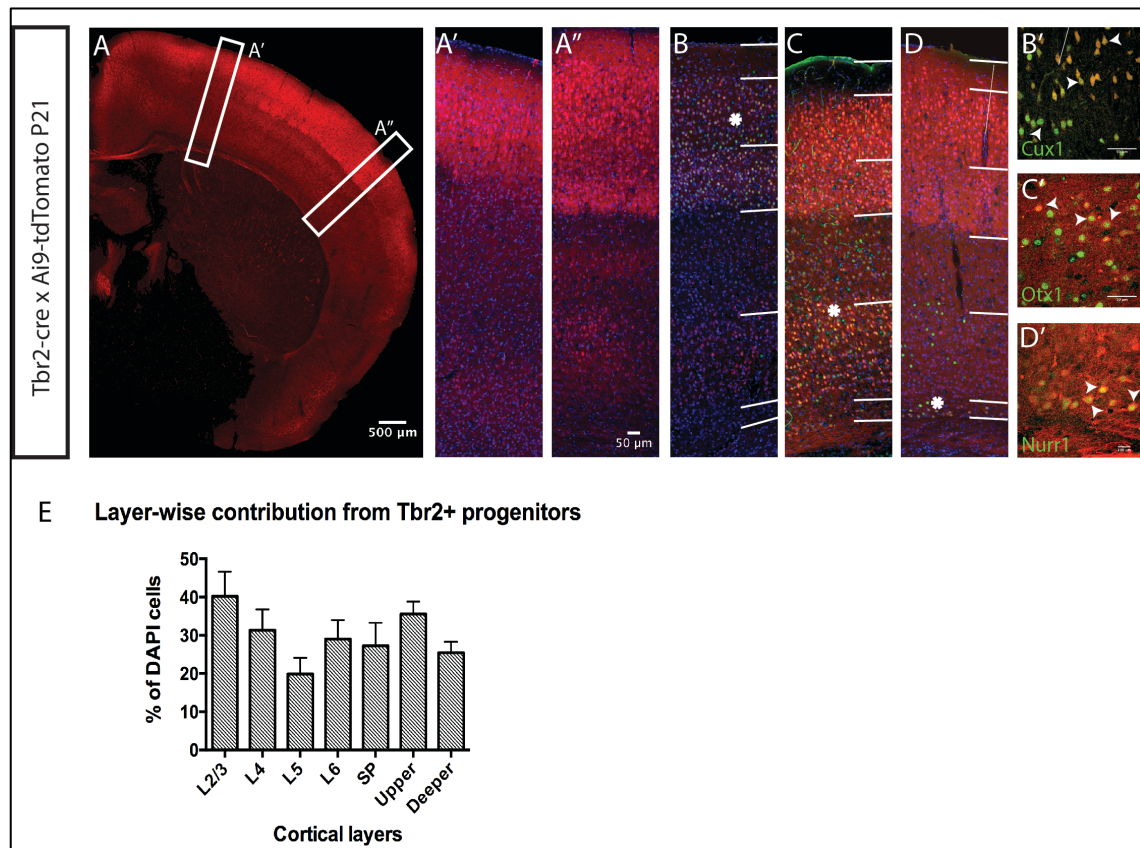


Figure 3.6: Tbr2 positive IPCs derived neurons contribute to all layers of the cortex

(A) Coronal section through a P21 brain derived from breeding Tbr2-cre and Ai9-tdTomato mouse. A' and A'' were taken with higher power from regions indicated in A. The cortical segment indicated in A'' was stained and imaged for Cux1 (B), Otx1 (C) and Nurr1 (D) immunoreactivity. Asterisks indicate the region in layers 2-3 (B'), layer 5-6 (C') and subplate (D') where the high power images were taken. Arrowheads in B'-D' indicate examples of double positive neurons.

(E) The percentage of Ai9-tdTomato expressing cells from all DAPI stained cells in P21 sections (shown in A'') shows that 20-40% of all layers is derived through Tbr2+ IPCs in S1. Layers 2-3 shows the highest (40.2%) while layer 5 is the least (19.8%).

It was previously reported that *Tbr2* conditional knockout cortices showed a large decrease in the Cux1+ population of neurons with no significant decrease observed for Er81+ and FoxP2+ neurons (Arnold et al., 2008b). However as these markers do not stain all pyramidal cells in the respective layers, we undertook an unbiased layer-wise cell count (neurons and glia on Nissl stained sections) on *Sox1^{Cre} x Tbr2^{fl/fl}* conditional knockouts in the somatosensory cortex (Figure 3.7A) at P8. Surprisingly, the decrease in cell numbers was observed only for layer 4 and layer 6 of the S1 in P8 cortex (Figure 3.7B). The decrease in layer 4 neurons was large (~ 43% of wild-type, p=0.009) while that in layer 6 neuronal count was 14.5% (p=0.08). None of the other layers showed any appreciable decrease while there was a minor increase in the layer 5 cell count (~ 5%, p=0.068) (Figure 3.7B). Thus the decrease in supragranular layers seems to stem from a decrease in layers 4 and 6.

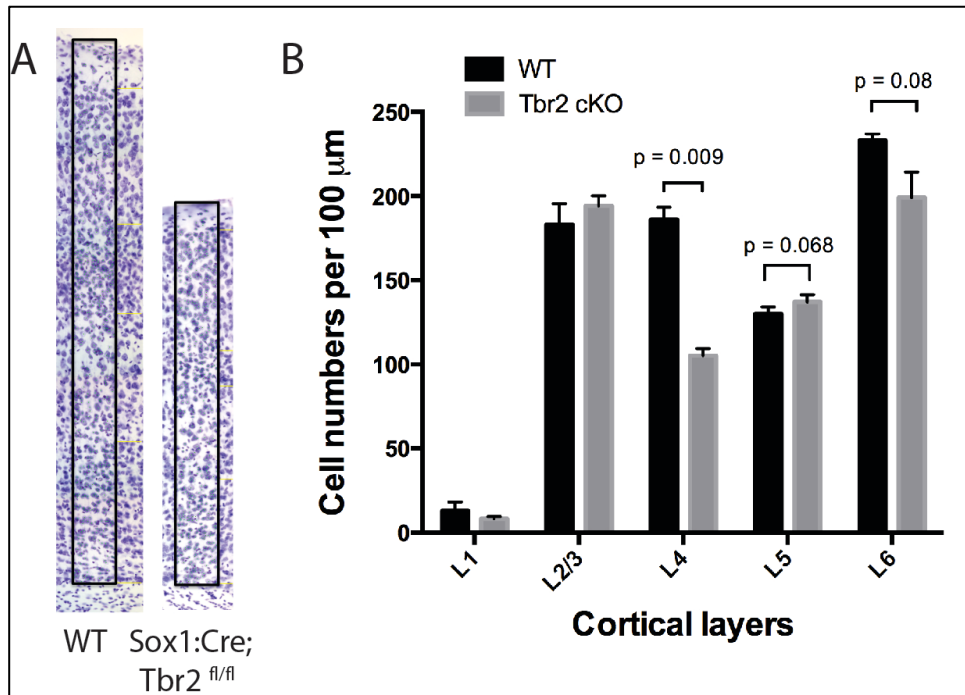


Figure 3.7: Comparison of layer-wise neuron counts in WT vs Tbr2 cKO

(A) Images of Nissl stained somatosensory cortex from coronal sections of wild type (WT) and Tbr2 conditional KO (*Sox1^{Cre}::Tbr2^{fl/fl}*) indicate reduced thickness. (B) Comparison of neuronal numbers in individual layers of wild type (WT) and Tbr2 conditional KO S1 cortex revealed significant reduction of layer 4 and 6 (43% and 14.5% respectively) and marginal increase in layer 5 (~5%).

It is widely accepted that IPCs contribute towards upper-layer genesis (Tarabykin et al., 2001; Wu et al., 2005; Zimmer et al., 2004). However, the cell counting showed that while there was a significant difference between the contribution towards supragranular ($35.55\% \pm 3.24$) and infragranular layers, the latter received a substantial contribution through IPCs ($25.44\% \pm 2.84$) (Figure 3.6E). Looking at the cortical thickness as a whole, the contribution from IPCs

was found to be $30.03\% \pm 1.95$ in S1 showing that contribution towards cortical layers is more homogeneous than previously thought.

To examine whether cells derived from Tbr2⁺ IPCs were glial or GABAergic, I stained for glial fibrillary acidic protein (GFAP) and gamma-amino butyric acid (GABA) in the P7 cortex (Figure 3.8A-E). Expression of GFAP was largely limited to the white matter with some expression in layer 1 and 5 (Figure 3.8A). Looking closely at the white matter, some overlap could be found between Ai9⁺ cells with GFAP⁺ processes in the corpus callosum and rostral migratory stream (RMS). This is in keeping with previous observations of Tbr2⁺ neuroblasts migrating to the olfactory bulb where they differentiate to glutamatergic interneurons (Brill et al., 2009) (Figure 3.8B-D). On the other hand, GABA immunoreactivity did not overlap with Ai9⁺ cells in the cerebral cortex (Figure 3.8E) maintaining that cells generated through the Tbr2⁺ lineage were glutamatergic as previously asserted (Hevner et al., 2006).

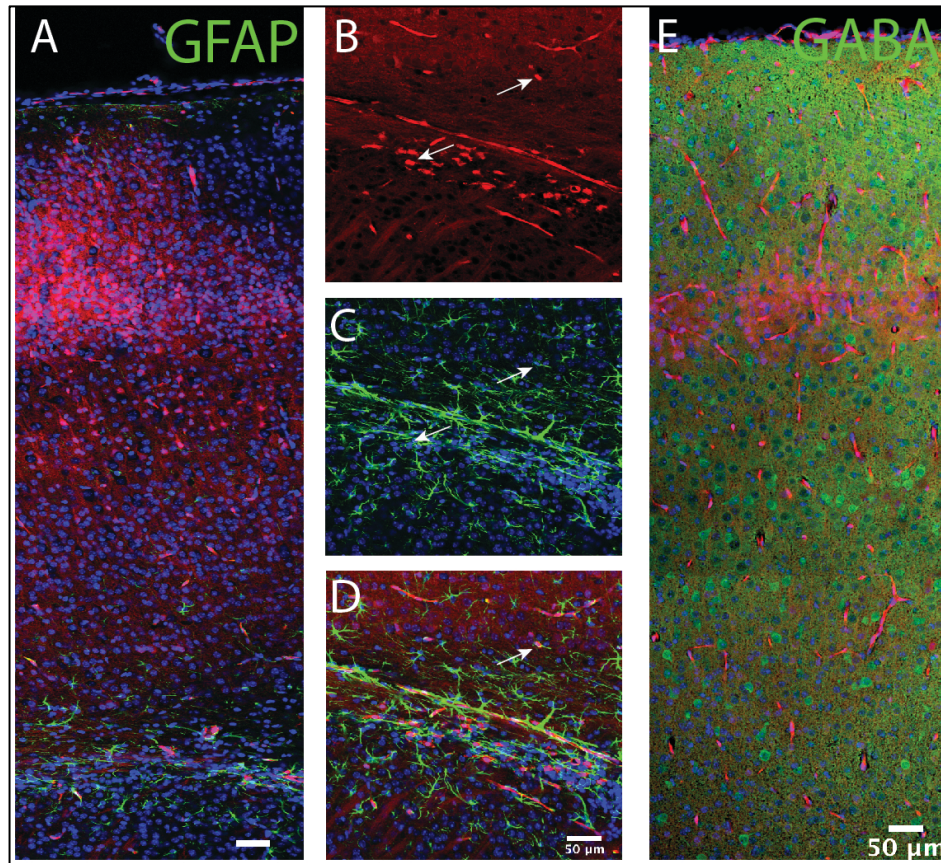


Figure 3.8: Tbr2+ IPC derived neurons do not express GFAP or GABA

(A) GFAP immunohistochemistry on Ai9 brain sections at P7. Reactivity can be seen mostly in the white matter (boxed) and not other regions of the cortex. (B-D) Higher power images from the boxed region in H shows clusters of Ai9+ cells (B; arrows) in close proximity with GFAP+ cells (C-D; arrows) in the white matter of P7 brains. (E) Immunohistochemistry for GABA did not show any co-localisation with Ai9+ cells. Scale bars: 50 μ m

To further validate these observations, fate mapping was also performed by in-utero electroporation of a Cre inducible fluorophore in *Tbr2^{Cre}* cortex. For this, a mix of CAG-STOP-EYFP and mPB (transposase) plasmids was electroporated into the dorsal cortex at E12.5 and E15.5 followed by perfusion of born pups at P8. When electroporation was performed at E12.5, the resultant neurons

generated were situated throughout the thickness of the cortex (Figure 3.9A). Majority of cells were present in layers 2-5, correlating with their birthdates being at the time of electroporation (Molyneaux et al., 2007) or afterwards. This also shows that the fluorophore cassettes were integrated in the radial glial progenitor genome (Fraser et al. 1983). However, only few cells were observed in layer 6 as expected (Figure 3.9A). In contrast pups collected from electroporations at E15.5 had labelled cells only in layers 2-4 (Figure 3.9B) and a strong band of fibre labelling could be observed in layer 5 which is known to be an output target for layer 2-3 neurons (Burkhalter, 1989).

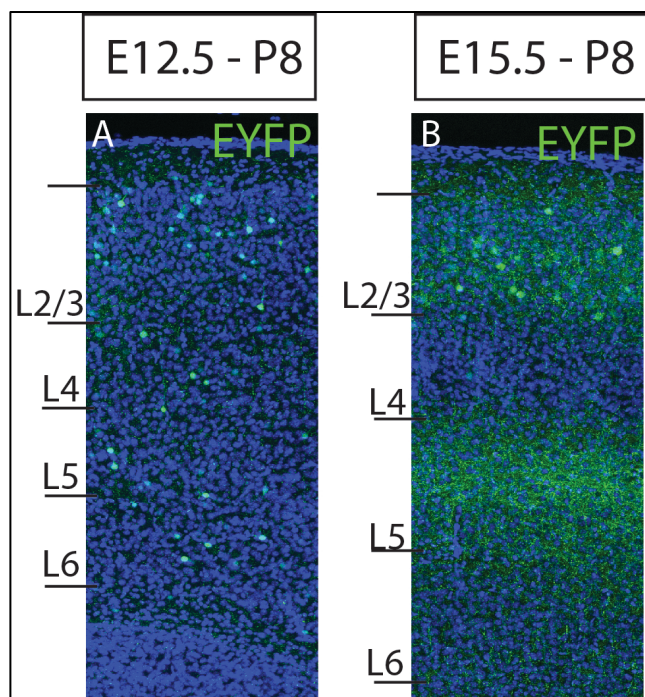


Figure 3.9: Fate mapping of Tbr2+ IPCs by stable transfection of fluorophore

(A-B) Single fluorophore (STOP-EYFP) labelling of clones born at E12.5 (A) and E15.5 (B). Early fate mapping (A) shows Tbr2+ IPCs give rise to cells across layers 2-6 while at a later stage (B) cells can only be seen in the supragranular layers. Scale bars: 50 μ m.

3.3.3 Individual clonal analysis in the *Tbr2*^{Cre} mouse with stable transfection of genetically encoded fluorophores reveals laminar and areal distribution

I was also interested in describing the clonal output from individual IPCs to the different cortical layers. For this I performed in-utero electroporations as described above with the inclusion of plasmids carrying different fluorophores (mCherry, EYFP, EGFP and mSapphire) targeted to different cellular locations (cytoplasmic, membrane and nuclear) at E12.5 and E15.5 and collected at P8 for analysis.

As the labelling vectors contain a loxP-STOP-loxP fragment, expression of the fluorophore is not possible in the absence of Cre recombinase. However, these plasmids are taken up by RGCs during electroporation and inserted into the progenitor genome by action of the transposase (Figure 3.2). As the insertion is random it leads to generation of a unique combination of fluorophores in each cell depending on the number of copies of a particular labelling plasmid and the different types (colour and subcellular location) of labelling plasmids inserted. Upon expression of Cre, the STOP fragment is excised leading to expression of the respective fluorophores. All IPCs derived from the same RGC will show the

same combinatorial label and further all neurons born from such IPCs will inherit the same combination. Thus by analyzing similar coloured cells in the cortex, one can identify clonal populations generated from individual IPCs.

In order to detect and analyse clones expressing the same combination of fluorophores a histogram-based thresholding algorithm (Arora et al., 2008) was used. The algorithm works by dividing a given image histogram into several thresholded values (the number of values is empirically determined) based on weighted means of pixel values in a given interval (intervals are determined by the mean and standard deviation of the histogram). The output generated is a matrix of discrete values of all pixels of the image (Figure 3.10A).

Multifluorescent images generated above (Figures 3.10B-C) were run through this algorithm and measured the distances between pixels holding similar values (representing clonally related cells). To aid understanding of relation between cells, they were divided into those present in the same layer and those present in different layers. Related cells in the same layer were separated by a mean distance of $36.8 \pm 20.5 \mu\text{m}$ ($n=23$ cell pairs across 4 brains) while those in different layers had a separation of $142.1 \pm 76.8 \mu\text{m}$ ($n=19$ cell pairs across 3 brains) (Figure 3.10D). Additionally, clonally related cells separated by cortical

layers were clustered closer than unrelated ones ($142.1 \pm 76.8 \mu\text{m}$ vs $294.9 \pm 105.4 \mu\text{m}$; $p < 0.0001$) (Figure 3.10D). However this did not apply to clonal clusters within the same layer or to the sum of the two. In all cases between 2-4 identically labelled cells were found in an arbitrary $100 \mu\text{m}$ column indicating that they were derived from symmetric division of 1-2 IPCs. Calculating backwards, this would indicate either the same IPC undergoing a symmetric renewal or two different IPCs being labelled identically. As the probability of the latter happening is low with this method (given the permutations and combinations brought about by the mixture of fluorophores and transposase) it suggests that daughter cells arising from the same IPC tend to remain in close association and within a defined region of space.

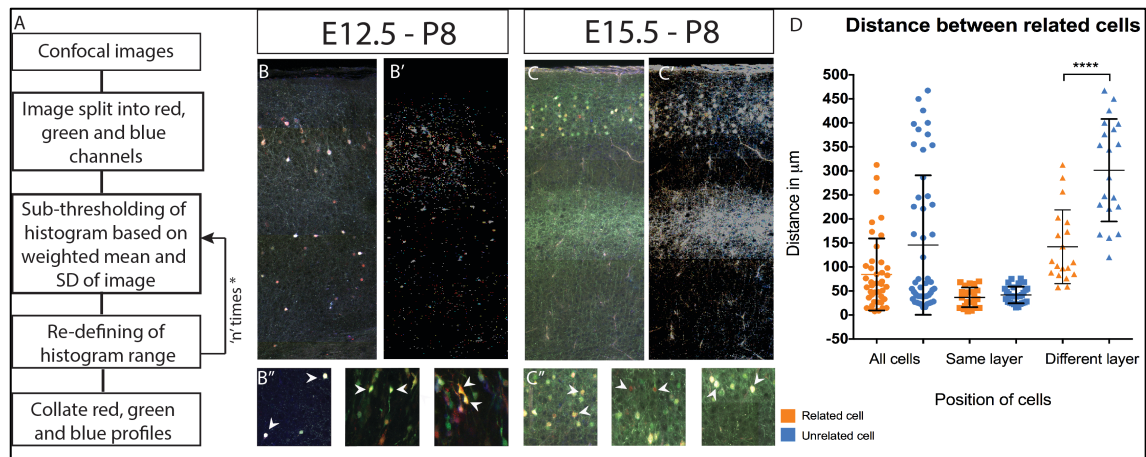


Figure 3.10: Individual clonal analysis in the *Tbr2*^{Cre} mouse with stable transfection of fluoroproteins reveals laminar and areal distribution.

(A) Algorithm for histogram based image clustering and segmentation. This algorithm was used to identify fluorescent cells that showed similar RGB properties and could be said to be clonal in origin. (B) Example of a E12.5 electroporated brain labelled using CLoN used for thresholding. Fluorescent cells of various hues can be seen in different layers. (B') Image after thresholding by the algorithm. Thresholded values of cells in B were read from the matrix of values obtained. (B'') Examples of clonally related cells identified using the algorithm. (C) Example of a E15.5 electroporated brain labelled using CLoN used for thresholding. Cells are positioned in the same layer (2/3) as compared to B due to birthdate. (C') Thresholded output image after processing. (C'') Examples of clonally related cells identified. (D) Graph representing distance between clones and unrelated cells in same layer, different layers and total. Only cells clustered in different layers show significant difference as compared to unrelated cells ($p < 0.0001$, Mann-Whitney test).

3.3.4 Extracortical contributions of Tbr2+ IPCs

In addition to fate mapping in the cortex, Tbr2 expression was also observed in previously undescribed regions. Tbr2 immunoreactive cells were observed in the septum and lateral olfactory tract (lot) (Figure 3.11A) as well as in a small population of migratory-shaped cells along the margins of the rostral medial telencephalic wall proceeding ventrally towards the septum. A similar migration has been previously described (Ceci et al., 2012) though the nature of the progenitors was not commented on. A large number of cells were also observed in the ventro-medial cortex and septum at E14.5 in coronal sections of Ai9 brains (Figure 3.11B) a large proportion of which colocalised with Tbr1 (Figure 5B' and 5B'', arrowhead) indicating that they are glutamatergic in nature. Ai9+ cells in the lot were also positive for calbindin (Figure 3.11D inset) as reported previously (Ceci et al., 2012).

Ai9 labelled cells were also observed at thalamic eminence and the pre-thalamus at E14.5 and appeared to cross the diencephalon-telencephalon boundary (DTB) seemingly emanating from the ventricular zone of the third ventricle (Figure 3.11C-D). It has been reported that cells from this region

migrate and contribute towards the posterior accessory olfactory bulb (pAOB) (Huilgol et al., 2013). Thus, it seems that Tbr2⁺ cells contribute towards the development of the vomeronasal system both by generating neurons that set up the lot and also by contributing towards the stream of neurons migrating from the pre-thalamus towards the pAOB.

I also examined sections from postnatal Ai9 brains (P7). As expected, we found labelled cells in the lot and the olfactory tubercle (Figure 3.11E, inset).

Our analysis of labelled cells also revealed Tbr2⁺ derived cells in other regions. We observed expression of Ai9 in the hypothalamus and pre-thalamus (Figure 3.11E'-E").

In addition, Ai9 staining could also be seen in the cells of indusium griseum, bed nucleus of anterior commissure (BAC) and the anterior commissure itself (Figure 3.11F-H'). Cells of the indusium griseum are born between E11 and E15 and are involved in the histogenesis of the corpus callosum via Slit-Robo and Fgfr1 signaling (Shu and Richards, 2001; Smith et al., 2006; Sturrock, 1978a; 1978b). On the other hand, cells of the bed nucleus of anterior commissure (BAC) are born starting E14 and are partly pallially derived (Bayer, 1987; Sturrock, 1977).

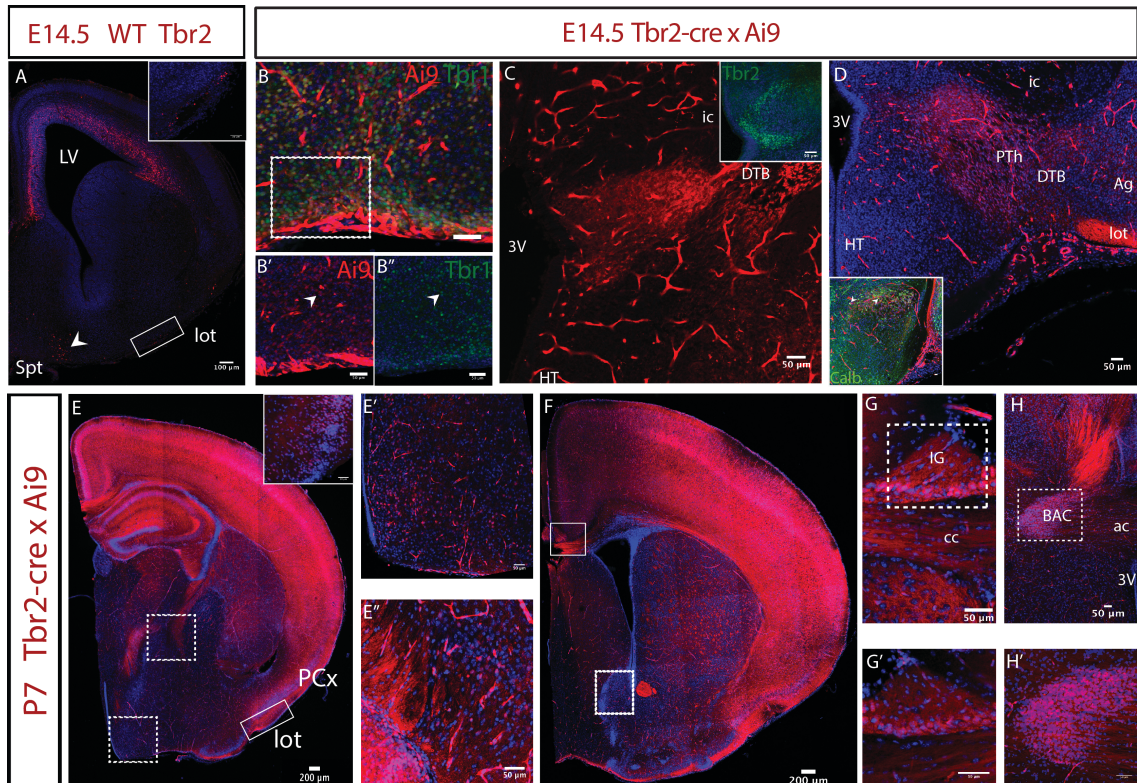


Figure 3.11: Extracortical contributions of Tbr2+ IPCs

A: Tbr2 immunoreactivity is detected at E14.5 in the septum (Spt, arrowhead) and in the lateral olfactory tract (lot). Inset shows expression in the lot from boxed area. LV, lateral ventricle.

B: Ai9+ (red) cells in the septal regions colocalise with Tbr1 (green) in the *Tbr2^{Cre}* bred with Ai9-tdTomato. Tbr1 expression (B'', arrowhead) overlaps with Ai9 (B', arrowhead) indicating glutamatergic nature of these cells. B' and B'' are taken from the boxed region of an adjacent section.

C: Tbr2 is also expressed in the pre-thalamus (PTh) as indicated by Ai9+ cells at E14.5. Cells can be seen on either sides of the diencephalon-telencephalon boundary (DTB). Inset shows expression of Tbr2 in the thalamic eminence.

D: On a more posterior section Ai9+ cells can be seen crossing the DTB and also in the lot at that level. Inset shows that some cells in the pre-thalamus co-express calbindin (arrowheads). 3V: 3rd Ventricle, HT: Hypothalamus, ic: internal capsule, Ag: Amygdala, lot: lateral olfactory tract.

E: Coronal section from a brain derived from the breeding of Ai9-tdTomato and *Tbr2^{Cre}* at P7 shows expression is maintained in the lot. Inset shows labelled cells in higher

magnification in lot. PCx: piriform cortex. E' and E' show that cells in the hypothalamus and pre-thalamus also continue to express Ai9 at postnatal ages.

F: Cell of the indusium griseum (IG; G-G') and bed nucleus of anterior commissure (BAC; H-H') express Ai9 in a P7 *Tbr2*^{Cre} x Ai9 section. G,H show expression in a corresponding section.

G: Expression of Ai9 can be seen in the cells of the IG next to the midline and above the fibres of corpus callosum (also labelled by Ai9). Inset shows higher magnification.

H: Ai9 expression can be seen in BAC as well as in the anterior commissure. Inset shows higher magnification from the boxed region.

Scale bars: A: 100 µm, E,F: 200 µm while rest are 50 µm.

3. 4 DISCUSSION

I studied the contribution from *Tbr2*⁺ progenitors towards the cerebral cortical layers directly and systematically in this chapter. While its origin has been well described (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004), the fate of its progenies has not been equally looked into. Ambiguity persists regarding the contribution towards the upper and lower layers. A study utilizing conditional knockout (Arnold et al., 2008b) describes a decrease of immunoreactivity for an upper layer marker while another report (Sessa et al., 2008) describes a loss in both upper and lower layers. In the latter case however, the use of *Foxg1*^{Cre} can be questioned as haploinsufficiency at the *Foxg1* locus is known to decrease the specification of IPCs (Siegenthaler et al., 2008) and is associated with a decrease in supragranular layer neurons (Eagleson et al., 2007; Siegenthaler et al., 2008).

In contrast, transgenics with a single copy of *Tbr2* develop normally and do not show reduced expression (Arnold et al., 2008b; Arnold et al., 2009). Thus the *Tbr2*^{Cre} mouse line is better suited for fate mapping of IPCs. Using this line, I show that *Tbr2*⁺ IPCs give rise to both upper and lower layer neurons but in

different proportions. Importantly, I describe that a sub-population of subplate neurons are also derived from IPCs thus describing the contribution towards early corticogenesis and circuit formation. As IPCs are an acquired progenitor group during cortical evolution, it suggests that the expansion of the cortex has been more homogeneous amongst all layers than previously thought (Tarabykin et al., 2001).

3.4.1 All layers are not affected in *Tbr2* cKO

Layer-wise cell counts from the conditional knockout as well as from the Ai9 brains indicates that while neurons born from IPCs come to lie across all cortical layers, the decrease in the conditional knockout is only seen in layers 4 and 6 (Figure 3.7). This further emphasises the redundancy in *Tbr2* function observed in relation to other layers during cortical neurogenesis (Arnold et al., 2008b).

However, the number of *Tbr2* derived cells in different layers is amenable to variation depending on the region of cell counting. Here, I counted cells from the S1 somatosensory cortex in order to have comparable data with previous studies (Arnold et al., 2008, Sessa et al., 2008). However, counting from cingulate or other regions might provide different results.

It is reported that the rodent somatosensory cortex contains ~36% glia and ~64% neurons (Ren et al., 1992). Further, amongst neurons, ~25% is constituted by GABAergic interneurons with excitatory cells making the majority of cells (Ren et al., 1992). In simpler terms, for every 100 cells counted in the somatosensory cortex of rodents, 64 cells are neurons of which 16 cells would be interneurons. Considering from results shown above that Tbr2+ IPC derived neurons account for 30% of all cortical cells, it implies that ~62.5% of excitatory neurons (30/48) are produced via Tbr2+ IPCs with the number being even higher in upper layers and remaining constant for lower layers (exact calculations are difficult since astrocytes and oligodendrocytes show layer specific clustering). This number differs from the predictive studies that use pH3+ cell numbers in the SVZ to calculate the contribution from IPCs (Martínez-Cerdeño et al. 2006) perhaps due to difference in the region used for analysis.”

Single cell profiling has described gene clusters (instead of single genes) that are expressed in the SVZ and might be involved in specifying progenitor and neuronal fate (Kawaguchi et al., 2008). In addition, *Tbx1*, another T-box gene that is correlated with DiGeorge Syndrome (Meechan et al., 2009), has been described to affect proliferation of IPCs leading to a decrease in supragranular

layer neurons. This leads to a conundrum where Tbr2 is used to label the IPC population despite lack of knowledge of it representing the entire population. This problem might be magnified in animals with larger brain that might have a wider genetic variety of intermediate progenitors leading to a greater diversity of cortical neuronal populations.

Previously, fate mapping of NEX+ progenitor cells of the SVZ suggested that they gave rise to pyramidal neurons of the upper layers (Wu et al., 2005). However, it is interesting to note that NEX+ cells at E13 were not immunoreactive for pH3 (a mitotic phase marker). Even at E15, only a small fraction of NEX+ cells expressed pH3 or Ki67 (a S-phase marker) indicating their non-dividing state (Wu et al., 2005). Finally, gene expression analysis of the *Tbr2::GFP* mouse line shows that NEX is expressed in a subset of Tbr2+ cells (Cameron et al., 2012). Taken together, this indicates NEX+ cells representing a post-mitotic state of IPCs. Regardless, long-term fate mapping of this line showed labelled neurons to be present across the cerebral cortex from layers 2-6 (Goebbels et al., 2006) similar to the findings above.

3.4.2 Characterisation of clones derived from Tbr2+ IPCs

Analysis of clonally generated cells from Tbr2+ IPCs shows that they do not disperse long distances but are in close proximity with one another. Also, I show that clonal clusters are located within limits that suggest their arrangements in columns as suggested previously (Mountcastle, 1997). It will be interesting to further study whether the clonally related cells in this study are also functionally related (Li et al., 2012; Yu et al., 2009).

The method used to label clonally related populations is contingent on certain conditions. Firstly, the expression of Cre-recombinase must be transient and specific to the IPCs. Secondly, the integration of the labeling vectors must occur prior to completion of the cell cycle to label the entire lineage and also so that the plasmids are not unequally divided between daughter cells. It would be beneficial though not necessary that excision of the STOP cassette occurs after integration of the plasmid in order to prevent episomal expression of the fluorophores. In my studies, I found that when using the fluorophore plasmids with defective transposable recognition sites, no episomal expression was observed in the postnatal cortex (data not included). This could be due to dilution/loss of the plasmids during division and cell maturation.

Finally, in order to obtain true representations of the hue, it is necessary that the imaging setup be calibrated and corrected for flat-field errors.

3.4.3 *Tbr2* is also expressed in extracortical regions during development

Finally, our study identified extracortical contributions of *Tbr2*⁺ expressing progenitors that are involved in the generation of glutamatergic (but not GABAergic neurons) of the lateral olfactory tract (lot), the olfactory tubercle and ventromedial hypothalamic nucleus.

I have described expression of *Tbr2* in regions outside of the cerebral cortex namely in the pre-thalamus and septum and contribution from *Tbr2* expressing cells towards the hypothalamus. The septal expression is particularly interesting as it aids in the development of the lot (Ceci et al., 2012). I have shown that rostro-medial electroporation labels cells that seem to migrate ventrally and tangentially through the septum and towards the lot. Previously it has been shown that development of the lot is affected in the conditional *Tbr2* knockout (Arnold et al., 2008b) thus arguing for a role of IPCs in extracortical regions. I also show expression in the pre-thalamus during embryonic development. Migration of cells from the thalamic eminence towards the olfactory bulb has

been described where cells cross the diencephalon-telencephalon boundary (DTB) and move ventro-laterally towards the accessory olfactory bulb (Huilgol et al., 2013). Thus, it seems that *Tbr2*⁺ IPCs are also involved in specification and development of these regions and ultimately to the development of the brain. However it remains to be seen as to whether the transcriptional regulation in these regions is similar to that studied in the SVZ (Cameron et al., 2012; Elsen et al. 2013; Sessa et al., 2008). In addition, I also show expression in the bed nucleus of anterior commissure and the indusium griseum, regions that are closely located with major fibre tracts of the brain i.e. the anterior commissure and corpus callosum both of which are absent in the conditional *Tbr2* knockout (Arnold et al., 2008b).

3.4.4 Role of *Tbr2*⁺ IPCs in evolutionary expansion of the cortex

Intermediate progenitors have been hypothesized to be an important addition in the evolutionary expansion of the cerebral cortex (Cheung et al., 2007; 2010; Martinez-Cerdeno, 2006). It is also known that a mutation spanning the *TBR2* locus in humans causes microcephaly with polymicrogyria (Baala et al., 2007). However, from mouse models of loss of function of *Tbr2* it is not clear as to which populations of cortical neurons are affected. This chapter shows the pan-

cortical contribution from IPCs and to several other regions, crucial for the development of the brain. The evolutionary impact of IPCs will thus have to be revisited to study if the expansion is limited to the neocortex or if it applies to these other regions of expression as well. At a clonal level, it would again be interesting to compare differences amongst mammals with an expanded germinal zone (Fietz et al., 2010; García-Moreno et al., 2012; Kelava et al.; Reillo et al., 2010) as well as with avian and reptilian counterparts. However, this might need designing of new experimental strategies.

3. 5 APPENDIX

A.5 MATLAB algorithm for multi-level thresholding and image analysis

```
clc; clear all;
a11 = imread('Brain #1 Sec1 20x z-stack MP.tif');
a11 = medfilt3(a11);

max_level = 15    % increase max_level till there is substantial increase in psnr

% Naive way to improve contrast - make every pixel having a value less than
% a certain threshold as being 0. - next three lines can be removed from
% code
threshold = 40;
indx = find(a11(:,:,:) < threshold);
a11(indx) = 0;

[mm,nn,oo] = size(a11);

a_thresh_all = zeros(mm,nn,oo);

for channel = 1:1:3

    a = (a11(:,:,channel));

    a_orig_gray = a;
    [m,n] = size(a_orig_gray);

    a1 = double(a);
    a_thresh = a1;

    high_assign = zeros(max_level);
    low_assign = zeros(max_level);

    for level = 1:1:max_level

        [m1,n1] = size(a1);
```

```

maxa = max(max(a1))+1;
mina = min(min(a1));
a1_mean = mean(mean(a1));

variance = (a1 - a1_mean).^2;
sum_par = sum(sum((a1-a1_mean).^2))/(m1*n1);
std1 = sqrt(sum_par);

high = a1_mean + std1;
low = a1_mean - std1;

if level == 1
    high_assign(level) = (high + 255)/2;
    low_assign(level) = low/2;
end

if level > 1 & level < max_level
    high_assign(level) = (high + high_assign(level-1))/2;
    low_assign(level) = (low + low_assign(level-1))/2;
end

if level == max_level
    high_assign(level) = (a1_mean + high_assign(level-1))/2;
    low_assign(level) = (a1_mean + low_assign(level-1))/2;
end

flag = length(find(a1 > low & a1 < high));

if level == 1
    count = 1;
    b = zeros(1,flag);
    for i = 1:m
        for j = 1:n
            if a_thresh(i,j) > high
                a_thresh(i,j) = high_assign(level);
            end
            if a_thresh(i,j) < low
                a_thresh(i,j) = low_assign(level);
            end
        end
    end
end

```

```

        end
        if (a_thresh(i,j) > low & a_thresh(i,j) < high)
            b(1,count) = a_thresh(i,j);
            count = count + 1;
        end
    end
end
end
end

if level > 1 & level~= max_level
    count = 1;
    b = zeros(1,flag);
    for i = 1:m
        for j = 1:n
            if a_thresh(i,j) > high & a_thresh(i,j) < high_assign(level-1)
                a_thresh(i,j) = high_assign(level);
            end
            if a_thresh(i,j) < low & a_thresh(i,j) > low_assign(level-1)
                a_thresh(i,j) = low_assign(level);
            end
            if (a_thresh(i,j) > low & a_thresh(i,j) < high)
                b(1,count) = a_thresh(i,j);
                count = count + 1;
            end
        end
    end
end

if level == max_level & max_level > 1
    for i = 1:m
        for j = 1:n
            if a_thresh(i,j) > a1_mean & a_thresh(i,j) < high_assign(level-1)
                a_thresh(i,j) = high_assign(level);
            end
            if a_thresh(i,j) < a1_mean & a_thresh(i,j) > low_assign(level-1)
                a_thresh(i,j) = low_assign(level);
            end
        end
    end
end

```



```

        end
    end

    clear a1;
    a1 = b;

    clear high; clear low;
    clear std1; clear a1_mean; clear a1_max; clear a1_min;
end

a_thresh_all(:, :, channel) = a_thresh;

clear a_thresh; clear high_assign; clear low_assign;
end

% original image
figure(1)
imshow(a11);
title('original image');

% thresholded image
figure(2)
imshow(uint8(a_thresh_all));
title('thresholded image');

% Save thresholded image as 'jpeg'
imwrite(a_thresh_all, 'ThresholdedImageJPEG', 'jpeg');

% Save thresholded image as 'data file'
save ThresholdedImageDataFile.mat a_thresh_all;

% List of different pixel values used in all 3 channels to threshold the
% image
ListPixelValue = unique(a_thresh_all(:, :, :))

% Compute PSNR between original and thresholded rgb image. Higher PSNR is
% better.
PSNR_COMPUTE = PSNR_RGB(double(a11), (a_thresh_all))

```

Chapter 4

Evolutionary Development of Cortical Germinal Zones

4.1 INTRODUCTION

The evolutionary expansion of the cerebral cortex has been correlated with substantial changes in the proliferative compartments of the telencephalon (Rakic, 2003; Martínez-Cerdeño et al., 2006). For example, progenitors in the telencephalon of reptiles only divide in the ventricular zone (VZ), while birds present a well-developed secondary germinal compartment, the subventricular zone (SVZ), in the subpallium and to a lesser extent in the dorsal cortex (Striedter and Charvet, 2009; Cheung et al., 2007). In mammals an innovative dorsal SVZ has evolved, likely related to an increased cortical thickness (Cheung et al., 2007). However, in the adult cortex of the non-eutherian mammal; *Monodelphis domestica* (Gray short-tailed opossum), considerably fewer neurons exist in an arbitrary unit column of their cortex than in mice (Cheung et al., 2010). Nevertheless during early postnatal development (opossums like other marsupials display precocial development i.e. their young ones are born underdeveloped) their SVZ reveals a similar gene expression pattern (Cheung et al., 2010; Puzzolo and Mallamaci, 2010), with an abventricular secondary layer of mitotic progenitors that appears relatively late and considered neurogenic (Cheung et al., 2010) or gliogenic (Puzzolo and Mallamaci, 2010). These findings suggest that although the proportions can vary largely, all species across the mammalian lineage, including marsupials, have a subventricular zone during cortical development. This evolutionarily acquired region is now thought to house two novel cell populations that contribute towards development of the cerebral cortex. First, the multipolar intermediate progenitor cell (IPC) that is not connected to

neuroepithelial surfaces and expresses specific markers including Tbr2 (Kowalczyk et al., 2009). The IPCs also have the capacity to undergo one or two subsequent symmetric divisions to amplify the number of neurons (Miyata et al., 2004; Noctor et al., 2004; Haubensak et al., 2004). Second, the recently described outer radial glial cells (oRGs), a bipolar progenitor that expresses Sox2, Pax6 and pVim and possesses a basal process extending to the pia (Hansen et al., 2010; Fietz et al., 2010; Reillo et al., 2010; Shitamukai et al., 2011; Wang et al., 2011). oRGs have been described to reside at the upper boundary of the SVZ, specifically the intermediate zone (IZ) of mice but the outer sub-ventricular zone (OSVZ) of larger brains, hence indicating a separate location for these progenitors. The ventricular zone, ISVZ and OSVZ can be clearly discerned based on differences in the cytoarchitecture (Smart et al., 2002). The OSVZ lies separated from the ISVZ by an inner fibre layer (IFL). Studies in macaque brains show that 30% of tritiated thymidine is incorporated into non-ventricular zone progenitors during periods of infragranular layer genesis (Lukaszewicz et al., 2006). Clearly, the ISVZ/OSVZ are major neurogenic regions in animals that possess this cytoarchitectonic separation. However, to date the OSVZ has been studied only in gyrencephalic species (such as macaques (Smart et al., 2002), ferret (Smart and McSherry, 1986) and pigs (Karsten B Nielsen, 2010) amongst others). Further, while oRG progenitors have been identified in the developing mouse cortex, they are estimated to be a small percentage (5.6% of electroporated cells retaining only their basal process at E15.5) in mice (Shitamukai et al., 2011) while the oRG population reaches nearly 50% in human or ferret brains (Fietz et al., 2010; Hansen et al., 2010; Reillo et al.,

2010). Hence, some studies suggest a correlation between the enlarged oRG progenitor populations and gyrencephaly (Fietz et al., 2010; Reillo et al., 2010). While this might be debatable (Figure 4.1), it may be said that compartmentalisation of the sub-ventricular zone (into inner and outer SVZ) has been a milestone in the evolution of cortical neurogenesis and the expansion of the neocortex (Smart et al., 2002; Dehay and Kennedy, 2008).

Figure 4.1: Existence of gyrencephaly and lissencephaly across multiple orders

(A) Phylogenetic tree showing various orders and sub-classes to depict diversity in brain sizes and folding pattern. (B) Corresponding histological sections of animals to show varying degrees of gyrification present in eutherians. Examples where a OSVZ has been described is labelled as oRG. (Reproduced from Lui et al., 2011).

This chapter hence attempts to pursue if indeed oRG cells are hallmarks of gyrencephaly (Fietz et al., 2010, Fietz et al., 2011). Additionally a comparative description of a lissencephalic primate brain and a gyrencephalic rodent brain is also described here.

4.2 MATERIALS AND METHODS

4.2.1 Animal Tissue

The usual trend for primate brains is to be gyrencephalic while that for rodents is to be lissencephalic. However, there are exceptions to this trend that begets the question as to whether specific progenitor cells contribute towards such phenotype. We thus examined embryos and neonates of the New World marmoset monkey (*Callithrix jacchus*) at different developmental stages (three brains at E60, two each at E88 and E110; gestational period lasts 145 days).

All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Monash University Animal Ethics Committee, which also monitored the welfare of the animals. Individuals were overdosed with the anaesthetic pentobarbitone sodium (100 mg/kg) and transcardially perfused with 0.1 M heparinized phosphate buffer (PB; pH 7.2) followed by 4% paraformaldehyde in 0.1 M PB. Cerebral tissues were immediately removed and postfixed for 24 hours in 4% paraformaldehyde in 0.1 M PB containing 10% sucrose. Cryoprotection was achieved by serially transferring the tissue through solutions of 20% and 30% sucrose in 0.1 M PB before being frozen in OCT (Sakura Finetek ®) embedding compound. Coronal frozen sections of the hemispheres (20-25 µm) were cut on a cryostat (Leica) and mounted on superfrost glass slides (VWR International).

Dasyprocta agouti were obtained from the Museum Emilio Goeldi (Belem, Para, Brazil) and kept at the Biological Institute of Federal University of Para. All animals were handled in accordance with “Principles of Laboratory Animal Care” (NIH). Timed-pregnant dams were anesthetized with a solution of ketamine (100g/kg) and xylazine (10mg/kg) (Konig Laboratories), and transcardially perfused with heparinized saline (5000 UI/L) followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2-7.4). E52 embryos (gestational period lasts approximately 110 days) were removed and the brains fixed by immersion in 4% paraformaldehyde at 4°C, cryoprotected with 30% sucrose overnight and then frozen in OCT compound. Sections were cut at 20µm on a cryostat (Leica), air dried, and stored at -20°C.

4.2.2 Immunohistochemistry

Different progenitor cells have been studied mostly by using antibodies against proteins specific to their cell type. Thus to describe progenitor composition in the brains of marmosets and agouti I used immunohistochemistry. Immunohistochemistry was performed on cryosections using the primary antibodies tabulated below. Slides were removed from the freezer and allowed to thaw at room temperature. Sections were washed with PBS (3 times for 5 minutes each) following which antigens were blocked with 5% goat or donkey serum diluted in PBS containing 0.1% Triton X-100 (PBST, BDH, UK). Primary antibodies diluted in PBST and 1% goat or donkey serum (Sigma, UK) was added and sections incubated overnight at 4°C. After several rinses, sections were incubated with fluorescent secondary antibodies diluted in the same solution as the primary antibodies. Alexa fluor conjugated secondary antibodies were used for fluorescent detection. DAPI (4',6-diamidino-2-phenylindole; Invitrogen) and Nissl's substance (Cresyl violet) were used for counterstaining tissues. Sections were mounted with 70% glycerol diluted in PBS and coverslipped. Slides were sealed using clear nail-varnish and allowed to dry before being stored at 4°C till imaging was completed.

ANTIBODY	SOURCE	CONCENTRATION
Mouse α - phospho-Vimentin	MBL D076-3	1:200
Mouse α -phospho-Histone H3	Abcam ab14955	1:1000
Rabbit α -phospho-Histone H3	Upstate 06-570	1:500
Rabbit α -Tbr2	Prof. Robert Hevner	1:2000
Rabbit α -Sox2	Abcam ab97959	1:1000

Rabbit α -Ki67	Abcam ab15580	1:500
Mouse α -Snap25	Covance SMI81R	1:500
Mouse α -srGAP1	Abcam ab57504	1:100
Donkey α -Mouse Alexa 488	Invitrogen	1:500
Donkey α -Mouse Alexa 568	Invitrogen	1:500
Donkey α -Rabbit Alexa 488	Invitrogen	1:500
Donkey α -Rabbit Alexa 568	Invitrogen	1:500

Table 4.1: Details of primary and secondary antibodies used during immunohistochemistry

4.2.3 Imaging and Image Analysis

Sections were first viewed under a fluorescent microscope (Leica DMR, Leica) and selected for confocal imaging. Confocal images were taken on a LSM710 confocal microscope (Carl Zeiss Microimaging). Basal processes of suspected oRG cells were confirmed by capturing Z-stacks using the ZEN software (Carl Zeiss).

All images were converted to TIFF format for post-processing and analysis using ImageJ (NIH) and Adobe CS3 (Adobe Inc.) software.

Plane of division measurements were taken from sections stained with pH3 on single confocal stacks using the angle tool in ImageJ. All measurements were done parallel to the ventricular surface while all angles were calculated between 0-90 degrees. Furthermore, in all cell counts, the number of cells counted is represented by *n* value within parentheses.

Figure 4.2: Calculation of the mitotic angle

Mitotic angles were calculated against the ventricular surface between 0-90 degrees. Depending on the plane of division, the angles could largely be divided into vertical (0-30), Oblique (30-60) and Horizontal (60-90). (Modified from LaMonica et al., 2013).

4.3 RESULTS

The agouti is considered a gyrencephalic rodent and the marmoset a lissencephalic non-human primate. In terms of gyrification indices (GI): the agouti has a GI of 1.23 (Pillay and Manger, 2007) while the marmoset varies slightly with a GI of 1.2 (Rogers et al., 2010). This is in contrast to brains of more convoluted species such as ferret with GI of 1.8 (Neal et al., 2007) and humans with a GI of 2.99 (Pillay and Manger, 2007).

4.3.1 Comparison between agouti and marmoset brains during development

However, the marmoset brain is strictly speaking not entirely smooth and neither is the agouti clearly gyrencephalic. The adult agouti possesses large sulci extending parallel to the midsagittal position and another at the ventrolateral aspect of the brain (Figure 4.3B). In the adult marmoset a prominent lateral sulcus is apparent (Figure 4.3C), however a large proportion of the convexity of the marmoset telencephalic vesicle shows no obvious sulci or gyri until birth (Figure 4.3C).

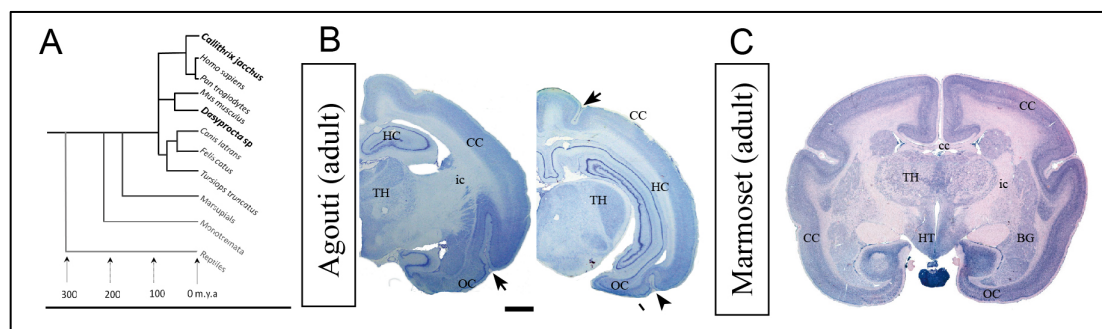


Figure 4.3: Gyrification in the adult agouti and marmoset brains

(A) Phylogenetic tree showing the relationship between marmoset (*Callithrix jacchus*) and agouti (*Dasyprocta agouti*). The two species would have evolved from a common ancestor 100 million years ago (mya). (B) Histological section of the adult agouti

brain. As can be noted, the agouti has two prominent gyri at the midsagittal and ventrolateral aspects. (C) The adult marmoset shows only a single gyrus at a lateral aspect. Scale bar: 250 μ m (BG: basal ganglia; CC: cerebral cortex; cc: corpus callosum; ic: internal capsule; HC: hippocampus; HT: hypothalamus; OC: olfactory cortex; TH: thalamus)

Sulci in the agouti are already apparent at midgestation, although some further maturation occurs subsequently (Figure 4.4A). Similarly, the marmoset brain is lissencephalic during development (atleast until E88), and it remains largely so until birth, when the calcarine sulcus is evident on the medial surface (Figure 4.4B panels).

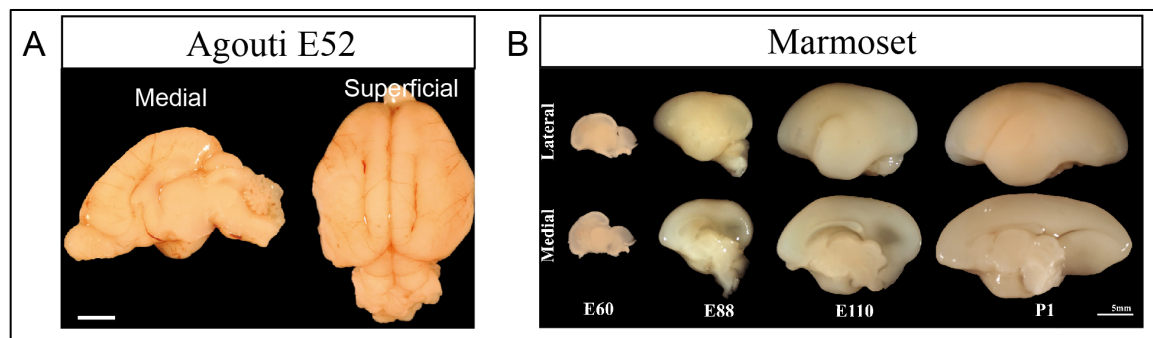


Figure 4.4: Pattern of gyrification in agouti and marmoset during development

(A) The agouti brain at E52 (midgestation) already shows the presence of midsagittal gyrus (superficial view). (B) Maromset brains during development remain largely lissencephalic, except for the appearance of the calcarine fissure at E110 (Lateral view). Scale bars: 5mm

In order to study the similarities and differences in their cortical architecture coronal sections of embryonic brains of both species were studied.

On Nissl's substance and DAPI counterstained coronal sections of E52 agouti and E88 marmoset parietal cortex the compartmentalisation of the germinal zone is clearly apparent (Figure 4.5A-B and E-F, respectively). Phospho-

Histone H3 (pH3) immunohistochemistry revealed ventricular and abventricular mitotic profiles in both species (Figure 4.5C,G).

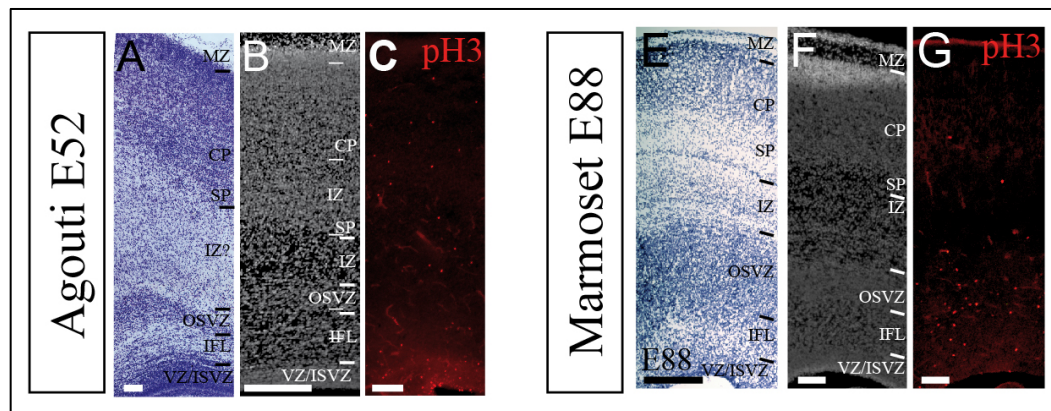


Figure 4.5: Germinal zone cytoarchitecture in developing agouti and marmoset brains

Nissl's and DAPI labelling of the developing agouti (E52) and marmoset (E88) cortical sections shows that the germinal zone is subdivided into different regions. A,E: Nissl staining, B,F: DAPI labeling and C,G: Phospho-histone H3 (pH3) immunohistochemistry of the developing cerebral cortex.

VZ: ventricular zone; ISVZ: inner subventricular zone; IFL: inner fibre layer; OSVZ: outer subventricular zone; IZ: intermediate zone; SP: subplate; CP: cortical plate; MZ: marginal zone. Scale bars: 50µm.

4.3.2 Development of the Inner fibre layer in the developing marmoset

Acetylcholine-esterase (AChE) activity and Snap25 (Synaptosomal associated protein 25) immunoreactivity have both been associated with early telencephalic fibre systems, including the periventricular fibres in the cortical germinal zone (Ulfig et al., 2000; Smart et al., 2002; Vasung et al., 2011). Snap25 immunohistochemistry in combination with pH3 and DAPI counterstaining was employed to further characterize the position of the inner fibre layer in relation to the mitotic profiles and the cytoarchitecture of the cortical germinal zones of the marmoset. Snap25 expression revealed a broad band of fibre fascicles extending through the internal capsule (below the ganglionic eminence close to the anlage of the basal ganglia) towards the

intermediate zone of the dorsal cortex (Figure 4.6A). The pH3 immunoreactive mitotic profiles were abundant both in the ventricular and subventricular zones. At E60 the intermediate and subventricular zones contained dense Snap25 immunoreactive fibres, but the ventricular zone and the cortical plate showed no or very little fibre labelling (Figure 4.6B). By E88 a fibre bundle, the inner fibre layer (IFL) became apparent between the ISVZ and OSVZ. All of these layers contained numerous pH3 immunoreactive mitotic profiles (Figure 4.6C and D). With the exception of the lower part of the OSVZ, the Snap25 immunoreactive fibres extended with parallel trajectories to the pial and ventricular surfaces. The ventral part of the OSVZ contained fibres with perpendicular trajectories to the ventricular surface (Figure 4.6D). These observations further demonstrate that marmoset cortical germinal zone is compartmentalised in a very similar manner as previously described for macaque and human (Smart et al., 2002; Vasung et al., 2011).

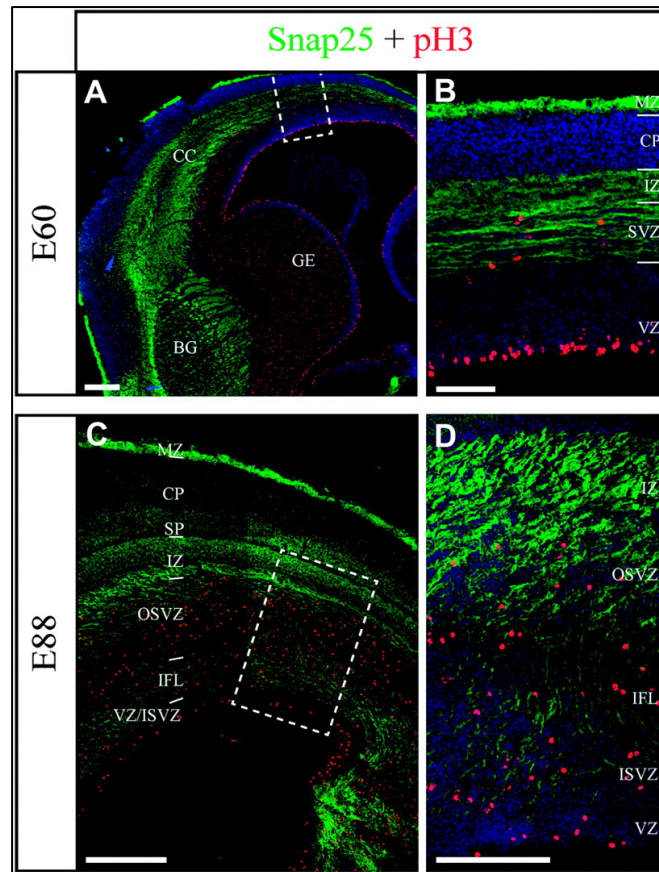


Figure 4.6: Fibre staining with Snap25 antibody

Snap25 reactivity showed an extensive fibre layer at E60 (A-B) and E88 (C-D) marmoset neocortex. A: At E60 Snap25 (green) reactivity showed a broad band of fibre fascicles crossing below the ganglionic eminence (GE) presumptive the basal ganglia (BG). B: The SVZ contained pH3+ nuclei (red). C: Snap25+ fibres varied in density in different layers. SP, IZ, upper part of OSVZ and IFL contained fibres with parallel trajectories to the pial and ventricular surfaces. The fibres were perpendicular to the ventricular surface with the lower part of the OSVZ. The VZ contained relatively few labelled fibres. Scale bars: 200 µm

4.3.3 Germinal zone cytoarchitecture changes during development

Having shown similar cytoarchitectonic distinctions on the germinal compartments in agouti and marmoset embryonic brains, neocortical development of marmoset was studied at three stages (E60, E88 and E110) in order to know the distribution and proportion of the several sub-ventricular progenitor subtypes. Marmoset embryos aged E60 did not present any sulci or gyri at the telencephalic surface (Figure 4.7A). Cortical layering at this early

stage of neurogenesis revealed a densely packed VZ, a loosely packed zone at the SVZ, the incipient origins of the fibre rich IZ and the cortical plate with the marginal zone lying most superficially below the pial surface. Further molecular characterisation of the various progenitors at E60 revealed no obvious distinctions within the SVZ at this stage (Figure 4.7C-E). Ki67 staining was observed in both the VZ and SVZ representing the germinal zones at this age (Figure 4.7C). In the SVZ, Ki67 demonstrated a dispersed expression with few immunoreactive cells located at the outer margins of the SVZ. As with Ki67, pH3 immunoreactive (pH3+) cells were observed mostly in the VZ and SVZ (Figure 4.7D). To identify the recently described oRG cells phospho-vimentin (pVim) immunohistochemistry was employed (Figure 4.7E). pVim expression followed a pattern very similar to pH3 on account of both being mitotic cell markers (Figure 4.7E inset; Kamei et al., 1998; Weissman et al., 2003).

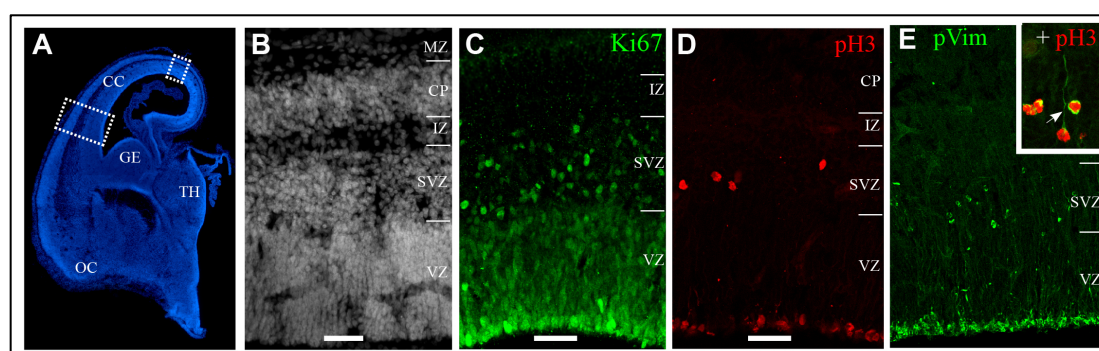


Figure 4.7: Cytoarchitecture of the proliferative domains in E60 marmoset brain. A: Coronal section of the forebrain stained with DAPI. Rectangles indicate the region where images were taken. B: DAPI staining to show cortical layering. Ki67, pH3 and pVim (C-E) immunohistochemistry demarcate two domains of cellular division, the VZ and SVZ. Inset in E shows the double labelling of SVZ progenitors with pVim and pH3, arrowhead points to the basal process of a presumptive oRG. Scale bars: 50 µm

4.3.4 Quantitative analysis of progenitor distribution in the marmoset germinal zones at E60

To probe the identity of oRG cells in marmoset, double-immunohistochemistry for Sox2 and phospho-vimentin (pVim) immunoreactivity was used (Figure 4.8A,C). Sox2 expression at E60 was mostly confined to the ventricular zone with a few labeled cells in the SVZ (Figure 4.8A). In the VZ (n=109), 87.2% (± 7.9 SED, $p < 0.005$, N=3 brains) of pH3+ cells were Sox2 immunoreactive (Figure 4.8A,E). Interestingly, already by E60, 16.19% of Sox2+ cells were located in the SVZ demonstrating heterogeneity in the progenitor population at this age (Figure 4.8A,E). Of the pVim+ cells studied in the SVZ (n=47), 40.4% (± 23.6 SED, $p < 0.005$, n=19) were Sox2+ demonstrating that a significant population of Sox2+ cells divided away from the ventricular zone. This population represents the recently discovered oRG cells that have been demonstrated to express Sox2 and Pax6, while being negative for the intermediate progenitor cell (IPC) marker Tbr2 (Hansen et al., 2010, Fietz et al., 2010, Reillo et al., 2010, Shitamukai et al., 2011, Wang et al., 2011). Consistent with these findings, oRG cells double-labelled for Sox2 and pVim at the outer border of the SVZ (Figure 4.8C). In addition to staining for radial glial and oRG progenitors Tbr2 immunohistochemistry was also performed to study IPCs in the developing neocortex (Figure 4.8B). Tbr2 immunoreactivity was restricted largely to the SVZ with a few cells also labelled in the VZ and IZ. This further represents that at E60, the VZ and SVZ are the only major germinal zones in the marmoset. To study the proportion of dividing IPCs, sections were double-stained for Tbr2/pH3 (Figure 4.8D,G). Of all pH3+ cells observed, no co-labelling was observed with Tbr2 in the VZ (n=92) while

86.2% (± 10.9 SED, $p < 0.05$ across 3 brains) of pH3+ cells in the SVZ co-labelled for Tbr2.

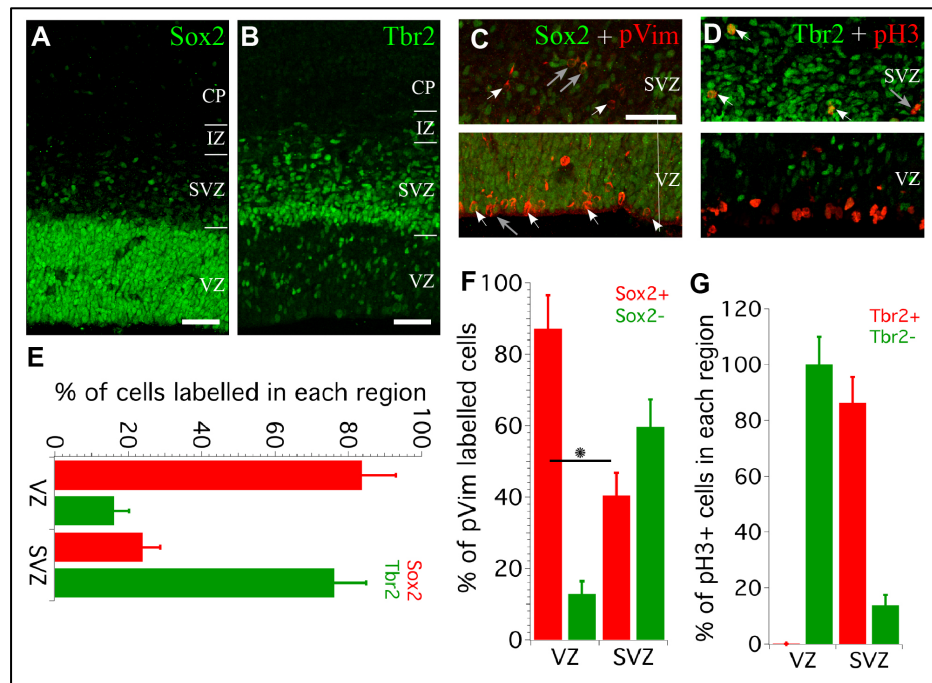


Figure 4.8: Quantitative description of progenitors in E60 marmoset cortex

(A) Sox2 staining revealed a strong labelling at the VZ while only some SVZ progenitors expressed it. (B) Tbr2 staining showed a majority of immunoreactive cells at the SVZ. (C) Double labelling with Sox2 (green) and pVim (red) at the SVZ (top) and the VZ (bottom). (D) Double labelling with Tbr2 (green) and pH3 (red) at the SVZ (top) and the VZ (bottom). White arrows represent double positive cells while grey arrows show Sox2- cells. (E) Graph presenting the percentage of Sox2+ and Tbr2+ cells in each domain. (F) Quantification of pVim+/Sox2+ cells at E60. (G) Graph showing the percent of pH3+ cells expressing Tbr2 in each proliferative domain. Asterisks indicate p values (one-tailed Student's t-test). Scale bars: 50μm.

Having found Sox2+ oRGs and Tbr2 immunoreactive IPCs at E60, it may be said that the appearance of these progenitors preceded the split of the subventricular zone into inner and outer zones.

Specific changes taking place in the developing neocortex at the level of the germinal zone were next analysed. In contrast to E60, Ki67 immunoreactivity, at E88 was observed in all germinal layers from the VZ to the OSVZ including the inner fibre layer (IFL) (Figure 4.9E). This was further confirmed by pH3

(Figure 4.9F) and pVim expression (Figure 4.9G), indicating that the subdivision of the SVZ takes place during the epoch between E60 and E88, creating an additional sector within the germinal zone. Sox2 expression was found in all germinal layers including the IFL (Figure 4.9H). Tbr2 expression at E88 was similar to Sox2 except that Tbr2⁺ cells were absent in the VZ (Figure 4.9I). Additionally, while expression in the ISVZ was in the form of a discernible band of cells parallel to the VZ, expression in the other regions was more diffuse similar to Sox2 expression in these regions. Compared to Sox2 expression, there was greater Tbr2 immunoreactivity in the OSVZ and ISVZ (Figure 4.9I).

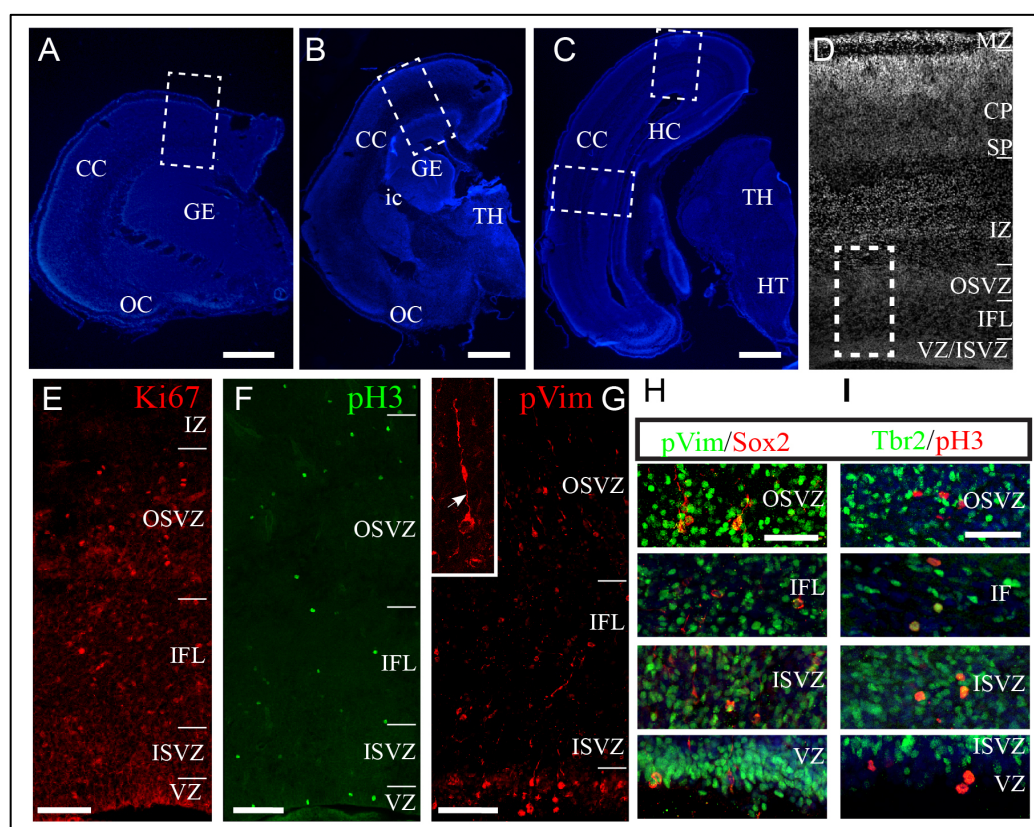


Figure 4.9: Proliferative domains in the E88 marmoset dorsal cortex

(A-C) Coronal sections of the forebrain stained with DAPI, from rostral to caudal. Images were taken at the level indicated by rectangles (D) Cortical layering at mid neurogenesis. (E-G) Ki67, pH3 and pVim labelling revealed proliferative domains.

Inset in G shows a presumptive oRG with a basal process (arrow). (H-I) Sox2/pVim (H) and Tbr2/pH3 (I) double-labelling shows distribution of progenitors in different layers. Scale bars: A-C: 1mm, rest are 100 μ m.

4.3.5 Quantitative analysis of progenitor distribution at E88

To understand the distribution of the different progenitors, I counted cells in the different domains. More Sox2⁺ cells were seen in the IFL and OSVZ compared to the VZ, where most of the radial glial population is believed to reside (17.09% VZ; 20.52% ISVZ; 29.16% IFL and 33.23% OSVZ, respectively, p values <0.05, minimum of 500 cells counted in each zone) (Figure 4.10A,C). Thus more than half of the Sox2⁺ population resides outside the confines of the VZ/ISVZ by E88, suggesting that the VZ is no longer the major germinal zone at this stage. Pattern of Tbr2 expression at E88 was similar to Sox2 except that Tbr2⁺ cells were absent in the VZ (0.46% VZ, n=11, 43.57% OSVZ, 32.34% ISVZ, p<0.005, minimum of 500 cells counted in each zone except the VZ) (Figure 4.10B,D) where most of the expression was found, while Sox2 expression was more equitable across the different zones. I also quantified pVim⁺/Sox2⁺ cells and pH3⁺/Tbr2⁺ cells in the different domains (Figure 4.10E,F) and found significant overlap in Sox2 and Tbr2 positive cells in the all domains except VZ where Tbr2 progenitors are not usually found at E88. This points towards a transitory nature of expression in progenitors where cells express both the markers. This has previously been shown by Hansen and colleagues (Hansen et al., 2010).

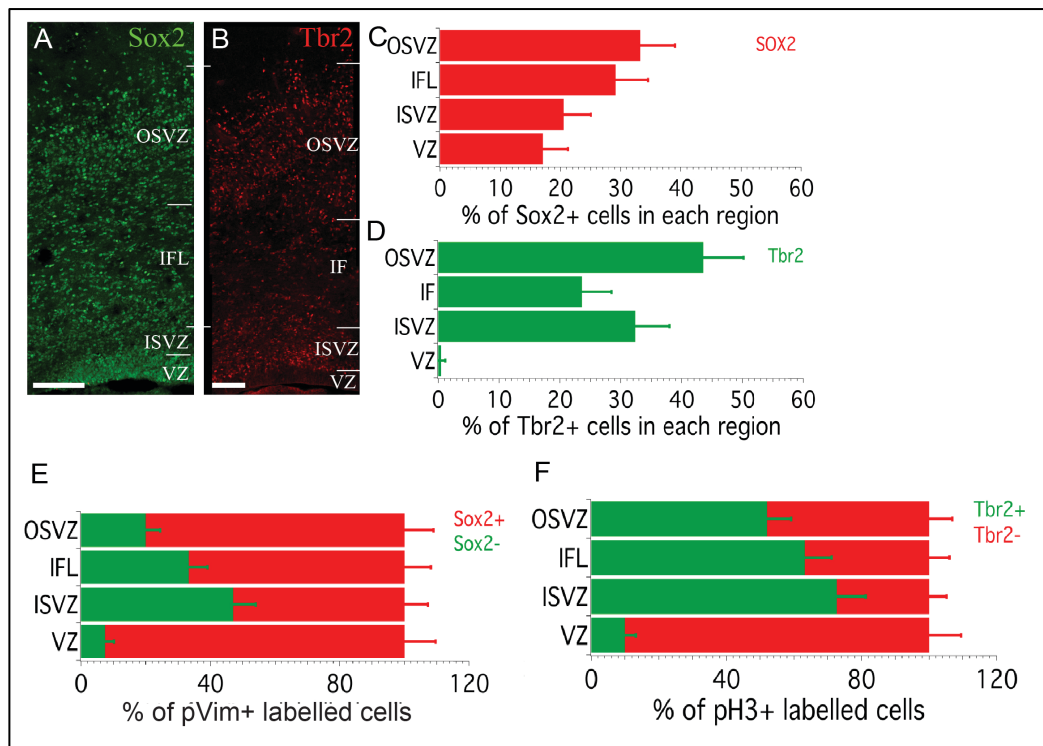


Figure 4.10: Quantitative description of progenitor distribution in E88 marmoset cortex

(A-B) Sox2 (A) and Tbr2 (B) labelling in E88 cortex shows distribution of both progenitors across the proliferative domains. (C-D) Graphs presenting the percentage of Sox2 (C) and Tbr2 (D) positive cells in each domain. (E) Quantification of pVim progenitors expressing Sox2 at E88. (F) Graph presenting the percentage of pH3+ cells expressing Tbr2 in each proliferative domain. Scale bars: A-B: 100µm.

Cumulatively, this indicates that oRG progenitors in the marmoset are not selectively located in the OSVZ but are also present in the ISVZ, albeit at lower levels. Alternatively, the oRG cells observed in the ISVZ and IFL could also be ones newly born from the radial glial progenitors and in the process of translocating to their final location in the OSVZ (as suggested by Wang et al., 2011).

The plane of division in progenitors has been shown to regulate the choice between proliferative and neurogenic division (Fish et al., 2006; Kosodo et al., 2004). It is also known that most intermediate progenitors (~90%) divide

symmetrically to generate a pair of neurons (Noctor et al., 2004) while most oRG cells undergo either proliferative divisions or generate intermediate progenitors (Fietz and Huttner, 2011; Hansen et al., 2010). I undertook measurement of mitotic planes in these progenitors to look for possible differences from human brains that might underline differences in gyrification.

The plane of division of pH3+ cells (n= 111 cells across 3 brains) at E60 at the ventricular epithelium was largely either perpendicular (60-90°, n=28) or horizontal (0-30°, n=32) with reference to the ventricular surface. In the SVZ, divisions were either horizontal (n=12) or oblique (n=13) with fewer cells dividing perpendicular to the ventricular surface (n=7) (Figure 4.11A).

At E88 however, cell divisions in the ventricular zone (n=16 cells across 2 brains) were mainly perpendicular (n=8) as compared to horizontal (n=5) with the rest showing an oblique plane. However, the majority of OSVZ divisions (n=25 cells) were oblique (16). Description of division planes in the ISVZ and IFL could not be made due to the small number of pH3+ cells counted across different sections (n=10 and 12 respectively out of 63) (Figure 4.11B).

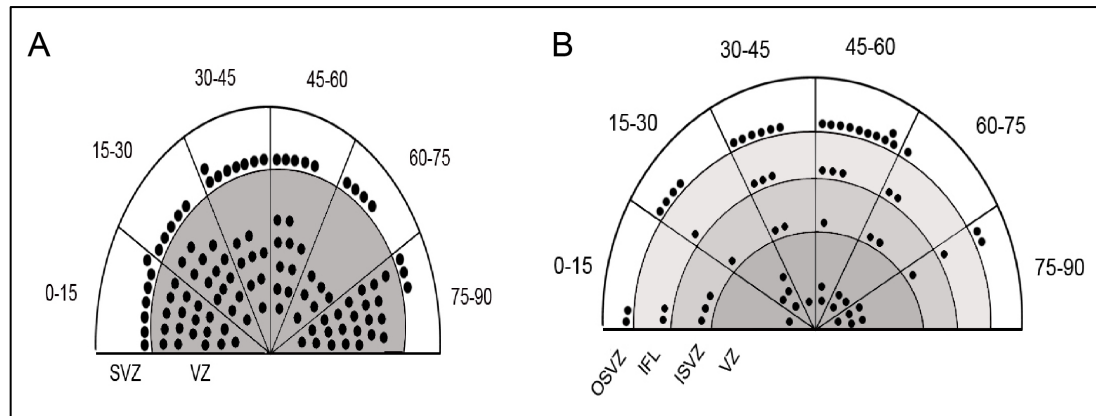


Figure 4.11: Orientation of division planes in E60 (A) and E88 (B) cortex
 (A) At E60, the mitotic planes in the VZ are mostly either perpendicular ($n=28$) or horizontal ($n=32$) in comparison to SVZ where the division was random. (B) The E88 mitotic planes in VZ were similarly perpendicular or horizontal while that of OSVZ progenitors was largely oblique.

4.3.6 Developing agouti brains show similar germinal zone architecture during development

In order to further compare the cortical germinal zones between the lissencephalic primate and a gyrencephalic rodent some markers of progenitor subtypes were examined in embryonic agouti brains. Sox2 and Snap25 immunohistochemistry in E52 agouti cortices revealed a similar expression pattern as that seen in marmoset. Sox2 immunoreactivity was dense in the VZ with a diffuse pattern in the remaining SVZ extending up to the IZ (Figure 4.12C). This pattern recaps that seen in marmoset and that reported in other species (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2010) and demonstrates that by E52 a large amount of Sox2 progenitors are present outside the VZ.

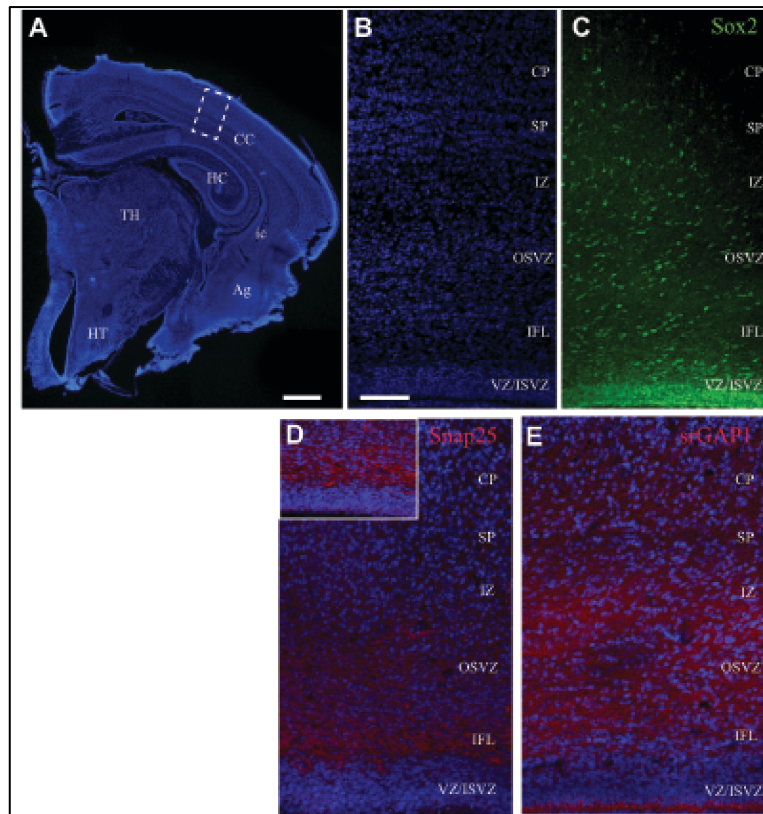


Figure 4.12: Germinal zone architecture in developing Agouti brain

(A) DAPI stained coronal section of E52 Agouti brain showing various brain regions (B) High power image from the dotted area in (A) showing gross cytoarchitecture of the developing cortical plate. (C) Sox2 staining of E52 agouti cortex shows dense group of cells in the VZ and a diffuse band in the IFL, OSVZ and IZ. Some staining can also be seen in the CP perhaps due to non-specific nature of the antibody. (D-E) Fibre staining with Snap25 (D) and srGAP1 (E) shows that fibres of the IFL are positive for both markers suggesting possible roles for these molecules and thalamocortical axons in mediating progenitor behaviour. Scale bars: 50 μ m

We also used Snap25 and srGAP1 (Slit-Robo Rho GTPase activating protein 1) immunohistochemistry to examine the fibre layers. srGAP1 was recently demonstrated to be expressed in fetal human cortices in the SVZ and later in the neocortical layers (Ip et al., 2011). Additionally, srGAP1 immunoreactivity in the human fetus is strong in the IFL by 12PCW (post-conception week) and broader in the IFL, IZ and subplate by 15PCW (Ip et al., 2011; Molnár and Clowry, 2011). In line with this, we observed both Snap25 and srGAP1 immunoreactive fibres in the IFL in the agouti germinal zone (Figure 4.12D-E),

however, compared to Snap25 the srGAP1 immunohistochemistry pattern was more diffuse. Taken together, the cytoarchitecture revealed by DAPI and Nissl's staining, the pattern of progenitors revealed with Sox2, and the fibre distribution that was revealed with Snap25 and srGAP1 immunoreactivity all point towards a clear compartmentalisation of the SVZ in the developing agouti cortex. This also affirms the presence of fibres in the developing germinal zones in species with an expanded SVZ. The anatomical placement of this fibre bundle might indicate its role in regulating the cell cycle, and thus its origin and detailed pattern of interaction will need further investigation. Limitations of tissue availability from other stages and the differences in antibody reactivity in agouti tissue prevented us from studying several other markers in both agouti and marmoset.

4.4 DISCUSSION

This chapter aims to demonstrate that the developmental program for neocortical neurogenesis is largely conserved between representatives of the Rodent and Primate Order, regardless of the extent of gyrification of the neocortex. Both, the marmoset and agouti SVZ appear compartmentalized due to the presence of IFL fibres and possess a large population of oRG. Understanding how an enlarged cortical plate with an increased capacity for establishing new patterns of connectivity is a central issue for developmental and evolutionary neurobiology (Molnár, 2011). One of the first models linking cortical expansion during evolution was the founder cell hypothesis (Rakic, 1995). According to this model, cortical expansion is the result of changes in proliferation kinetics that increase the number of radial columnar units without changing the number of neurons within each unit significantly. Therefore the detailed study of timing, control and ratio of symmetric and asymmetric modes of cell divisions in the proliferative zone is of major interest for understanding evolution.

The contribution of the multiple progenitor population towards development of the neocortex is currently not known. There is a self-renewing progenitor cell population, namely the radial progenitors (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001), that generate a single neuron per division and a basal progenitor pool that amplifies neuronal output by dividing symmetrically to provide two neurons (Noctor et al., 2004; Haubensak et al., 2004). Evolution of the neocortex can be achieved by changes to this dual

progenitor system. That is to say by increasing the proliferative activity of the progenitors at the behest of cell cycle parameters (Martinez-Cerdeño et al., 2006; Pilaz et al., 2009) or by an increase in progenitors as noted during evolution between sauropsids and therapsids (Cheung et al., 2007; Striedter and Charvet, 2008).

The progenitor pool termed as oRGs (Hansen et al., 2010, Fietz et al., 2010; Reillo et al., 2010) and the transient progenitor pool close to the pallial-subpallial boundary (Tessier et al., 2010) might suggest that the evolution of the cortex has proceeded by means of additional progenitor subtypes. These progenitor populations are now better defined and pVim, Sox2 and Tbr2 can distinguish populations in the SVZ. However, their contribution towards the development and evolution of the neocortex are yet to be described.

Recently, compartmentalisation and diversification of the SVZ has been proposed to be a key step in evolution of cortical neurogenesis while it has been specifically related to gyrencephalic brains (Fietz et al., 2010; Reillo et al., 2010) as well as nonhuman primates (Smart et al., 2002; Dehay and Kennedy, 2008). The cytoarchitectonic distinctions in the germinal zone and the OSVZ was initially described to be primate (including human) specific in the presumptive primary visual cortex (Smart et al., 2002) and later described to contain both Pax6 and Tbr2-expressing progenitor cells, thereby indicating its dual nature (Bayatti et al., 2008). OSVZ progenitors or oRGs (or intermediate radial glia), but not the cytoarchitectonic distinctions between OSVZ and ISVZ, have also been demonstrated to be present in the ferret

Chapter 5

Conclusions

5.1 Evolutionary development of progenitor cells

Comparative analysis clearly shows that abventricular mitosis and presence of a well-defined SVZ in the dorsal cortex is a characteristic of mammalian cortices (Cheung et al., 2010; Martinez-Cerdeno, 2006). However until now, it has not been possible to address how progenitor cells have evolved.

In order to appreciate this, the evolution of any progenitor cell (IPCs here) must be viewed from a similar ontogenic and phylogenic angle that is used for evolution of whole organisms and for genes (briefly discussed in Hughes, 1994). The same principles of functional segregation, divergence and acquisition of new function could hence occur in cell development as it occurs in gene evolution (reviewed in Arendt, 2008). Following these principles, one must address the substrate from which novel progenitor cell type originate. In the case of the nervous system this would undoubtedly have to be neuroepithelial cells that are conserved and required for the development of a forebrain. Having identified the substrate, the next major challenge is to address the mechanism to produce a cell capable of terminal divisions thereby amplifying the output and responding to changes in internal and external environmental pressures (Lander et al., 2009).

However it is prudent to remember that evolution occurs primarily by accruement of changes and is non-directional (Gould and Eldredge, 1977). Owing to this, comparative analysis of development shows us many examples where abventricular mitosis is absent (Cheung et al., 2007) or where despite

the presence of *Tbr2*⁺ IPCs, these cells do not contain a proliferative potential (Puzzolo and Mallamaci, 2010; Suzuki et al., 2012). Comparing the potential of IPCs from rodent or primate species with such examples will provide answers to the mechanisms behind generation of IPCs and also regarding changes in fate restriction and determination.

Thus combined with modern tools of sequencing and transcriptomic analysis, comparative studies is capable of addressing the changes during the evolution of the cortex and in the process also uncover underlying basis of disease and development (Belgard et al., 2011; 2013; Bernard et al., 2012).

5.2 Review of findings

In this thesis, I set out to probe questions centered on the role of intermediate progenitors in the developing cortex and their evolutionary significance. I also attempted to address the mechanistic basis of regulation of proliferation in intermediate progenitors.

I briefly list the results obtained in each chapter and will conclude with overarching questions regarding evolution of progenitor cells as well as cerebral cortices.

In Chapter 2, I used an over-expression protocol to increase *Tbr2* levels during cortical development. Using this I show that in response there is an upregulation of cell-cycle, spindle assembly and Wnt signaling genes. The

upregulation of both *Cdk4* and *Aspm* is particularly interesting as both genes have a direct role in influencing cell proliferation and renewal. Multiple reports argue that overexpression of *Cdk4* (and/or appropriate cyclins) leads to an increase in cell-cycle re-entry leading to an increased production of neurons (Lange et al., 2009; Pilaz et al., 2009) and even macrocephaly (Nonaka-Kinoshita et al. 2013). Indeed, the decrease in re-entry is also noted during normal development of brains (Calegari et al., 2005; Lukaszewicz et al., 2005).

Similarly, the interaction with *Aspm* is also a novel interaction discovered. Due to its role as a centrosomal protein that stabilizes mitotic spindle formation (Fish et al., 2006), *Aspm* could shed new knowledge on the mechanism in the small proportion of intermediate progenitors that undergo self-renewing division (Attardo et al., 2008; Noctor et al., 2004).

I also describe a direct and reciprocal interaction between *Tbr2* and the Wnt signaling pathway genes such as *Wnt5a* and β -*catenin*. Even though *Wnt5a* mRNA levels were not detected in the ventricular or subventricular zones, a recent publication shows that disrupting expression of a receptor (*Ror1* or *Ror2*) binding to *Wnt5a* during development leads to an increase in the number of neurons generated at the expense of progenitor cells (Endo et al., 2012). This shows that despite low levels of *Wnt5a* being expressed (or secreted), it has a physiologically relevant effect on the development of the cortex.

In Chapter 3, I studied the contribution from *Tbr2*⁺ progenitors towards the cerebral cortical layers. My study indicates that while neurons born from IPCs come to lie across all cortical layers, there is a layer-specific decrease in the conditional knockout (only seen in layers 4 and 6). This further emphasizes the redundancy in *Tbr2* function observed in relation to other layers during cortical neurogenesis. Analysis of clonally generated cells from *Tbr2*⁺ IPCs shows that they do not disperse long distances but are in close proximity with one another. Also, I show that clonal clusters are located within limits that suggest their arrangements in columns. However, I am still pursuing the contributions from a single IPC towards the cerebral cortex including the angles of dispersion of neurons. This shall allow comparison with other species on whether the angle also changes in addition to neuron production thus increasing the surface area naturally.

I have also identified contributions of *Tbr2*⁺ expressing progenitors that are not involved in the generation of GABAergic neurons of the lateral olfactory tract (lot), the olfactory tubercle and ventromedial hypothalamic nucleus in addition to the indusium griseum.

In Chapter 4, I have shown and discussed how an enlarged cortical plate with greater neuronal numbers can be constructed by addition of progenitor cell populations such as the outer radial glia. However, despite the identification of this new progenitor subtype, there is little difference in its behavior as compared to RGCs. Thus it begs the debate as to whether this is a mere

duplication of progenitor cells to achieve a larger cortical structure or truly a different progenitor class.

I have argued using the examples of agouti and marmoset that the cytoarchitectonic subdivision of the neocortical germinal zone into OSVZ and ISVZ by IFL and the presence of the large population of oRG are evolutionary trends in larger brains and not primate or gyrencephalic brain specific features. Undoubtedly, diverse brain structures (Lui et al., 2011) including mice (Shitamukai et al., 2011; Wang et al., 2011) contain oRG cells despite having smooth brained surfaces. I thus argue the need to include models other than those based on progenitor cells to describe the causes of gyrencephaly including but not limited to Van Essen's tension based model of morphogenesis (Essen, 1997; Iva Kelava, 2013; Lewitus et al., 2013; Ronan et al., 2013)

5.3 Unresolved questions and future prospects

The work presented and discussed in this thesis raises some important questions regarding the development of the brain and the role played by intermediate progenitors.

Firstly, transit-amplifying cells such as IPCs, show a small degree of self-replication (Attardo et al., 2008; Noctor et al., 2004). However, this self-renewal must be adequately controlled to achieve proper organ growth and failure to do so could result either in an uncontrolled expansion or a premature extinction of progenitor cells (Lander et al., 2009). Thus a precise control not

just on the renewal of IPCs is needed but also a control on their rate of generation as well as the rate of generation of neurons. This requires a reciprocal system of feedback between IPCs and RGCs. In recent times, a number of molecules have been suggested that control the rate of generation of IPCs such as *Tcfap2g* (Pinto et al., 2009), *Insm1* (Farkas et al., 2008) and *Ngn2* (Ochiai et al., 2009). However in each of these cases the control is one way (i.e. via transcriptional regulation in the RGCs). Knowledge of a reciprocal step of regulation is missing (from IPCs towards RGCs). The importance of this step has been mathematically highlighted by Lander and colleagues (Lander et al., 2009) who argue that having a feedback loop from terminally differentiating cells (or IPCs) onto stem cells (or RGCs) reduces the perturbations of the system without changing the output or other characteristics significantly (Figure 5.1). This provides a strong argument in favour of a reciprocal feedback controlling the rate of RGC renewal from IPCs. Even though *Wnt5a* does not change in response to change in *Tbr2*, there might be other molecules that perform this role. One such example has been suggested to be *Dll1*; a ligand for Notch signaling that is expressed in the multipolar processes of IPCs (Nelson et al., 2013). Other ligands or secreted factors might exist and require study to fully understand the control of neurogenesis.

A recent study shows the bimodal role of *Axin* in regulating both IPC generation and differentiation (Fang et al., 2013). This dual function is brought about by shifting the localization of *Axin* caused due to phosphorylation of one of its residues. In this, the example provides a switch to bring about an increase in IPC numbers without compromising the radial glia. While the

validity of this switch is yet to be determined at an evolutionary scale, it cannot be ruled out that such a regulation would be crucial in increasing the neuronal population and ultimately the size of the cerebral cortex.

Figure 5.1: Regulation of stem cell proliferation and effect on output

Graphical results of simulated experiments are shown where different parameters influence the rate of expansion of 10^5 progenitors (solid lines) or 5×10^4 (dashed lines). Analysis is followed till an output of 10^8 cells is achieved. Intermediate progenitors lines are shown in red.

(A) Simple expansion model where progenitor cells continue to expand until the desired output. Halving the progenitors (dashed blue line) reduces output.

(B) Nowakowski-Caviness system where progenitors undergo proliferative and differentiative divisions with a decreasing proliferation rate (p_0). Again a decrease in initial progenitor pool decreases the output.

(C) Negative feedback mechanism where the proliferation rate (p_0) is inhibited by the product of the reaction. In this scenario, a halving of progenitor pool produces no change in output apart from a one-cycle increase in reaching it. (Reproduced from Lander et al., 2009)

Franco and colleagues (Franco et al., 2012) have demonstrated the presence of fate-restricted progenitors during development. They show that *Cux2*⁺ radial progenitors specifically generate upper layer neurons at early periods of neurogenesis when infragranular layer neurons are being produced. This questions the long standing finding that *Tbr2*⁺ progenitors produce upper layer neurons (Tarabykin et al., 2001). In the light of new findings shown and discussed in this thesis, a re-assessment of the contribution from both *Tbr2*⁺ IPCs and fate-restricted *Cux2*⁺ RGCs towards supragranular layer neurons is required using genetic tools and transgenic mice. It is equally important to note that *Cux2* is also a marker of some IPCs (Cubelos et al., 2008) and might define a separate subset of IPCs with properties differing from *Tbr2*⁺ IPCs in determining cell fate.

This understanding also indicates the redundancy in function in IPCs. While I have shown that *Tbr2*⁺ IPCs contribute towards all layers of the cerebral cortex, the conditional knockout does not indicate a similar degree of decrease in the different layers. This probably stems from expression of other genes in IPCs that compensate the loss of *Tbr2*. Finding the compensatory genes and their mechanisms would shed light on the heterogeneity in IPCs. In addition, it will also clarify whether distinct sub-populations of IPCs contribute towards different cortical layers or if the pan-cortical contribution persists. Alternatively, it would also be prudent to study the role of entire genetic networks in the regulation of progenitor cell fate rather than only a handful of genes. As suggested by Waddington (Siegal and Bergman, 2002), the apparent redundancy might actually be fallout of developmental stability stemming from a robustness of transcriptional networks (Masel and Siegal, 2009). The role of microRNAs and long non-coding RNAs can hence not be ruled out and such examples has been described with regard to *Tbr2* (Nowakowski et al. 2013; Sasaki et al., 2008)

Finally, studying the emergence of IPCs would answer many questions in evolutionary development. Understanding the cellular, genetic and transcriptomic changes leading to the generation of IPCs will provide an insight into the evolutionary changes required for the emergence of a new progenitor type. It is imperative that such studies be coupled with comparative analysis of brains that lack IPCs or where IPCs do not function similarly as in primates. It will also be crucial to understand the evolution of brains from reptiles and amphibians (rudimentary cortices having only 3 layers) to avian

brains (that lack a laminar structure) to hexalaminar mammalian brains such as rodents and primates to understand fate restriction and determination of each progenitor type. In addition, it would also indicate conserved mechanisms in generation of neurons and of specific brain areas.

Thus the findings presented in this thesis plugs important gaps in our understanding of cortical development and address other lacunae that would aid in describing the formation and functioning of a normal cerebral cortex.

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neocortex, a gyrencephalic canid (Fietz et al., 2010), and more recently in mice (Wang et al., 2011; Shitamukai et al., 2011). Analysis shown here demonstrates that in marmoset the pVim+ oRGs and Tbr2 positive IPCs are not restricted to ISVZ or OSVZ but present in both in varying proportions.

The examples of agouti and marmoset in this study suggest that the cytoarchitectonic subdivision of the neocortical germinal zone into OSVZ and ISVZ by IFL and the presence of the large population of oRG are evolutionary trends in larger brains and not primate or gyrencephalic brain specific features. Instead, the compartmentalisation of germinal zones (ISVZ/OSVZ) and a concomitant increase in the oRG and IPC population is a more prudent indicator of the evolutionary mechanism leading to an expansion of the neocortex regardless of the folding pattern of the brain.

In the marmoset increases in both oRG and IPC populations are seen during development. Since IPCs can amplify the output of neurons, the presence of an increased Tbr2+ population may lead to an increase in the neuronal population and eventually to cortical thickness. Further lineage studies are required to test the hypothesis that certain intermediate progenitors contribute to particular layers or cell types (Molnár, 2011). Moreover quantitative analysis of neocortical cell types in different regions in the adult cortex and quantitative assessment of various progenitors in the germinal zone during embryonic development are required by using isometric fractionation in combination with markers of progenitors and neocortical cell types (see methods used by Collins et al., 2010).

There are possible mechanisms how the increase of these populations might stimulate the increased generation and migration of interneurons. Broccoli and colleagues (Sessa et al., 2010) demonstrated that Tbr2 directs migration of interneurons through chemokine receptors. It is conceivable that an additional Tbr2+ positive germinal zone would attract more interneurons also leading to an increase in neuronal numbers and cortical thickness.

Keeping in line with previous studies correlating the presence of a SVZ with a larger cortex in birds (Streidter and Charvet, 2009, Cheung et al., 2010), this raises the possibility that the evolution of the OSVZ with oRG progenitors might be a more potent indicator towards the evolution of a larger brain with faster development irrespective of the order or folding pattern. Since both subtypes of progenitors (IPCs and oRGs) are present in the OSVZ, it indicates that the OSVZ is neither a novelty nor a trigger in terms of cortical evolution but rather a proliferative layer similar to the ISVZ, only different in terms of progenitor proportions and location.

The relevance of the inner fibre layer (IFL) that separates the ISVZ from the OSVZ is also unknown. A suggested possibility could be the earliest thalamocortical projections constitute the outer fibre layer (OFL) while some later ones contribute to the IFL (Lukaszewicz et al., 2005). If some of the fibres in the IFL indeed originate from the thalamus, then species with cytoarchitectonic distinctions between the OSVZ and ISVZ would have thalamic fibres in closer association with the germinal zone than in species without such distinctions. The concept that the ascending pathways influence

rates of proliferation in the cortex and ultimately contribute to setting up distinct proliferative programmes in the germinal zone and possibly determine cortical cytoarchitecture is not new (Dehay et al., 2001; Lukaszewicz et al., 2005; Carney et al., 2007). Actually, it has been demonstrated *in vitro* that thalamic explants can alter cell-cycle parameters in embryonic progenitor cells (Dehay et al., 2001; Dehay and Kennedy, 2007) and accelerate neuronal migration *in vitro* (Edgar and Price, 2001). However, cortical neurons are generated in roughly the same number in mouse models of *cerveau isole* (Zhou et al., 2010), indicating that this mechanism of control might have originated only in larger cortices with a cytoarchitectonic subdivision of the germinal zones as seen in the present study. Alternatively, the IFL could accommodate fibres from newly generated projection neurons. Time-lapse recordings of newly born neurons in mouse neocortex reveal that they extend projections into the SVZ and VZ during early cortical development (Noctor et al., 2004; Lickiss et al., 2011). This mechanism could contribute to the regulation of neuronal production by means of such interactions.

The extent of gyrencephaly varies tremendously across species and orders. Both smooth and folded brains appear in many orders adding to the diversity in brain sizes and structures (Cheung et al., 2007). Additionally, it has been shown that gyrencephaly is correlated to the brain weight and that the measure of gyrencephaly follows an allometric scaling specific to each order (Pillay and Manger, 2007). In keeping with such studies, the appearance of convolutions during development might be due to the increased tension from white matter and other axonal fibre bundles (van Essen, 1997), or due to a

thinning of the cortex (Pillay and Manger, 2007; Rakic, 1988) rather than simply the heterogeneous expansion of abventricular progenitors. A morphogenetic model of gyrification has been proposed that incorporates the inherent structure of the neocortex along with the elastic forces and growth of the cortex (Toro and Burnod, 2005). This model uses computer simulations to suggest that convolutions are a natural consequence of cortical growth and occurs due to geometric asymmetry in the brain shape coupled with mechanical asymmetry due to differential gene expression and differential growth dynamics. In accordance with this study, it can be argued how species belonging to an otherwise gyrencephalic order violate the trend and reveal contradictory folding patterns. This might also explain why the extent of gyrencephaly varies among species.

Extending the number of species studied by adding agouti and marmoset provides evidence of compartmentalisation of the SVZ and the abundant presence of SVZ progenitor subtypes. Hence, we can conclude that these features are not related to a specific form of brain or mammalian order. On the contrary, the cortical developmental program remains relatively constant across the mammalian lineage.

While this study does not answer the mechanisms of folding itself, it provides pertinent examples to warn against generalisations based on folding or taxa. More comparative work is needed to understand why adult brains are so diverged taking into consideration variations in cortical size, folding, cell cycle parameters and developmental patterns.

I demonstrate here that the gyrencephalic amazonian rodent, agouti and a lissencephalic non-human primate, the marmoset monkey both possess oRGs from around midgestation and a well-organized and positionally distinct OSVZ during neocortical neurogenesis. Moreover, the presence of different subventricular progenitors is not associated with specific germinal layers as pVim⁺ oRGs and Tbr2⁺ IPCs are present both in ISVZ and OSVZ, but in different proportions. Interestingly, these proportions are not different from the observations made in other gyrencephalic brains. Thus the association of these discerning cytoarchitectonic features of the germinal zone and increased oRG populations with gyrencephaly requires re-evaluation.

Chapter 5

Conclusions

5.1 Evolutionary development of progenitor cells

Comparative analysis clearly shows that abventricular mitosis and presence of a well-defined SVZ in the dorsal cortex is a characteristic of mammalian cortices (Cheung et al., 2010; Martinez-Cerdeno, 2006). However until now, it has not been possible to address how progenitor cells have evolved.

In order to appreciate this, the evolution of any progenitor cell (IPCs here) must be viewed from a similar ontogenic and phylogenic angle that is used for evolution of whole organisms and for genes (briefly discussed in Hughes, 1994). The same principles of functional segregation, divergence and acquisition of new function could hence occur in cell development as it occurs in gene evolution (reviewed in Arendt, 2008). Following these principles, one must address the substrate from which novel progenitor cell type originate. In the case of the nervous system this would undoubtedly have to be neuroepithelial cells that are conserved and required for the development of a forebrain. Having identified the substrate, the next major challenge is to address the mechanism to produce a cell capable of terminal divisions thereby amplifying the output and responding to changes in internal and external environmental pressures (Lander et al., 2009).

However it is prudent to remember that evolution occurs primarily by accruement of changes and is non-directional (Gould and Eldredge, 1977). Owing to this, comparative analysis of development shows us many examples where abventricular mitosis is absent (Cheung et al., 2007) or where despite

the presence of *Tbr2*⁺ IPCs, these cells do not contain a proliferative potential (Puzzolo and Mallamaci, 2010; Suzuki et al., 2012). Comparing the potential of IPCs from rodent or primate species with such examples will provide answers to the mechanisms behind generation of IPCs and also regarding changes in fate restriction and determination.

Thus combined with modern tools of sequencing and transcriptomic analysis, comparative studies is capable of addressing the changes during the evolution of the cortex and in the process also uncover underlying basis of disease and development (Belgard et al., 2011; 2013; Bernard et al., 2012).

5.2 Review of findings

In this thesis, I set out to probe questions centered on the role of intermediate progenitors in the developing cortex and their evolutionary significance. I also attempted to address the mechanistic basis of regulation of proliferation in intermediate progenitors.

I briefly list the results obtained in each chapter and will conclude with overarching questions regarding evolution of progenitor cells as well as cerebral cortices.

In Chapter 2, I used an over-expression protocol to increase *Tbr2* levels during cortical development. Using this I show that in response there is an upregulation of cell-cycle, spindle assembly and Wnt signaling genes. The

upregulation of both *Cdk4* and *Aspm* is particularly interesting as both genes have a direct role in influencing cell proliferation and renewal. Multiple reports argue that overexpression of *Cdk4* (and/or appropriate cyclins) leads to an increase in cell-cycle re-entry leading to an increased production of neurons (Lange et al., 2009; Pilaz et al., 2009) and even macrocephaly (Nonaka-Kinoshita et al. 2013). Indeed, the decrease in re-entry is also noted during normal development of brains (Calegari et al., 2005; Lukaszewicz et al., 2005).

Similarly, the interaction with *Aspm* is also a novel interaction discovered. Due to its role as a centrosomal protein that stabilizes mitotic spindle formation (Fish et al., 2006), *Aspm* could shed new knowledge on the mechanism in the small proportion of intermediate progenitors that undergo self-renewing division (Attardo et al., 2008; Noctor et al., 2004).

I also describe a direct and reciprocal interaction between *Tbr2* and the Wnt signaling pathway genes such as *Wnt5a* and β -*catenin*. Even though *Wnt5a* mRNA levels were not detected in the ventricular or subventricular zones, a recent publication shows that disrupting expression of a receptor (*Ror1* or *Ror2*) binding to *Wnt5a* during development leads to an increase in the number of neurons generated at the expense of progenitor cells (Endo et al., 2012). This shows that despite low levels of *Wnt5a* being expressed (or secreted), it has a physiologically relevant effect on the development of the cortex.

In Chapter 3, I studied the contribution from Tbr2⁺ progenitors towards the cerebral cortical layers. My study indicates that while neurons born from IPCs come to lie across all cortical layers, there is a layer-specific decrease in the conditional knockout (only seen in layers 4 and 6). This further emphasizes the redundancy in *Tbr2* function observed in relation to other layers during cortical neurogenesis. Analysis of clonally generated cells from Tbr2⁺ IPCs shows that they do not disperse long distances but are in close proximity with one another. Also, I show that clonal clusters are located within limits that suggest their arrangements in columns. However, I am still pursuing the contributions from a single IPC towards the cerebral cortex including the angles of dispersion of neurons. This shall allow comparison with other species on whether the angle also changes in addition to neuron production thus increasing the surface area naturally.

I have also identified contributions of Tbr2⁺ expressing progenitors that are not involved in the generation of GABAergic neurons of the lateral olfactory tract (lot), the olfactory tubercle and ventromedial hypothalamic nucleus in addition to the indusium griseum.

In Chapter 4, I have shown and discussed how an enlarged cortical plate with greater neuronal numbers can be constructed by addition of progenitor cell populations such as the outer radial glia. However, despite the identification of this new progenitor subtype, there is little difference in its behavior as compared to RGCs. Thus it begs the debate as to whether this is a mere

duplication of progenitor cells to achieve a larger cortical structure or truly a different progenitor class.

I have argued using the examples of agouti and marmoset that the cytoarchitectonic subdivision of the neocortical germinal zone into OSVZ and ISVZ by IFL and the presence of the large population of oRG are evolutionary trends in larger brains and not primate or gyrencephalic brain specific features. Undoubtedly, diverse brain structures (Lui et al., 2011) including mice (Shitamukai et al., 2011; Wang et al., 2011) contain oRG cells despite having smooth brained surfaces. I thus argue the need to include models other than those based on progenitor cells to describe the causes of gyrencephaly including but not limited to Van Essen's tension based model of morphogenesis (Essen, 1997; Iva Kelava, 2013; Lewitus et al., 2013; Ronan et al., 2013)

5.3 Unresolved questions and future prospects

The work presented and discussed in this thesis raises some important questions regarding the development of the brain and the role played by intermediate progenitors.

Firstly, transit-amplifying cells such as IPCs, show a small degree of self-replication (Attardo et al., 2008; Noctor et al., 2004). However, this self-renewal must be adequately controlled to achieve proper organ growth and failure to do so could result either in an uncontrolled expansion or a premature extinction of progenitor cells (Lander et al., 2009). Thus a precise control not

just on the renewal of IPCs is needed but also a control on their rate of generation as well as the rate of generation of neurons. This requires a reciprocal system of feedback between IPCs and RGCs. In recent times, a number of molecules have been suggested that control the rate of generation of IPCs such as *Tcfap2g* (Pinto et al., 2009), *Insm1* (Farkas et al., 2008) and *Ngn2* (Ochiai et al., 2009). However in each of these cases the control is one way (i.e. via transcriptional regulation in the RGCs). Knowledge of a reciprocal step of regulation is missing (from IPCs towards RGCs). The importance of this step has been mathematically highlighted by Lander and colleagues (Lander et al., 2009) who argue that having a feedback loop from terminally differentiating cells (or IPCs) onto stem cells (or RGCs) reduces the perturbations of the system without changing the output or other characteristics significantly (Figure 5.1). This provides a strong argument in favour of a reciprocal feedback controlling the rate of RGC renewal from IPCs. Even though *Wnt5a* does not change in response to change in *Tbr2*, there might be other molecules that perform this role. One such example has been suggested to be *Dll1*; a ligand for Notch signaling that is expressed in the multipolar processes of IPCs (Nelson et al., 2013). Other ligands or secreted factors might exist and require study to fully understand the control of neurogenesis.

A recent study shows the bimodal role of *Axin* in regulating both IPC generation and differentiation (Fang et al., 2013). This dual function is brought about by shifting the localization of *Axin* caused due to phosphorylation of one of its residues. In this, the example provides a switch to bring about an increase in IPC numbers without compromising the radial glia. While the

validity of this switch is yet to be determined at an evolutionary scale, it cannot be ruled out that such a regulation would be crucial in increasing the neuronal population and ultimately the size of the cerebral cortex.

Figure 5.1: Regulation of stem cell proliferation and effect on output

Graphical results of simulated experiments are shown where different parameters influence the rate of expansion of 10^5 progenitors (solid lines) or 5×10^4 (dashed lines). Analysis is followed till an output of 10^8 cells is achieved. Intermediate progenitors lines are shown in red.

(A) Simple expansion model where progenitor cells continue to expand until the desired output. Halving the progenitors (dashed blue line) reduces output.

(B) Nowakowski-Caviness system where progenitors undergo proliferative and differentiative divisions with a decreasing proliferation rate (p_0). Again a decrease in initial progenitor pool decreases the output.

(C) Negative feedback mechanism where the proliferation rate (p_0) is inhibited by the product of the reaction. In this scenario, a halving of progenitor pool produces no change in output apart from a one-cycle increase in reaching it. (Reproduced from Lander et al., 2009)

Franco and colleagues (Franco et al., 2012) have demonstrated the presence of fate-restricted progenitors during development. They show that *Cux2*⁺ radial progenitors specifically generate upper layer neurons at early periods of neurogenesis when infragranular layer neurons are being produced. This questions the long standing finding that *Tbr2*⁺ progenitors produce upper layer neurons (Tarabykin et al., 2001). In the light of new findings shown and discussed in this thesis, a re-assessment of the contribution from both *Tbr2*⁺ IPCs and fate-restricted *Cux2*⁺ RGCs towards supragranular layer neurons is required using genetic tools and transgenic mice. It is equally important to note that *Cux2* is also a marker of some IPCs (Cubelos et al., 2008) and might define a separate subset of IPCs with properties differing from *Tbr2*⁺ IPCs in determining cell fate.

This understanding also indicates the redundancy in function in IPCs. While I have shown that *Tbr2*⁺ IPCs contribute towards all layers of the cerebral cortex, the conditional knockout does not indicate a similar degree of decrease in the different layers. This probably stems from expression of other genes in IPCs that compensate the loss of *Tbr2*. Finding the compensatory genes and their mechanisms would shed light on the heterogeneity in IPCs. In addition, it will also clarify whether distinct sub-populations of IPCs contribute towards different cortical layers or if the pan-cortical contribution persists. Alternatively, it would also be prudent to study the role of entire genetic networks in the regulation of progenitor cell fate rather than only a handful of genes. As suggested by Waddington (Siegal and Bergman, 2002), the apparent redundancy might actually be fallout of developmental stability stemming from a robustness of transcriptional networks (Masel and Siegal, 2009). The role of microRNAs and long non-coding RNAs can hence not be ruled out and such examples has been described with regard to *Tbr2* (Nowakowski et al. 2013; Sasaki et al., 2008)

Finally, studying the emergence of IPCs would answer many questions in evolutionary development. Understanding the cellular, genetic and transcriptomic changes leading to the generation of IPCs will provide an insight into the evolutionary changes required for the emergence of a new progenitor type. It is imperative that such studies be coupled with comparative analysis of brains that lack IPCs or where IPCs do not function similarly as in primates. It will also be crucial to understand the evolution of brains from reptiles and amphibians (rudimentary cortices having only 3 layers) to avian

brains (that lack a laminar structure) to hexalaminar mammalian brains such as rodents and primates to understand fate restriction and determination of each progenitor type. In addition, it would also indicate conserved mechanisms in generation of neurons and of specific brain areas.

Thus the findings presented in this thesis plugs important gaps in our understanding of cortical development and address other lacunae that would aid in describing the formation and functioning of a normal cerebral cortex.

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