The Development of Inhibitory Cortical Interneurons in the Early Fetal Human Telencephalon

Ayman Mohammad Alzu'bi

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Abstract

Higher order processing by circuits in the cerebral cortex is highly dependent on inhibitory interneurons; failure in their generation and/or functions is thought to lead to neurodevelopmental conditions. In rodents, interneurons are almost entirely generated in the ganglionic eminences (GE) and migrate into the cortex; however, it is still contentious to what degree this applies to the more complex human cerebral cortex.

In this study, immunohistochemical analysis of early fetal human ventral telencephalon showed distinct (although overlapping) expression domains for several interneuron precursor transcription factors revealing the complex subdivisions of the GE; including three compartments for the CGE (medial, lateral, ventral) and septum (MGE-like, LGE-like, pallial septum). Two migratory pathways of interneurons from the CGE (anteriorly via LGE) and septum (medially) into the cortex, not previously reported in rodents, were also described. Cortically-derived cultures of fetal human neuroprogenitors contained considerable numbers of GABA+ and calretinin+ cells; significantly more so in anterior- versus posterior-derived cortical cultures. Many cells expressed either COUP-TFI or COUP-TFII, but not NKX2.1, characteristic of MGE-derived cells. Furthermore, RNA sequencing data from fetal human cortical samples found mRNA levels for *DLX1, DLX2, GSH2, ASCL1, ARX, OLIG2* and *CALB2*, genes characteristic of GABAergic interneurons and their progenitors, to be significantly higher in samples derived from anterior than posterior cortical regions.

As in mice, SP8 and COUP-TFI were expressed in counter-gradients across the cortex, but unlike in rodents, expression overlapped extensively in the ventricular zone (VZ) of parietal, occipital and dorso-temporal cortex. COUP-TFII was expressed widely throughout the ventral temporal and ventral posterior cortex overlapping extensively with COUP-TFI. VZ OLIG2 expression was confined to anteromedial cortex at early stages. Arealised expression of transcription factors in the cortical wall may, in turn, control expression of signalling pathways that attract migrating cells expressing the same transcription factors, setting up various pathways into the cortex for interneurons arriving from the GE.

In conclusion, the early fetal human brain shares the fundamental mechanisms of protomap formation and interneuron generation with rodents; however, there may also be specific differences. The much larger human cortex may require additional migratory pathways for interneuron precursors. Interneuron generation in the anterior regions of developing human cortex in particular, along with more complex interplay between arealisation genes in the formation of the protomap, could be two mechanisms by which association cortex has become expanded and more specialised in human.

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Abbreviations

ANOVA	Analysis of variance
ANR	Anterior neural ridge
Ant	Anterior (= Rostral in rodent studies)
ARX	Aristaless related homeobox
ASCL1	Achaete-scute family bHLH transcription factor 1
BMP	Bone morphogenetic protein
CalR	Calretinin
Cb	Calbindin
cDNA	Complementary deoxyribonucleic acid
CGE	Caudal ganglionic eminence
CMS	Caudal migratory stream
CNS	Central nervous system
COUP-TFI/II	Chicken ovalbumin upstream promoter transcription factor 1/2
CP	Cortical plate
CR	Cajal-Retzius
Crx	Cortex
CS	Carnegie stage
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DBX1	Developing Brain Homeobox 1
dLGE	Dorsal lateral ganglionic eminence
DLX	Distal-less homeobox
dMGE	Dorsal medial ganglionic eminence
DNA	Deoxyribonucleic acid
dPallium	Dorsal pallium
E	Embryonic
EGF	Epidermal growth factor
EMX1/ 2	Empty spiracles homeobox1/2
ER81	E-twenty six (Ets) variant 1
FBS	Fetal calf serum
FGF	Fibroblast growth factor

GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
GE	Ganglionic eminence
GFAP	Glial fibrillary acidic protein
GLI	GLI Family Zinc Finger
GSH1/2	Genetic screened homeobox 1/2
H&E	Haematoxylin and eosin
HBSS	Hank's balanced salt solution
HDBR	Human Developmental Biology Resource
hIPSC	human Induced pluripotent stem cell
HRP	Horseradish peroxidase
IC	Internal capsule
ICC	Immunocytochemistry
ID	Identificatation number
IGM	Institute of Genetic Medicine
IHC	Immunohistochemistry
ISVZ	Inner sub-ventricular zone
IZ	Intermediate zone
KO	Knock-out
LGE	Lateral ganglionic eminence
LHX	LIM homeobox
LPallium	Lateral pallium
MGE	Medial ganglionic eminence
ml	Millilitre
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
mPallium	Medial pallium
MT	Middle temporal
MZ	Marginal zone
n	Number
NGN2	Neurogenin2
NKX2.1	Nk2 homeobox 1
NOS1	Nitric oxide synthase 1

NPY	Neuropeptide Y
Nr2f1/2	Nuclear receptor subfamily 1/2
OLIG2	Oligodendrocyte transcription factor 2
OSVZ	Outer sub-ventricular zone
PAX6	Paired-box 6
PCW	Post conceptional weeks
PFA	Paraformaldehyde
POA	Preoptic area
Pos	Posterior (= Caudal in rodent studies)
PP	Preplate
PROX1	Prospero Homeobox 1
PTCH1	Transmembrane receptor Patched 1
Pv	Parvalbumin
RNA	ribonucleic acid
RNA Seq	Ribonucleic acid sequencing
ROBO	Roundabout
RPKM	Reads per kilo base per million
RRID	Research Resource Identifier
RTO	Rostral telencephalic organiser
SFRP2	Secreted frizzled-related protein 2
Shh	Sonic hedgehog
SMO	Smothened
SOX6	Sex determining region Y (SRY) box 6
SP	Subplate
SP8	Specificity protein 8
Sst	Somatostatin
SUFU	Suppressor of fused
SVZ	Subventricular zone
TBR1/2	T-box brain 1/2
TBS	Tris based buffer
vCGE	Ventral caudal ganglionic eminence
VIP	Vasoactive intestinal peptide
vLGE	Ventral lateral ganglionic eminence

vMGE	Ventral medial ganglionic eminence
vPallium	Ventral pallium
VZ	Ventricular zone
Wnt	Wingless
µg	Microgram
µl	Microliter
µm	Micrometre
µM	Micro molar

Chapter 1: Introduction

The six-layered cerebral cortex is considered the most complex structure in the mammalian nervous system. Although the cortex is known to be heterogeneous at the cellular level, it contains two principal types of neurons: projection neurons and interneurons. The interconnectivity and synchronicity between these two types of neurons form the key role of cerebral cortex functions in memory and cognitive abilities. The more numerous pyramidal projection neurons are excitatory in action and release glutamate (also known as glutamatergic neurons). The interneurons are mainly inhibitory and use the neurotransmitter gamma-aminobutyric acid (GABA, also known as GABAergic interneurons). The proper and complex function of the neocortical circuits largely depends on maintaining the balance between the excitatory and inhibitory inputs delivered by the glutamergic neurons and GABAergic interneurons, respectively (Marín and Rubenstein, 2001; Klausberger and Somogyi, 2008). Evidence presented suggests that the developmental origins of these two classes are distinct; while the glutamergic neurons are generated from the radial glia cells in the proliferative zone of the dorsal telencephalon and radially migrate within the cortex, the GABAergic interneurons appear to be derived from a separate type of progenitor cell in the ventral telencephalon and follow distinct tangential pathways of migration to the dorsal telencephalon (Lavdas et al., 1999; Anderson et al., 2001; Anderson et al., 2002; Nery et al., 2002). In rodents, interneurons comprise almost 20% of all cortical neurons (Wonders and Anderson, 2006). Despite their relatively small proportion, GABAergic interneurons play essential roles in orchestrating higher cognitive functions in the cerebral cortex and it has been widely suggested that defects in the generation, migration and function of these interneurons are a cause of neurodevelopmental conditions such as autism and schizophrenia (Marín, 2012; Le Magueresse and Monyer, 2013).

GABA is the major inhibitory neurotransmitter in GABAergic interneurons where it is synthesized from glutamate using two isoforms of L-glutamic acid decarboxylase (GAD) 65 and 67. Each one of these isoforms has distinct distribution; GAD65 is mainly found in nerve endings and responsible for vesicular GABA production, GAD67 is more distributed in cytoplasm and seems to be responsible for cytoplasmic GABA synthesis (Le Magueresse and Monyer, 2013). GABA is known as the first neurotransmitter active in the immature brain,

$GABA_{A/B}$ receptors are also known to be expressed in radial glia and migrating neurons early in the developing brain. Unlike in the adult, where this neurotransmitter acts synaptically to inhibit the target neurons, this early form of GABA signalling during development may act as paracrine signalling molecules and play essential role in regulating cortical development (Manent and Represa, 2007, Wang and Kriegstein, 2009). GABA can depolarize progenitor cells and their progeny due to their high intracellular chloride concentration, thus providing the main excitatory drive for the immature cortical network (Li et al. 2002; Wang et al. 2002). GABA is also required to serve as an ideal signal to coordinate corticogenesis (Wang and Kriegstein, 2009) via decreasing the net DNA synthesis and cortical progenitor proliferation, this effect is likely to be due to its ability to depolarize the cell and activate voltage-gated Ca2+ channels that in turn regulate DNA synthesis (LoTurco et al. 1995; Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005). Furthermore, activation of specific GABA receptors is instrumental in cell migration by acting as a motility promoting, acceleratory, or stop signal (Manent and Represa, 2007).

The recent advances in studying the development of cortical interneurons and the network of transcription factors that regulates their production, migration, and sorting these neurons into diverse subtypes will be addressed in this chapter. Although most studies cited in this chapter used rodents as an experimental model, the latest published work on human tissue is also reported in order to understand the different aspects of GABAergic interneurons production and specification in developing human cortex.

1.1 The early human brain development

The process of human brain development begins in the third gestational week and extends through late adolescence. The gestational period in human is divided into the embryonic and fetal periods; the embryonic period begins from the time of fertilization (at conception) and last approximately for 60 days (8 weeks) (Stiles, 2008; O'Rahilly and Müller, 2010; Stiles and Jernigan, 2010). Based on the morphologic features of the embryo (not the age or the size) this period is divided into 23 stages which are known as Carnegie stages (CS) (Hamilton, 1974). By the end of the embryonic period, the primary rudimentary structures of the brain are established and well defined (Stiles, 2008). After 8 weeks, the term embryo is usually replaced with the term fetus, indicating the beginning of the following fetal period which extends from

the 9 gestational week through to the end of gestation. In this period, the brain continues to grow and the primary structures differentiate into the adult brain structures (Stiles and Jernigan, 2010).

By the end of the third week of development, the embryo is a three-layered structure: the inner endodermal stem cell layer, the intermediate mesodermal stem cell layer, and the external ectodermal stem cells layer. Brain development begins by the formation of the neural tube, which arises from the neuroectoderm (the neural progenitor cells located along the rostral-caudal midline of the ectodermal layer and referred to as the *neural plate*). The neural plate develops two lateral ridges that fold inwards and fuse to create the hollow neural tube structure (Copp *et al.*, 2003). Following the complete closure of the neural tube (CS12, approximately embryonic day 30), the rostral end of the neural tube expands forming the three primary brain vesicles, while the caudal part develops into the spinal cord. The three vesicles aligned along rostral-caudal axis are as follow: the prosencephalon (forebrain), the mesencephalon (midbrain), and the rhombencephalon (hindbrain). Later and by the end of the embryonic period, the prosencephalon and rhombencephalon further subdivide, whereas the mesencephalon does not divide. The prosencephalon divides into the telencephalon and the diencephalon, and the rhombencephalon subsequently divides into the metencephalon and myelencephalon, ending up with fives secondary vesicles which establish the primary organization of the developing brain (Stiles, 2008; Stiles and Jernigan, 2010). The early development and organization of the telencephalon will be discussed further below from data that are mostly from rodent studies.

1.1.1 Development of the telencephalon

The telencephalon, the most rostral part of the developing central nervous system, is partitioned into structurally and functionally distinct dorsal and ventral regions, the neuroepithelium of these two regions are called pallium and sub-pallium, respectively. The pallium (dorsal telencephalon) is the primordium of cerebral cortex, whilst the sub-pallium gives rise to the structures of the basal ganglia (caudate, putamen, globus pallidus and nucleus acumbens). In rodent, the pallial and sub-pallial regions are mainly identified by characteristic expression of certain transcription factors across each region (Figure 1.1), their expression controlled by

gradients of soluble morphogens (such as FGFs, BMPs and SHH) released from four discrete forebrain signalling centres (Figure 1.2A). The pallium is characterized by the expression of Paired-box 6 (PAX6), Empty spiracles homeobox 1 and 2 (EMX1, EMX2), and T-box brain 1 (TBR1), whereas transcription factors like Glutathione synthetase homeobox (GSH), Distal-less homeobox (DLX), and Nk2 homeobox (NKX) are solely expressed in the sub-pallium (Anderson *et al.*, 1997b; Pabst *et al.*, 2000; Monuki *et al.*, 2001; Flames *et al.*, 2007). The boundary between the pallium and sub-pallium made by these transcription factors does not strictly correlate with the morphological boundary between these domains. The expression boundary is located ventral to the apparent anatomical sulcus between these two regions (Figure 1.1).

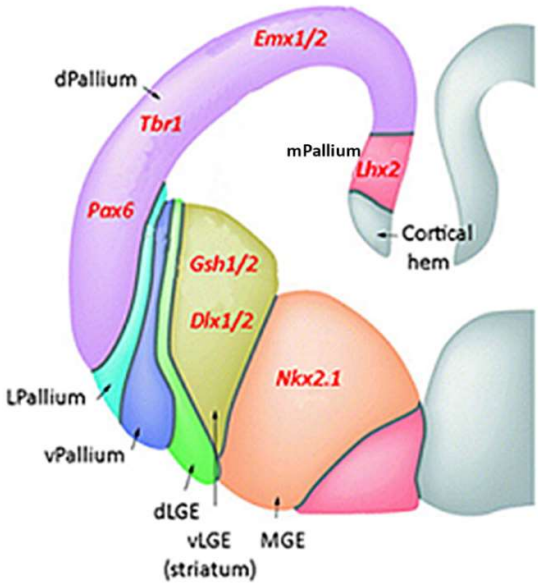

Figure 1.1: The subdivisions of telencephalon. Schematic coronal section showing the subdivisions of telencephalon identified by the expression of certain transcription factors. The pallium is divided into medial, dorsal, lateral, and ventral pallial regions; the sub-pallium is divided into medial and lateral ganglionic eminences (MGE and LGE). Adapted from (Schuurmans and Guillemot, 2002). Abbreviations in this figure legend and those following are given in Table of abbreviation.

The discrete or graded expression of either dorsally or ventrally expressed transcription factors in the pallium or sub-pallium further subdivide these two regions into various progenitor domains (Figure 1.1) that give rise to wide diversity of specific neuronal subtypes. Along the medio-lateral axis, the proliferative zones of pallium are partitioned into four distinct regions (medial, dorsal, lateral, and ventral pallial regions). The medial pallium corresponds to the hippocampal primordium, dorsal pallium gives rise to the neocortex, lateral pallium generates the priform cortex, ventral pallium gives rise to the claustroamygdaloid complex (Campbell, 2003). Similarly, the proliferative zones of the sub-pallium are divided into distinct regions called the ganglionic eminences (GE). Corresponding to their anatomical positions, the GE are known as the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE) and caudal ganglionic eminence (CGE); Gene expression and fate mapping studies have shown that these three sub-pallial domains can be also further subdivided into smaller domains which increases the neuronal diversity produced in these regions (Wonders and Anderson, 2006; Flames *et al.*, 2007). Unlike the pallium, the GE is source of neural types that populate not only the sub-pallial structures (like striatum, globus pallidus, and parts of the amygdala) but also give rise to multiple interneuronal subtypes that tangentially migrate into the cortex and olfactory bulb. The MGE is known as the source of projection neurons of the pallidum but is also the major source of cortical interneurons (Lavdas *et al.*, 1999; Anderson *et al.*, 2001; Butt *et al.*, 2005; Wonders and Anderson, 2006); whereas the LGE is the source of projection neurons of striatum and interneurons of the olfactory bulb (Stenman *et al.*, 2003). Although the CGE is defined only as caudal extensions of the MGE and the LGE, several studies have demonstrated that the CGE has its own identity and is the source of neuronal types that are discrete from those generated in the MGE and the LGE (Nery *et al.*, 2002; Corbin *et al.*, 2003; Wonders and Anderson, 2006; Miyoshi *et al.*, 2010).

1.1.2 The early patterning of the telencephalon

The areal organization (the protomap hypothesis) of telencephalon begins early in the embryonic period and prior to the arrival of thalamic inputs which have also been postulated to contribute to determining the layout of the cerebral cortex and its maturation (Rakic, 1988). Coordinated interactions between intrinsic and extrinsic signals results in dividing the ventral and dorsal telencephalon into molecularly distinct proliferative zones that generate the neuronal diversity in the adult brain (Campbell, 2003; Hébert and Fishell, 2008). Work on rodent

development has identified the presence of certain organizers (signalling centres) that regulate the early pattering of the telencephalon (Figure1.2A). Cells in these centres release soluble morphogens (such as, Wnts, BMPs, FGFs and Shh) that usually act in concentration dependent manner to induce the expression of several specific transcription factors (Figure1.2B) (Monuki *et al.*, 2001; Campbell, 2003; Hébert and Fishell, 2008). In turn, transcription factors appear to control regional expression of secondary signalling molecules, cell adhesion molecules, and cell surface receptors, leading to, not only the formation of the pallial-sub pallial boundary, but also organizing the cerebral cortex into distinct functioning areas (Ericson *et al.*, 1995; Muzio *et al.*, 2002; López-Bendito and Molnár, 2003; O'Leary *et al.*, 2007; Rakic, 2009). Interference in transcription factor expression, for instance in transgenic mice (Figure1.2C) (Bishop *et al.*, 2000; Armentano *et al.*, 2007; O'Leary *et al.*, 2007; Borello *et al.*, 2013) or in intercellular signalling by applying exogenous morphogens (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003; Sahara *et al.*, 2007) leads to expansion or contraction of primary cortical areas. Similarly, the regional identity of the proliferative zones of the ventral telencephalon can be also shifted by manipulation of morphogens or transcription factor expression (Gutin *et al.*, 2006; Lodato *et al.*, 2011).

The ventral and dorsal identities of the telencephalon are regulated by the ventralizing and dorsalizing functions of the secreted signalling protein Shh and the zinc-finger gene Gli3, respectively. The antagonistic interplay between these two signals establishes the early pallial-sub pallial boundary (Ericson *et al.*, 1995; Rallu *et al.*, 2002; Campbell, 2003; Hébert and Fishell, 2008). The source of Shh that is involved in the telencephalic patterning is from the ventral midline of the diencephalon (Ericson *et al.*, 1995). Shh is required for the patterning of the ventral telencephalon by repressing the dorsalizing function of Gli3 and promoting the generation of the ventral cell types (Kohtz *et al.*, 1998; Rallu *et al.*, 2002). Shh induces the expression of NKX2.1 which is known as a characteristic marker for MGE. In Shh-null mice, NKX2.1 expression is lost and MGE fails to develop (Pabst *et al.*, 2000; Rallu *et al.*, 2002). However, the expression of other transcription factors that are expressed in both the MGE and LGE (like Dlx and Gsh2) is conserved in these mutants, suggesting that Shh is only required for patterning the MGE but not LGE (Rallu *et al.*, 2002). Conversely, Gli3 is expressed throughout the dorsal telencephalon and is required to repress the ventralizing signal of Shh; loss of Gli3 function leads to ectopic expression of Gsh2, a characteristic marker of the ventral telencephalon (Rallu *et al.*, 2002). The dorso-ventral patterning of the telencephalon in

Shh;Gli3 double mutants is improved over either that in *Shh* or *Gli3* single mutants (Rallu *et al.*, 2002) suggesting the presence of other signalling pathways (SHH-independent) may also act during the development of telencephalon (Rallu *et al.*, 2002; Campbell, 2003).

Other important signalling centres involved in the patterning of the telencephalon are the cortical hem, anterior neural ridge (ANR)/septum, and the antihem/ventral pallium (VP) (Figure 1.2A) (Furuta *et al.*, 1997; Grove *et al.*, 1998; Monuki *et al.*, 2001; Campbell, 2003). The cortical hem is located in the dorsal midline of the telencephalon (roof plate), it is known as the source of BMP and WNT signalling molecules (Furuta *et al.*, 1997; Grove *et al.*, 1998; Monuki *et al.*, 2001). BMPs and WNTs regulate the expression of several transcription factors like Emx1, Emx2, and Lhx2 that are involved in the development and expansion of the dorsal and medial pallium including the hippocampus (Porter *et al.*, 1997; Monuki *et al.*, 2001; Campbell, 2003). The ANR is known as the source of Fgf signalling, mainly Fgf8, which diffuses caudally in gradient antagonizing the effect of BMPs and Wnts released from the cortical hem (Fukuchi-Shimogori and Grove, 2001; Hébert and Fishell, 2008) suggesting a role of cortical hem and ANR released signalling molecules in patterning the rostro-caudal axis of the cerebral cortex. The antihem is positioned in the ventricular zone of the ventral pallium at the boundary between the LGE and the lateral neocortex. The rodent antihem is marked by expression of the Dbx1, Fgf7, and secretable WNT antagonist secreted frizzled-related protein 2 (SFRP2) (Assimacopoulos *et al.*, 2003; Kawano and Kypta, 2003; Subramanian *et al.*, 2009).

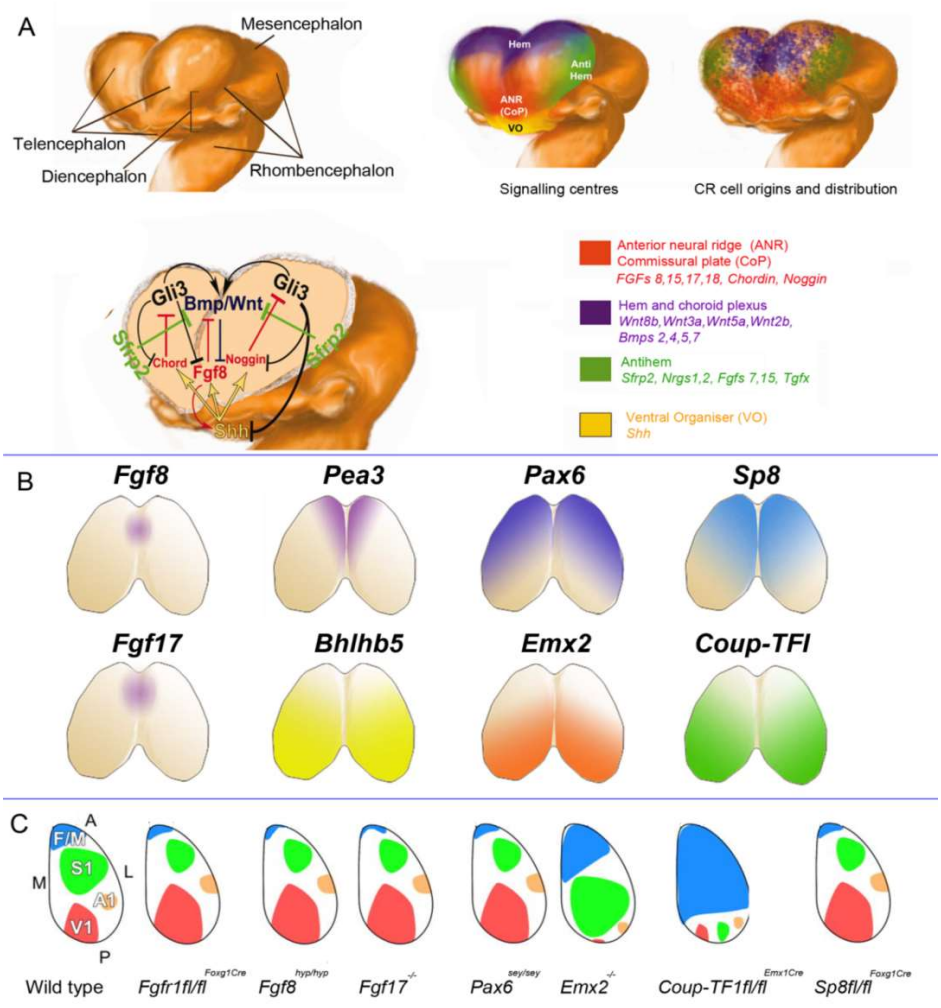

Figure 1.2: Generating the protomap in the rodent cortex. (A) Shows the location of the signalling centres of the forebrain and how Cajal-Retzius (CR) cells derived from these centres distribute across the cortical surface. It also lists the signalling molecules released from each centre and illustrates how these interact with each other. (B) Shows location of expression of Fgf8 and some of its downstream effectors, all of which show high anteromedial expression (Fgf17, Pea3, Sp8) along with transcription factors expressed in an opposing gradient (Coup-TFI, Bhblb5) and Pax6 and Emx2, expressed in opposing anterolateral to posteromedial gradients. (C) Summarises the effects of experiments down-regulating the expression of these morphogens or transcription factors upon the size and location of primary cortical areas, usually identified and delineated in perinatal animals by expression of specific cell adhesion molecules. Adapted from (O'Leary *et al.*, 2007; Alfano and Studer, 2013) .

1.1.3 The neurogenesis in the cerebral cortex

Following the complete closure of the neural tube (CS12, approximately E30), the number of the neural progenitor cells in the neuroepithelium is still far too small to produce the billions of neurons found in the normal human brain. Therefore, between E25 and E42, these progenitor cells undergo a symmetrical mode of cell division, in which two identical neural progenitor cells are produced (Stiles and Jernigan, 2010). When the pool of the neural progenitor cells is adequately expanded, the mode of cell division is shifted from symmetrical to asymmetrical producing one progenitor cell and one neuron for each division. The progenitor cell remains in the proliferative zones (the ventricular and subventricular zones) to go through another round of cell division, while the newly born neuron leaves the ventricular zone (VZ) and move radially into the neocortex (Figure 1.3) (Wodarz and Huttner, 2003). In human, the neurons production begins at approximately E32 in the lateral cortical wall and at E42 in the other parts of cortical wall and continues over the fetal period (Bystron *et al.*, 2008; Stiles and Jernigan, 2010).

As the neurogenesis continues, the first produced neurons migrate from the VZ into the neocortex via somal translocation (Nadarajah and Parnavelas, 2002; Stiles and Jernigan, 2010). However, as the cortex expands in size, the means of migration is changed in order to accommodate the greater distance that should be traversed by the neurons. Special population of cells called the ‘’radial glia cells’’ within the VZ extend basal processes to the pial surface of the brain, these processes provide the guidance scaffold for the neural migration, therefore this mean of migration is called “glia guided” (Figure 1.3) (Bystron *et al.*, 2008; Stiles and Jernigan, 2010). In addition to their role in cell migration, radial glia cells are also neural progenitor cells and divide to produce other progenitors and neurons (Noctor *et al.*, 2001; Noctor *et al.*, 2002). After a certain time of neurogenesis, distinct progenitor cells (“intermediate” progenitors) start to appear in a compartment located above the VZ, this compartment is called the sub-ventricular zone (SVZ). The intermediate progenitors exclusively express TBR2, not expressed in the VZ progenitors, and their symmetrical division produce two neurons. other types of progenitor cells found mainly in the outer sub-ventricular zone (oSVZ) called outer radial glial cells; these cells, unlike ventricular radial glia cells, maintain connection with pial surface only and divide asymmetrically to self-renew, and give

rise to an extended lineage of transit amplifying cells (Figure 1.3) (Bystron et al., 2008; Hansen et al., 2010; Lui et al., 2011).

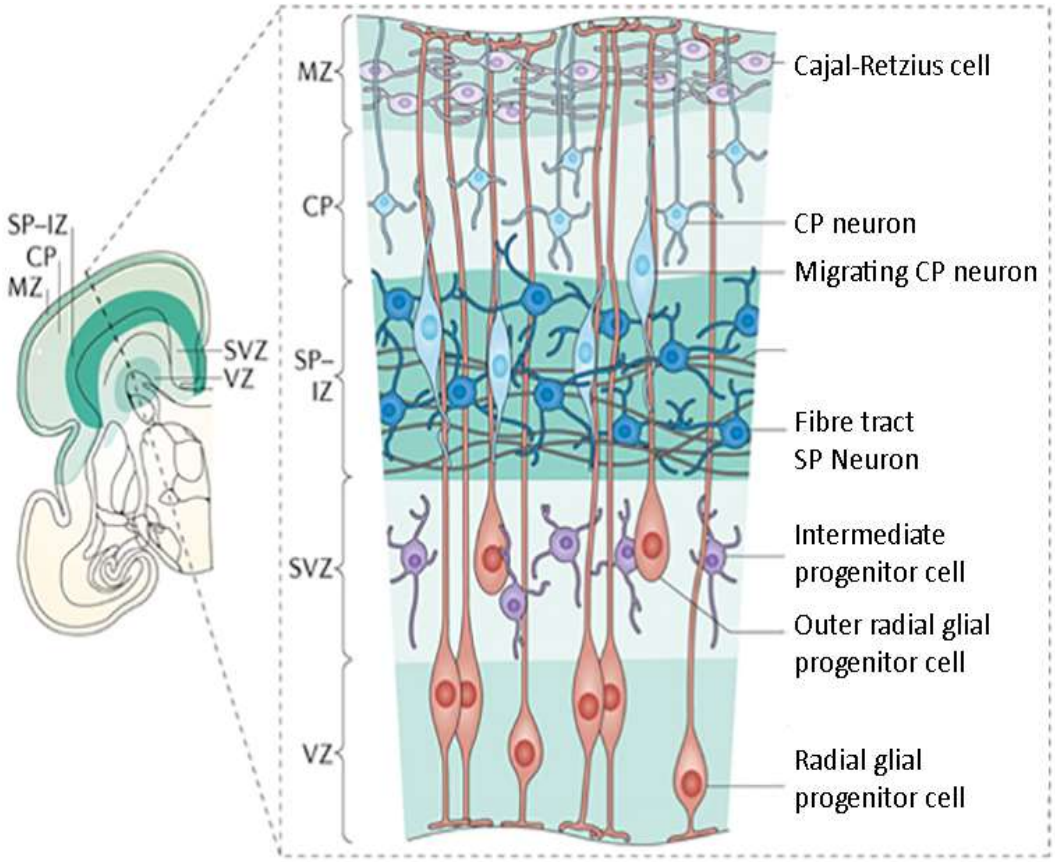

Figure 1.3: A schematic model of neurogenesis in the cerebral cortex. Radial glia cells provide the guidance scaffold for the migration of newly born neurons from ventricular zone (VZ) into the cortical plate (CP). The intermediate progenitors are only found in the sub-ventricular zone (SVZ). The Cajal-Retzius cells in the marginal zone (MZ) play vital roles in neuronal migration and cortical lamination. Cells of the subplate (SP) are important in the formation of cortical connections including the targeting of thalamocortical fibres to the cortex. Adapted from (Hoerder-Suabedissen and Molnár, 2015).

The first batch of neurons to arrive from the ventricular zone form a transitory structure called the preplate (PP) (Figure 1.3), the following arriving neurons split the preplate into two separate transient layers, the marginal zone (MZ) and subplate (SP). These new arrival cells form a new region called the cortical plate (CP) between the MZ and SP (Figure 1.3). Neurons in the CP are arranged in an "inside out" pattern, where first migrating cells occupy the deeper layers and late arriving cells form the superficial layers (Bystron *et al.*, 2008; Stiles and Jernigan, 2010). The marginal zone and SP play important roles in the development of the neocortex and both disappear by the end of the fetal period. Cells in the marginal zone called "Cajal-Retzius cells" release a signalling molecule called reelin which has a vital role in neuronal migration and cortical lamination (Bielle *et al.*, 2005; Huang, 2009). Cells of the SP are known to be important in the formation of cortical connections including the thalamocortical fibres into the cortex (Stiles and Jernigan, 2010). The neurogenesis in the ganglionic eminences and the mode of neural migration (of cortical interneurons) into the neocortex will be discussed below.

1.2 The origin of cortical GABAergic interneurons in mouse

There is general agreement that the MGE and CGE are considered the primary source of cortical GABAergic interneurons (Lavdas *et al.*, 1999; Nery *et al.*, 2002; Butt *et al.*, 2005; Wonders and Anderson, 2006). However, other subcortical structures like the LGE and preoptic area also known to be smaller contributors to the neocortical GABAergic interneuron population (Figure 1.5) (Wichterle *et al.*, 1999; Anderson *et al.*, 2001; Wichterle *et al.*, 2001). In primates, including human, in addition to the ventral telencephalon, a considerable amount of literature has revealed a potential origin of cortical interneurons from the dorsal telencephalon. However, the developmental origin of cortical interneurons in human will be discussed extensively later in this chapter (see 1.5).

1.2.1 Medial ganglionic eminence

The medial ganglionic eminence in rodents is the primary birthplace of GABAergic interneurons for the cortex. It is believed to be the origin of almost 50-60 % of neocortical GABAergic interneurons in rodents (Wonders and Anderson, 2006). The first experimental demonstration of cell migration from the GE to dorsal telencephalon was reported by De Carlos

et al. (1996) through injecting the LGE of live embryos from E12- E14 rat with lipophilic carbocyanine fluorescent tracers DiI and DiA, after two days, many labelled cells were found in the cortex. Anderson *et al.* (1997a) provided evidence that 20% of migrating DiI-labelled cells to the cortex are GABAergic interneurons and express the GABAergic interneuron neurotransmitter GABA. However, these studies did not distinguish whether these cells are derived from the LGE or from elsewhere in the ventral telencephalon.

Subsequent studies provided evidence that most of these cells are originally born in the MGE and they migrate through the LGE into the cortex. One day after the injection of Dil into the MGE of mouse slices (E14), large number of labelled cells were found in LGE and cortex (Wichterle et al., 1999). Similarly, reported that after fluorescent labelling of the MGE in slice cultures from E13-E19 rats, results showed a substantial stream of GABAergic interneurons migrating from the MGE to the cortex passing through the LGE. By comparing the migratory behaviour of MGE and LGE cells, MGE cells have also shown higher tendency to migrate into the cortex (Anderson et al., 2001). These findings were also supported by study on homeobox transcription factor Nkx2.1 (transcription factor that is necessary for the development of MGE). Nkx2.1 mutant mice have shown an almost 50% reduction of GABAergic interneurons in the cortex (Sussel et al., 1999).

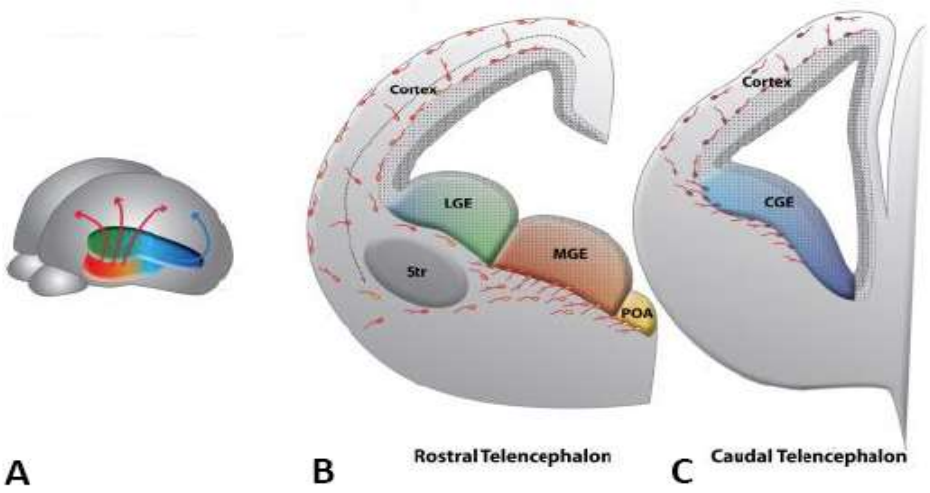

Figure 1.4: The origin of cortical GABAergic interneurons in rodents. (A) Diagram showing the spatial origins of cortical GABAergic interneurons from the MGE (red) LGE (green) POA (yellow) and CGE (blue). (B) Rostral and coronal sections through the telencephalon showing the migratory pathways of interneurons from their origins into the cortex. Adapted from (Laclef and Métin, 2017)

1.2.2 Caudal ganglionic eminence

The CGE is another neurogenic domain in the ventral telencephalon that is considered to be the second greatest contributor to the neocortical GABAergic interneurons. It is the source of almost 30% of all neocortical GABAergic interneurons in rodent (Nery *et al.*, 2002; Wonders and Anderson, 2006). Anatomically, the CGE is considered to be a caudal extension of both MGE and LGE (Fig 1.4). Further, it is a hybrid of MGE and LGE in accordance with the expression of specific transcription factors. The dorsal part (also called dorsal CGE) that protrudes to the lateral ventricle expresses Gsh2 and ER81 which are required for LGE patterning. The ventral part which lies directly opposing to the narrow ventral extension of lateral ventricle, is also called ventral CGE and it expresses the homeobox transcription factor *Nkx2.1* which is required for patterning of the MGE (Corbin *et al.*, 2003; Stenman *et al.*, 2003). By a fate mapping study via *in-vivo* homotopic and heterotopic transplantations of CGE and MGE cells at E13.5 mice, Nery *et al.* (2002) was the first group to show that CGE is a distinct origin of neocortical GABAergic interneurons. Subsequent *in-vivo* and *in-vitro* studies have

confirmed these results and referred to specific morphological and electrophysiological features of CGE–derived interneurons (Xu *et al.*, 2004; Butt *et al.*, 2005; Miyoshi *et al.*, 2010). In addition, the GABAergic interneuron precursor marker COUP-TFII is preferentially expressed in CGE, where it is required to drive CGE-derived interneuron migration into the cortex (Kanatani *et al.*, 2008).

1.2.3 Lateral ganglionic eminence

The LGE has been known as a source of striatal projection neurons and interneurons of the olfactory bulb (Stenman *et al.*, 2003). The possibility that the LGE is also a birthplace of neocortical GABAergic interneurons has been debated. After MGE and CGE, several studies provided evidence that LGE could be far smaller contributor to neocortical GABAergic interneurons (Wichterle *et al.*, 1999; Anderson *et al.*, 2001; Wichterle *et al.*, 2001). In a transplantation study of cell division marker bromodeoxyuridine (BrdU) labelled LGE cells from E 14.5 to E16.5 mice to a host tissue at the same age of development, small numbers of proliferated cells within the LGE migrated to cortex; however, not all migrating cells were also GABA positive (Anderson *et al.*, 2001). As discussed earlier, in a study on Nkx2.1 mutant mice, in which MGE fails to form, these mutants have shown only 50% reduction of GABAergic interneurons in the cortex. While MGE is the primary source of neocortical interneurons, that there was only a 50% reduction raises the possibility that other regions in the brain are also responsible for generating neocortical interneurons (Sussel *et al.*, 1999). Furthermore, cells migration from LGE to the cortex continued when MGE had been removed from explants taken from rat embryos (Jiménez *et al.*, 2002).

1.2.4 Preoptic Area

The preoptic area (POA) is a part of the hypothalamus; it is located immediately adjacent to the MGE in front of optic recess. Contribution of embryonic POA to neocortical GABAergic interneurons has been recently demonstrated by Gelman *et al.* (2009) via in-utero electroporation and fate mapping experiments. Results showed that cellular migration from POA to the cortex does exist, these cells were also found to express GABAergic interneuron

markers. Subsequently, another study showed that multiple subtypes of interneurons are generated from the POA and possibly make up 10% of all neocortical GABAergic interneurons as the third most important source after MGE and CGE (Gelman *et al.*, 2011).

1.3 Interneuronal subtypes

Wide diversity of cortical GABAergic interneurons was described in both developing and adult brains. This variety of interneuronal subtypes is thought to provide the means by which the cortex performs complex functions. However, the classification of GABAergic interneurons is still under debate, for the most part because of overlapping of several interneuronal subtypes. These subtypes are grouped on the basis of the physiological diversity including their synaptic targets and firing patterns. Additional heterogeneities are also provided by the immunohistochemical signature and the morphology for these interneurons (Markram *et al.,* 2004; Rudy *et al.,* 2011). GABAergic interneurons can be categorized into three major groups (Table 1.1) that express the calcium binding protein parvalbumin (Pv), the neuropeptide somatostatin (Sst), and the Serotonin receptor 3a (5-HT3aR) (Lee et al., 2010; Fogarty *et al.,* 2007; Rudy *et al.,* 2011). Transplantation and fate-mapping studies have established that the spatial and temporal origins of interneurons can determine their subtype. The early born Pv+ and Sst+ cortical interneurons are predominantly derived from MGE and migrate tangentially initially into the deep cortical layers of anterior two thirds of the cortex (Wichterle et al., 2001; Wonders and Anderson, 2006) before populating the whole cortex. Conversely, the CGE appears to be the main source of late born cells that express 5-HT3aR, which migrate first to the superficial cortical layers of caudal cortex (Nery et al., 2002; Butt et al., 2005). Further spatial segregation has been also observed within the MGE for the generation of Sst+ interneurons from the dorsal MGE whereas Pv+ interneurons tend to be derived from the ventral MGE (Figure 1.5) (Fogarty et al., 2007; Wonders et al., 2008).

Table 1.1: Cortical interneuronal subtypes and their characteristics in rodents.

Marker and origin	%total GABA+ cells	Morphology	Axonal targeting on projection neurons	Firing pattern	References
Parvalbumin vMGE	~ 40%	Large basket	Proximal dendrites and soma	Fast spiking	Gibson *et al.,* 1999: Cauli *et al.,* 1997: Kawaguchi and Kubota, 1997; Xu and Callaway, 2009; Rudy et al., 2011
		Chandelier	Axonal initial segment	Fast spiking	
Somatostatin dMGE	~30%	Martinotti	Distal dendrites	Bursting	Kawaguchi and Kubota, 1997; Markram et al., 2004; Wang et al., 2004; Uematsu et al., 2008; Rudy et al., 2011
5-HT3aR CGE	~30%	VIP+: Bipolar	Proximal dendrites	Irregular spiking	Cauli et al., 1997; Porter et al., 1998; Cauli et al., 2000; Férézou et al., 2002; Caputi et al., 2009; Lee et al., 2010; Miyoshi et al., 2010; Rudy et al., 2011;
		VIP-: Neurogliaform	Other GABA+ cells	Fast adapting	

1.3.1 Parvalbumin -expressing interneurons

Pv-expressing interneurons represent approximately 40% of all cortical GABAergic interneuron population (Rudy *et al.,* 2011). Physiological and morphological analysis of interneuronal subtypes have indicated that MGE derived Pv-expressing interneurons are mostly fast spiking, characterized by a high-frequency train of action potentials (Gibson *et al.,* 1999; Cauli *et al.,* 1997; Kawaguchi and Kubota, 1997; Xu and Callaway, 2009) and contain two morphological subtypes: basket and chandelier interneurons (Markram *et al.,* 2004;

Helmstaedter *et al.,* 2009; Uematsu *et al.,* 2008). Furthermore, these interneurons possess the lowest input resistance and the fastest membrane time constant of all interneurons (Ascoli *et al.,* 2008; Goldberg *et al.,* 2008; Markram *et al.,* 2004; Gibson *et al.,* 1999; Cauli *et al.,* 1997; Kawaguchi and Kubota, 1997). Basket cells are the most common and comprise almost 50% of all inhibitory interneurons that have multipolar morphology; they tend to make synapses at the soma and proximal dendrites of the target pyramidal neurons (Kawaguchi and Kubota, 1997; Markram *et al.*, 2004; Ascoli *et al.*, 2008). Fast-spiking basket neurons are considered the dominant inhibitory system in the cortex by mediating fast inhibition to target neurons (Rudy *et al.*, 2011; Kelsom and Lu, 2013). Unlike basket cells, chandelier cells are infrequent and target the axon initial segment of pyramidal neurons, therefore they are known as axo-axonic cells (Kawaguchi and Kubota, 1997; Ascoli *et al.*, 2008).

1.3.2 Somatostatin-expressing interneurons

Sst-expressing interneurons make up roughly 30% of all cortical GABAergic interneurons and represent distinct non-overlapping population with Pv-expressing interneurons (Fogarty *et al.,* 2007; Rudy *et al.,* 2011). Sst-expressing interneurons are associated with Martinotti-like morphology, these cells are found in cortical layers II–VI, most abundant in layer V, with ascending axons to layer I where they arborize and make synapses on the apical dendritic tufts of pyramidal neuron (Kawaguchi and Kubota, 1997; Markram *et al.*, 2004; Wang et al., 2004; Uematsu *et al.,* 2008; Rudy *et al.*, 2011). This interneuronal subtype mediate a regular adapting firing pattern, but also fire bursts of two or more spikes when depolarized from depolarized from hyperpolarized potentials (Xu *et al.,* 2013; McGarry *et al.,* 2010; Ma *et al.,* 2006).

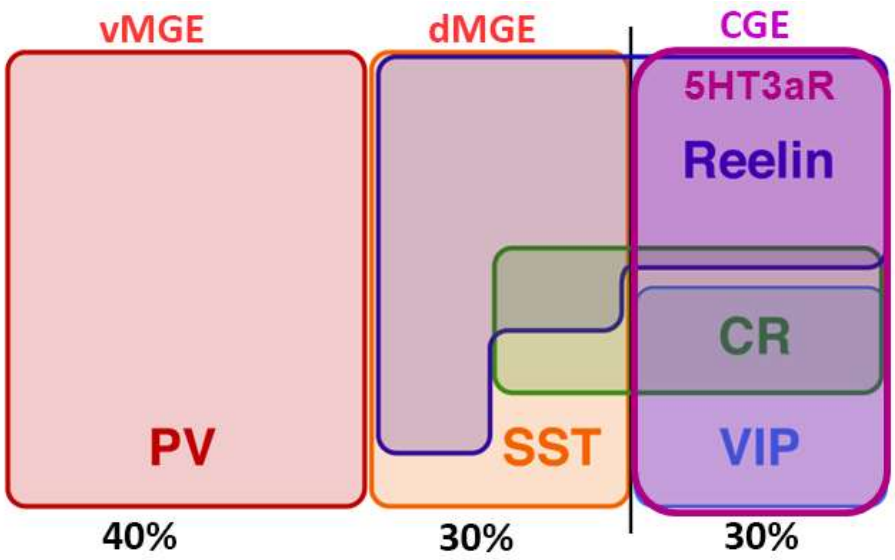

Figure 1.5: Origin of cortical interneuronal subtypes. Schematic diagram demonstrates the molecular expression profiles and developmental origins of three major interneuronal subtypes: Pv-, Sst-, and 5HT3aR- expressing interneurons. PV- and SST-expressing cells represent two non-overlapping populations that are exclusively derived from the Lhx6-expressing MGE lineage (Fogarty et al., 2007). All CGE-derived interneurons express the serotonin receptor 5HT3aR which includes subtypes express reelin, calretinin, and vasoactive intestinal peptide (VIP). Proportion of MGE derived Sst-expressing cells also coexpress Reelin and CR. (adapted from Myioshi et al., 2010).

1.3.3 5-HT3aR-expressing interneurons

The CGE derived 5-HT3aR expressing interneurons comprise 30% of all the cortical GABAergic interneuron population. These cells are grouped into vasoactive intestinal peptide (VIP)-expressing cells (40%) and non-VIP-expressing cells (60%) (Lee et al., 2010; Rudy et al., 2011). Most of VIP-expressing cells also express the calcium binding protein calretinin (CalR) and are mainly associated with a bipolar, or bitufted, morphology (Lee et al., 2010; Miyoshi et al., 2010; Caputi et al., 2009). These cells generally make synapses onto proximal dendrites of target neurons (Lee et al., 2010; Miyoshi et al., 2010; Cauli et al., 2000) and are characterized by an irregular spiking pattern (Cauli et al., 1997; Porter et al., 1998; Cauli et al., 2000; Férézou et al., 2002; Miyoshi et al., 2010). They are vertically oriented and found in layer II-IV with dendrites extending towards layer I and down to layer VI, (Markram *et al.*, 2004). However, the smaller proportions of VIP-expressing cells that do not co-express CalR are known to mediate fast-adapting firing pattern (Lee *et al.,* 2010; Miyoshi *et al.,* 2010).

The majority of non-VIP 5HT3aR-expressing interneurons are reelin-positive. Neurogliaform cells are one type belonging to this group and characterized by multiple dendrites radiating from a round soma. These cells are also known to co-express neuropeptide Y (NPY) (Oláh et al., 2007; Kawaguchi and Kubota, 1997). Neurogliaform cells function by activating slow $GABA_A$ and $GABA_B$ receptors in order to elicit long lasting inhibitory postsynaptic potentials onto pyramidal neurons and other interneurons (Tamás *et al.,* 2003; Oláh *et al.,* 2007;). Furthermore, these cells have a unique feature among other types of interneurons because they make synapses not only with each other but also with other interneuronal subtypes as well, while other interneurons can make synapses with homologous interneurons, thus solidifying their important role in regulating neural circuitry (Price *et al.,* 2005; Simon *et al., 2005*; Zsiros and Maccaferri, 2005).

1.4 Specification of cortical GABAergic interneurons

Large efforts have been made over the last 20 years to determine the genetic regulatory pathways that promote GABAergic interneurons production, migration, and how their functional diversity becomes established. It is now widely recognised that the molecular differences in the neural progenitors of these interneurons have strong implications with regards to their spatial and temporal development, and their specification into diverse subtypes. The neuroepithelium lining of each of the primary origins of cortical GABAergic interneurons (MGE, CGE, and POA) is identified by a distinct transcriptional network, resulting in distinct neuronal fates produced in these regions (Kelsom and Lu, 2013; Kessaris *et al.*, 2014)

1.4.1 Specification of GABAergic interneurons in the MGE

Several transcription factors were identified to play an essential role in regulating the production and specification of MGE-derived GABAergic interneurons (Figure 1.6). At the top of the molecular hierarchy in the MGE is the homeobox transcription factor *Nkx2.1*, which is considered as the key regulator of MGE-derived interneurons specification (Sussel *et al.*, 1999; Xu *et al.*, 2004; Butt *et al.*, 2008; Du *et al.*, 2008). *Nkx2.1* is the only transcription factor that characterizes MGE from other subcortical domains. In early loss of *Nkx2.1* function (Nkx2.1

mutants) the MGE acquires an LGE-like character (Sussel *et al.*, 1999), whereas late conditional loss of function switches MGE to CGE in character (Butt *et al.*, 2008). In contrast to Nkx2.1 null animals which are not viable postnatally, most likely because NKX2.1 is also essential for the organogenesis of lung and thyroid (Kimura et al., 1996), conditionally mutant mice are viable postnatally and show pronounced epileptic seizures and dyskinesia (Butt et al., 2008).

While MGE is the major source of cortical GABAergic interneurons, Nkx2.1 expression in this region is required for the specification of Sst+ and Pv+ interneurons derived from this region (Xu *et al.*, 2004; Butt *et al.*, 2008; Du *et al.*, 2008). Slice cultures in Nkx2.1 mutant mice have shown apparent failure in MGE progenitors to differentiate to Pv and Sst expressing interneurons and a three-fold reduction in their number (Xu *et al.*, 2004; Butt *et al.*, 2008; Du *et al.*, 2008). Significant increase in the production of VIP/CalR CGE- derived interneurons was observed in these mutants which suggested as a compensation process for the loss of Pv and Sst MGE- derived interneurons (Xu *et al.*, 2004; Butt *et al.*, 2008). However, rescued expression of Nkx2.1 (electroporation of Nkx2.1 cDNA) into the ventral telencephalon of slice cultures from Nkx2.1 mutants can rescue the loss of these interneuronal subtypes (Du *et al.*, 2008). Although NKX2.1 is broadly expressed through the MGE, it has been shown that the VZ of the MGE can be further subdivided into distinct progenitor domains which could encode the neuronal diversity produced in this region (Flame *et al.,* 2007; Flandin *et al.,* 2010); for example, Nkx6.2 is expressed in a restricted spatial pattern at the MGE/LGE boundary (interganglionic sulcus) and at the most dorsal aspect of the Nkx2-1-positive region in the MGE (Stenman *et al.,* 2003; Flame *et al.,* 2007). Nkx6.2+ domain has been shown to give rise to both Pv and Sst-expressing interneurons and in particular be enriched in a subpopulation of Sst/CalR expressing interneurons (Fogarty et al., 2007; Sousa *et al.,* 2009).

A second transcription factor which is downstream of Nkx2.1 is Lhx6. Nkx2.1 appears to directly induce the expression of Lhx6 which is required for specification of Pv+ and Sst+ MGE-derived interneurons by supressing the CGE-like identity in MGE cells (Liodis *et al.*, 2007; Du *et al.*, 2008; Vogt *et al.*, 2014). Lhx6 activity is also required for migration of these interneuronal subtypes from MGE to the cortex (Liodis *et al.*, 2007; Zhao *et al.*, 2008; Flandin *et al.*, 2011). While Nkx2.1 expression is downregulated as interneurons migrate out of MGE (Marín *et al.*, 2000), the onset of Lhx6 expression appears in these interneurons around the

time of their final cell cycle and as they exit the proliferative zone through to their maturity in the developing cortex (Lavdas *et al.*, 1999; Liodis *et al.*, 2007). Most interneurons in Lhx6 mutant mice failed to integrate into their appropriate cortical layer (Zhao *et al.*, 2008).

One factor that appears to be upstream of Nkx2.1 is Shh (Xu *et al.*, 2005; Xu *et al.*, 2010). Three crucial actions of Shh signalling were identified. First, at an early stage of neural development, Shh signalling is required for establishment of dorsoventral patterning in the telencephalon, as mentioned above, Shh signalling initiates the patterning of ventral telencephalon by promoting the induction of ventral transcription factors and repressing of Gli3 function (Gunhaga *et al.*, 2000; Rallu *et al.*, 2002; Fuccillo *et al.*, 2004). Second, Shh is also required for expanding the numbers of neuronal progenitors in ventral telencephalon (Machold *et al.*, 2003; Xu *et al.*, 2005). Third, it has become apparent that Shh signalling is required for maintaining Nkx2.1 expression in MGE, thereby, Pv+ and Sst + interneurons fate determination in this region (Xu *et al.*, 2005; Xu *et al.*, 2010). The concentration gradient of Shh signalling across the MGE has also been shown to preferentially promote one specific subtype over another, a higher level of Shh signalling in dorsal MGE promote the generation of Sst+ interneurons while the lower level in ventral MGE promotes the generation of PV+ interneurons (Xu *et al.*, 2010). Shh- mutant mice at embryonic day E 12.5 have shown reduced Nkx2.1 expression in MGE and pronounced reduction in Nkx2.1- dependent Pv+ and Sst+ interneurons in the postnatal cortex. In the same study exogenous Shh rescued the loss of Nkx2.1 expression and interneuron fate effects in slices from these mutants (Xu *et al.*, 2005). In addition to reduction in Nkx2.1 expression, downregulation of Shh signaling in MGE results in upregulation of Gsh2, a transcription factor enriched in CGE and contributes in production of bipolar CalR+ interneurons, which means that loss of Shh signaling leads to conversion of MGE-derived interneurons fate from Pv+ and Sst+ interneurons to CalR+ interneuron which are normally derived from CGE (Butt *et al.*, 2005; Xu *et al.*, 2010).

Lhx8 and Sox6 are other transcription factors that lie downstream of Nkx2.1 and have roles in the genetic regulatory pathway of interneuron production and migration in the MGE. Although the precise roles of Lhx8 in the specification of cortical MGE-derived interneurons need to be better delineated, it is known to work in conjunction with Lhx6 (Zhao *et al.*, 2003; Flandin *et al.*, 2011). In a study of Lhx8/Lhx6 double mutants (but not single mutants) there was reduction

in tangentially migrated interneurons to the cortex. The same study has also indicated that both Lhx8 and Lhx6 are important in promoting the expression of Shh signal in MGE (Flandin *et al.*, 2011). However, Lhx8 single mutants analysis has shown that Lhx8 plays a major role in the development of striatal interneurons and minor role in the development of GABAergic cortical interneurons (Zhao *et al.*, 2003). Sox6 acts downstream of Lhx6; it is primarily expressed in postmitotic MGE cells (Zhao *et al.*, 2003; Liodis *et al.*, 2007; Batista-Brito *et al.*, 2009) and mainly required for normal positioning and maturation, but not the specification, of MGE-derived interneurons (Azim *et al.*, 2009; Batista-Brito *et al.*, 2009).

The Dlx family of homeobox genes, mainly Dlx1/2, are believed to be functioning at the top of the genetic cascade of interneuron development, not only in the MGE, but also in the other subcortical regions (Anderson *et al.*, 1997a; Cobos *et al.*, 2005; Long *et al.*, 2009). Dlx1/2 are required to induce expression of several transcription factors in MGE progenitors as well as proteins involved in migration and integrations of MGE-derived interneurons into the cortex (Long *et al.*, 2009). For example, Dlx1/2 are required for the generation of interneuron precursors in the MGE and their migration to the cortex (Anderson *et al.*, 1997a), and are particularly involved in specification of Sst/CalR expressing interneurons (Xu *et al.*, 2004; Cobos *et al.*, 2005). Dlx1 is also essential for the preserving of functional interneurons in the cortex of adult brain (Cobos *et al.*, 2005). Additionally, Dlx1/2 are required to induce the expression of Dlx5/6 in the ventral telencephalon (Liu *et al.*, 1997), which are required for interneuron migration and differentiation, but not their generation; they are particularly required for the differentiation of Pv expressing interneurons (Wang *et al.*, 2010). The Arx homeobox transcription factor is highly expressed in the GE, it appears to be downstream of Dlx genes, and is required for interneurons migration from the ganglionic eminence to the cortex (Kitamura *et al.*, 2002). However, recent study has shown that Arx is direct downstream target for Lhx6 in MGE-derived interneurons, where it is required to determine their fate and laminar position in the cortex (Vogt *et al.*, 2014).

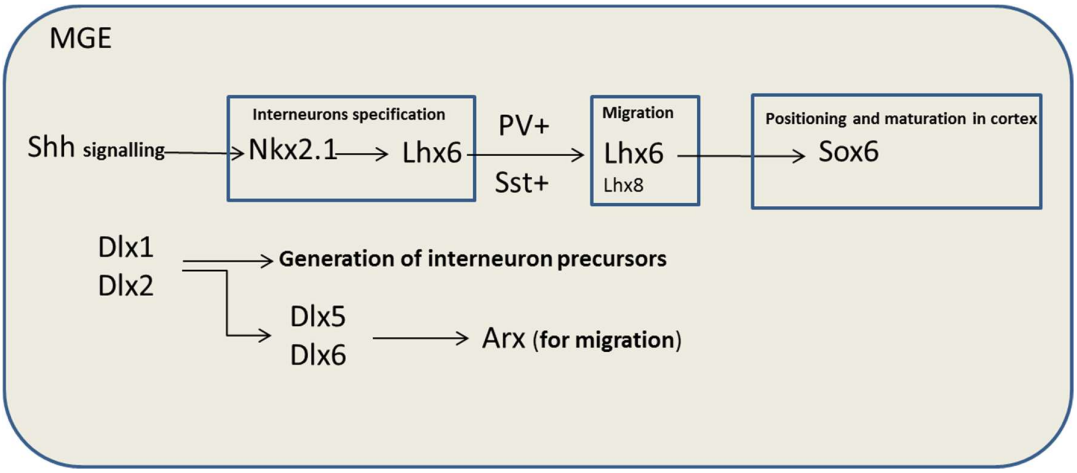

Figure 1.6: Production of cortical interneurons in the MGE. Adapted from (Kelsom and Lu, 2013).

1.4.2 Specification of GABAergic interneurons in the CGE

The CGE is a source of interneuronal subtypes that are different from those generated in the MGE; it is believed to be the main source for CalR and/or VIP- expressing interneurons (Xu *et al.*, 2004; Butt *et al.*, 2005; Fogarty *et al.*, 2007). The CGE is also known to be molecularly distinct, expressing separate set of transcription factors that regulate the interneurongenesis in this domain (Figure 1.7)**.** In addition to their role in production of striatal projection neurons and olfactory bulb interneurons from LGE (Stenman *et al.*, 2003) the homeobox transcription factor Gsx2 is at the top of the hierarchy governing the production and specification of CGE-derived cortical GABAergic interneurons (Xu *et al.*, 2010). It is also considered among the earliest transcription factors expressed within the LGE and CGE progenitors, but weakly expressed in MGE (Corbin *et al.*, 2003). Conditional null mutants of this transcription factor, where Gsx2 expression is eliminated in the majority of telencephalic progenitors, have shown dramatic loss of bipolar CalR-expressing interneurons (Xu *et al.*, 2010). A related gene is Gsx1; although they are co-expressed, the Gsx1/2 have been known to have antagonist functions, while Gsx2 maintains the undifferentiated state of progenitors, Gsx1 promotes the progenitors'

maturation and differentiation; however, downstream targets of Gsx1/2 seem to be involved in this process. (Pei *et al.*, 2011).

One of the well-known targets of Gsx genes is Ascl1 (also known as Mash1) (Wang *et al.*, 2009). Ascl1 is proneural basic helix-loop-helix transcription factors expressed in the progenitor zones of the ventral telencephalon, together with Dlx1/2 have a complementary role in regulating notch signalling, thereby controlling the temporal neurogenesis in the ventral telencephalon (Casarosa *et al.*, 1999; Marín *et al.*, 2000; Yun *et al.*, 2002). While Ascl1 is required for early neurogenesis in the ventral telencephalon (Casarosa *et al.*, 1999; Horton *et al.*, 1999). Dlx1/2 which are located downstream of both Gsx2/ Ascl1 are required to downregulate these two transcription factors to promote the differentiation of late born neurons (Anderson *et al.*, 1997b; Marín *et al.*, 2000; Yun *et al.*, 2002). Ascl1 promotes the expression of Notch Ligand Delta 1 and mediates lateral inhibition via Notch signalling pathway thus prevents precocious differentiation of neural progenitors (Casarosa *et al.*, 1999; Horton *et al.*, 1999; Yun *et al.*, 2002). Ascl1 mutants have shown a reduction in early- born neurons in the ventral telencephalon (E10.5) (Casarosa *et al.*, 1999; Yun *et al.*, 2002). These mutants have also shown molecular defects in both the proliferative and postmitotic zones, the presence of these defects suggests an accelerated differentiation and thereby production of neurons prematurely (Fode *et al.*, 2000; Yun *et al.*, 2002). Dlx1/Dlx2 mutants have shown defects in the late- born neurons in the ventral telencephalon (E12.5), which was attributed to the expanding of Notch signaling domain to SVZ, where Dlx1 and Dlx2 play crucial roles in down-regulating the Notch signaling and promoting the terminal differentiation of these later subset of neural progenitors (Marín *et al.*, 2000; Yun *et al.*, 2002). One more piece of evidence of interaction of Dlx1/2 with Ascl1 is that Dlx1/2 expression is up-regulated in most VZ cells in Ascl1 mutant ventral telencephalon (Casarosa *et al.*, 1999).

The nuclear receptors COUP-TFI and COUP-TFII (also known as Nrf2f1 and Nrf2f2, respectively) are important transcription factors that play essential roles in CGE-derived interneurons specification and migration. Both COUP-TFI and COUP-TFII are preferentially expressed in the CGE as well as in the migrating interneurons into the cortex (Kanatani *et al.*, 2008; Lodato *et al.*, 2011). CGE-derived interneurons preferentially migrate caudally to the most caudal part of telencephalon (Yozu *et al.*, 2005). COUP-TFII is essential to establish this caudal migratory stream of these interneurons (Kanatani *et al.*, 2008). COUP-TFI is required

to maintain the balance of generation of different interneuronal subtypes from MGE and CGE by regulating the rate of proliferation of progenitor cells in CGE. Conditional inactivation of COUP-TFI leads to significant decrease in the number of CGE-derived CalR/VIP interneurons (Lodato *et al.*, 2011). Another transcription factor, Prox1, is specifically important for migration and differentiation, but not production, of CGE-derived interneurons in rodents (Miyoshi *et al.*, 2015). However, the precise roles of COUP-TFs and Prox1 transcription factors in specification the CGE-derived interneurons are still unclear.

SP8 is another transcription factor that is widely expressed in the ventral and dorsal telencephalon in mouse (Waclaw et al., 2006; Sahara et al., 2007; Waclaw et al., 2010; Ma et al., 2012; Borello et al., 2013) it is a member of the Sp1 zinc finger transcription factor family (Bell et al. 2003; Treichel et al. 2003). Its expression in the ventral telencephalon regulates differentiation of LGE-derived interneurons that populate the amygdala and the olfactory bulb via the rostral migratory stream (Waclaw et al., 2006; Waclaw et al., 2010). However, Sp8 is also expressed in subpopulation of dorsal LGE/CGE-derived cortical interneurons that preferentially occupy superficial cortical layers (Ma et al., 2012). Its expression in the dorsal telencephalon regulates the cortical patterning where it is required to establish the rostral identity of the cerebral cortex (Sahara et al., 2007; Zembrzycki et al., 2007).

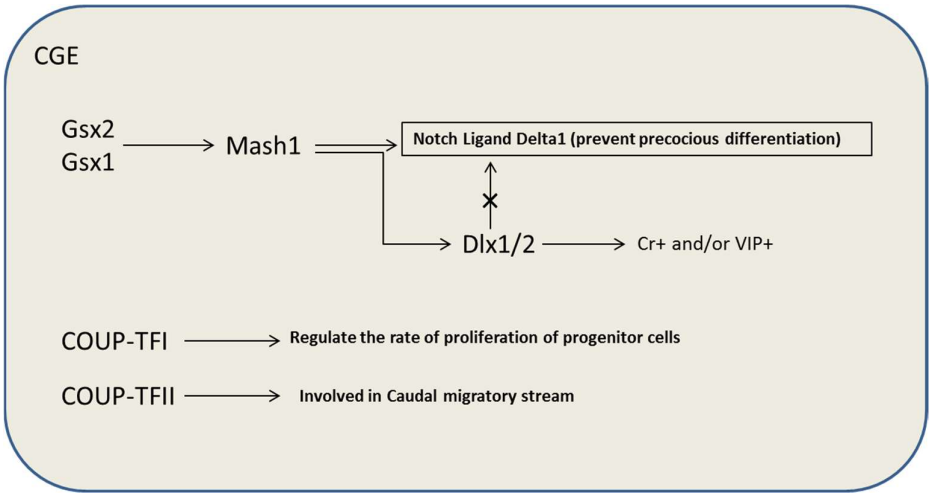

Figure 1.7: Production of cortical interneurons in the CGE. Adapted from (Kelsom and Lu, 2013).

1.4.3 Specification of GABAergic interneurons in the POA

The network of transcription factors that regulates the production of GABAergic interneurons in POA is not well defined yet (Figure 1.8). It is located immediately adjacent to the MGE and nearly all precursor cells in POA express Nkx2.1 but not its downstream effector Lhx6 (Flames *et al.*, 2007). Thus, the POA seems to be molecularly distinct from the MGE. In addition, transcription factors such as Dbx1, Nkx6.2, Nkx5.1, and Shh morphogen are expressed in POA progenitors but not in MGE progenitors (Gelman *et al.*, 2009). In a fate mapping and in utero transplantation study, using Cre line mice under the control of Nkx5.1 (exclusively expressed in POA), it has been shown that POA is a birthplace of cortical interneurons that express NPY and/or reelin. Neither of these cells co-express Sst, suggesting that NPY+/Sst- and Reelin+/Sst- interneurons are derived from both CGE and POA (Gelman *et al.*, 2009). In addition to Nkx5.1-derived cortical interneurons, it has been reported that another population of interneurons are derived from Dbx1 expressing progenitor cells. Diverse classes of interneurons are derived from this domain (Gelman *et al.*, 2011). Despite these findings, the network of transcription factors that plays role in specification of POA-derived interneurons remains unclear.

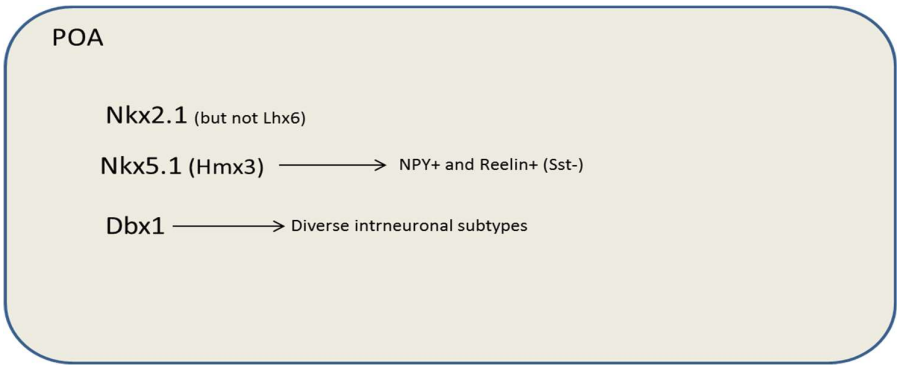

Figure 1.8: Production of cortical interneurons the POA. Adapted from (Kelsom and Lu, 2013).

1.5 The generation of cortical GABAergic interneurons in human fetal brain

Due to the availability of transgenic models, the neocortical development has been widely studied in rodents; however, it is less recognized to what degree the developmental rules in human can match those found in our experimental models which have a less complex and shorter developmental course (Rakic, 2009). Humans have considerably expanded cognitive abilities compared to all other species, which may be dependent on the remarkable increase in the number of neurons and cortical connections (DeFelipe, 2011; Buckner and Krienen, 2013). With no correlate in rodents, humans have expanded higher order associative areas which cover a large part of the human cortical surface (Uylings and van Eden, 1991), the deep layer III pyramidal neurons in these associative areas form crucial elements for substantial number of connections with other cortical areas (Barbas *et al.*, 2005; Yeterian *et al.*, 2012) which are important for higher cognitive abilities (Selemon *et al.*, 2003; Wang *et al.*, 2006; Verduzco-Flores *et al.*, 2009). The tremendous increase in cortical volume has been linked to the expanded outer subventricular zone (OSVZ) of the developing cortical wall in human (Rakic, 2009; Geschwind and Rakic, 2013; Sousa *et al.*, 2017).

The generation of interneurons may be also more complicated in primates, which have evolved an expanded OSVZ in the ganglionic eminences (Hansen *et al.*, 2013a). Proportionally more interneurons appear to be produced in the CGE, the majority of which populate the superficial layers of the cortex (Nery *et al.,* 2002; Butt *et al.*, 2005, Hansen *et al.*, 2013a; Ma *et al.*, 2013). Furthermore, several studies have reported that during the early stage of development, cortical GABAergic interneurons in primate, like in rodents, are mainly generated in the GE; whereas in the second trimester (15–24 PCW in human and E64–E75 in macaque monkey) the proliferative zone of the dorsal telencephalon could also contribute to a proportion of cortical GABAergic interneurons (Letinic *et al.*, 2002; Petanjek *et al.*, 2009a; Zecevic *et al.*, 2011; Radonjić *et al.*, 2014a). Unlike rodents, where Pv-expressing interneurons are the dominant subtype, the proportion of CalR-expressing interneurons increased three fold in human, where they become the dominant subtype and increase the proportion of cortical neurons that are interneurons by 50% in primate compared to the rodents (Condé *et al.*, 1994; Gabbott *et al.*, 1997; Zaitsev *et al.*, 2005; Barinka and Druga, 2010; Hladnik *et al.*, 2014). Almost half of all these CalR interneurons are found in the frontal associative area (Hladnik *et al.*, 2014). The distant anatomical positions of frontal associative area in the frontal lobe and the posteriorly

positioned CGE (which is the major source of CalR-expressing interneurons) suggest that proportion of these subtypes could be generated intra-cortically (Hladnik *et al.*, 2014).

The first indication for generation of cortical interneurons in the proliferative zone of the dorsal telencephalon was demonstrated by Letinic *et al.* (2002) using retroviral labelling of Dlx2 and Ascl1 expressing progenitors in slice cultures of human fetal brain. This study proposed the presence of two distinct sources of neocortical GABAergic interneurons; 65% of all cortical GABAergic interneurons are generated in the proliferative zone of the dorsal telencephalon, and only 35% are generated in the proliferative zone the ventral telencephalon. Similar findings were also reported in a study on the macaque monkey, suggesting that this can also occur in other primates (Petanjek *et al.*, 2009b). These two groups proposed that the generation of cortical GABAergic interneurons takes place in distinctive temporal profiles. For example, at earlier stages of development (10-13 PCW) in human and (E47-E55) in macaque monkey, the GE of ventral telencephalon is the birthplace of all cortical GABAergic interneurons. At later stages of development (15–24 PCW) in human and (E64-E75) in macaque monkey, in addition to the ventral telencephalon, cortical GABAergic interneurons are massively generated in the ventricular zone of the cortex (Letinic *et al.*, 2002; Petanjek *et al.*, 2009b). The presence of CalR+ interneurons that are double labelled with KI67 (cell division marker) in the VZ/SVZ of dorsal telencephalon of 20 PCW human fetal brain also supports these findings (Zecevic *et al.*, 2011). Moreover, subsequent studies have confirmed the presence of proliferative interneurons progenitor cells that express interneuronal markers, such as ASCL1, DLX2, and NKX2.1 in proliferative zone of dorsal human telencephalon which suggests the cortical origin of GABAergic interneurons (Radonjić *et al.*, 2014a; Al-Jaberi *et al.*, 2015).

Despite of all these findings, other groups have argued that interneuronogenesis in primate fetal brains is essentially the same as in rodent. In studies in human and monkey, they reported that all proliferative interneurons progenitor cells found in the cortex are originally derived in the GE but may retain proliferative capacity after migrating into the cortex (Hansen *et al.*, 2013a; Ma *et al.*, 2013). Furthermore, as there is general agreement that most of the calretinin-expressing interneurons in rodents are derived from the CGE (Nery *et al.*, 2002; Butt *et al.*, 2005), Hansen *et al.* (2013a) reported that the CGE in human generates a greater percentage of interneurons than in rodents, which could give explanation for higher prevalence of calretinin-

expressing interneurons in adult human brain than in rodents. However, the possibility of dorsal origins for GABAergic interneurons is still divisive and needs more investigation.

1.6 Aims of the study

The aims of this project were to:

- Identify the expression patterns of specific transcription factors expressed in GABAergic interneuron precursors in the ventral telencephalon and the cortex of 8–12 PCW human fetal brain (Chapters 3 and 4).

- Reveal the complex organization for the GE and septum into distinct neurogenic domains (Chapters 3 and 4).

- Demonstrate the distinct migration pathways, identified by the expression of specific transcription factors, of GABAergic interneurons from the ventral telencephalon into the cortex (Chapters 3 and 4).

- Explore the extent to which expression of COUP-TFs and SP8 the in the human forebrain mirrors that in the rodent models (Chapter 4).

- Explore the potentially expanded origins of GABAergic interneurons in human fetal brain (Chapters 3 and 5).

- Investigate the gradient of "GABAergic" genes expression in human fetal cortex. (Chapters 4 and 5).

- Explore the effect of exogenous sonic hedgehog (Shh) treatment on cortical cell cultures (Chapter 5).

Chapter 2: Materials and Methods

2.1 Human Fetal Brains and Ethical approval

Human fetal tissue from terminated pregnancies was obtained from the joint MRC/Wellcome Trust-funded Human Developmental Biology Resource (HDBR, http://www.hdbr.org; Gerrelli et al. 2015) based in Newcastle, UK. All tissue was collected with appropriate maternal consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee (REC reference 08/H0906/21+5). HDBR Newcastle is licensed as a tissue bank as part of Newcastle Biobanks (http://www.ncl.ac.uk/biobanks/) which is licensed by the UK Human Tissue Authority (licence number 12534). Fetal samples ranging in age from 8 to 12 PCW were used in this study. Ages were estimated from foot, and heel to knee length measurements according to Hern (1984).

2.2 Tissue Processing and Sectioning

Processing and sectioning of embryonic and foetal material for paraffin sections was performed by the HDBR Newcastle staff. Brains were isolated and fixed for at least 24 h at 4^{o}C in 4% paraformaldehyde dissolved in 0.1 M phosphate- buffered saline (PBS) (PFA; Sigma Aldrich). Once fixed, whole or half brains (divided sagittally) were dehydrated in graded ethanols (70% for 15 minutes, 100% for 45 minutes, 2 x 100% for 1 hour) at room temperature. The fixed brains were dissected into blocks of approximately equal size, with the number depending on the size of the brain. Blocks were then incubated in xylene (2 h) before embedding in paraffin (Shandon Pathcentre Tissue Processor, Thermo Scientific, Epsom, UK). Brain tissue blocks were cut at 8 um section thickness (Leica RM 2235 microtome) in three different planes; horizontally, sagittally, and coronally (Table 2.1), mounted on slides and used for haematoxylin and eosin staining (H&E) and immunostaining.

Table 2.1: Details of the human fetal samples used in this study for IHC and cell culture.

Brain No.	HDBR Sample ID Number	Age	Application	Sectioning Orientation
1	12506	8 PCW	IHC	Sagittal
2	11872	8 PCW	IHC	Horizontal
3	1975	8 PCW	IHC	Horizontal
4	11577	8 PCW	IHC	Horizontal
5	12294	8 PCW	IHC	Sagittal
6	13107	8 PCW	IHC	Sagittal
7	12294	8 PCW	Cell culture	-
8	13254	9 PCW	Cell culture	-
9	13642	9 PCW	Cell culture	-
10	12721	10 PCW	IHC	Horizontal
11	13136	10 PCW	IHC	Coronal
12	13081	10 PCW	IHC	Coronal
13	13168	10 PCW	Cell culture	-
14	13183	11 PCW	Cell culture	-
15	13405	11 PCW	Cell culture	-
16	13854	11 PCW	Cell culture	-
17	11610	12 PCW	IHC	Sagittal
18	11761	12 PCW	IHC	Coronal
19	11523	12 PCW	IHC	Coronal
20	11795	12 PCW	IHC	Sagittal

Note: the brain numbers in column 1 have been given in the figure legends in chapters 2-5 to identify the specific fetal sample used to illustrate methods or in the experiments described**.**

2.3 Haematoxylin & eosin histological staining

Paraffin sections were dewaxed in xylene for 5 minutes and rehydrated in a series of ethanol dilutions (100%, 100%, 95%, 70%). Sections were rinsed in tap water and placed into Harris' Haematoxylin solution (Raymond A Lamb Ltd., Eastbourne, UK) for 1 minute and rinsed in tap water afterward. The nuclei of cells were 'blued' in Scots tap water substitute (3.5g sodium bicarbonate, 20g magnesium sulphate, 1L distilled water (Sigma Aldrich), placed in eosin (1% aqueous, Raymond A Lamb Ltd) for 10 seconds to stain the cytoplasm and rinsed in tap water. Sections were then dehydrated by serial dilutions of ethanol (70%, 95% and 100%), immersed in two changes of xylene and mounted using DPX (Sigma-Aldrich, Poole, UK).

2.4 Immunohistochemistry (IHC)

2.4.1 Immunoperoxidase histochemistry

Paraffin sections were dewaxed by treatment with two changes of xylene for 5 minutes each and rehydrated via four changes of graded ethanol (100%, 100%, 95%, and 70%). Endogenous peroxidase activity was blocked by treatment with methanol peroxide (3ml hydrogen peroxide, Sigma Aldrich, 180 ml methanol) for 10 minutes. Sections were rinsed in tap water and boiled by microwave treatment in 10mM citrate buffer pH6 (Table 2.2) for antigen retrieval for 10 minutes. Sections were then incubated with the appropriate normal 10% blocking serum (species in which secondary antibody was raised, Vector Labs) in Tris buffered saline (TBS; Table 2.2) for 10 min at room temperature before incubation with the primary antibody (diluted in 10% normal blocking serum) overnight at 4 ^{o}C. Details of all the primary antibodies used in this study are found in Table 2.3. Then, sections were washed and incubated with the biotinylated secondary antibody for 30 minutes at room temperature (Vector Laboratories Ltd., Peterborough, UK) at 1:500 dilution in 10% normal serum in TBS followed by washing and incubation with avidin-peroxidase for 30 minutes (ABC-HRP, Vector Labs). The sections were developed with diaminobenzidine (DAB) solution for 10 minutes (Vector Labs) washed, dehydrated and mounted using DPX (Sigma-Aldrich). Positive signal is indicated by the brown stain.

Table 2.2: Components of commonly used solutions.

Solution	Components	pH	Concentration
TBS	8.75g NaCl 6.50g Trizma base 800ml distilled H2O	7.5	-
Citrate Buffer	5.88g of (C6H5Na3O7)2H2O tri-sodium citrate 2L distilled H20	6	0.1M

2.4.2 Immunofluorescence (double and triple labelling)

We used a novel immunofluorescent staining method, Tyramide Signal Amplification (TSA) that permits sequential double and triple staining using antibodies from the same species without cross-reactions (Goto *et al.*, 2015; Harkin *et al.*, 2016). Sections were treated as described above until the secondary antibody stage, then they were incubated with HRP-conjugated secondary antibody for 30 minutes (ImmPRESS™ HRP IgG [Peroxidase] Polymer Detection Kit, Vector Labs) washed twice for 5 minutes in TBS and incubated in the dark for 10 minutes with fluorescein tyramide diluted at 1/500 in 1X Amplification buffer (Tyramide Signal Amplification (TSA™) fluorescein plus system reagent, Perkin Elmer, Buckingham, UK). Tyramide reacts with HRP to leave fluorescent tags covalently bound to the section.

Prior to starting the second round of staining, sections were first washed in TBS and boiled in 10mM citrate buffer to remove all antibodies and unbound fluorescein from the first round. Sections were then incubated in 10% normal serum before incubating with the second primary antibody (Table 2.3) for 2 hours at room temperature. Following washing, sections were again incubated with ready to use HRP-conjugated secondary antibody then incubated with CY3 tyramide for 10 minutes (Tyramide Signal Amplification [TSA™] CY3 plus system reagent, Perkin Elmer). The same steps were repeated for the third round of staining (if triple labelling was needed) using CY5 Tyramide (Tyramide Signal Amplification (TSA™) CY5 plus system reagent, Perkin Elmer). Sections were washed before applying 4',6-diamidino-2-phenylindole

dihydrochloride (DAPI; Thermo Fisher Scientific, Cramlington, UK) and mounted using Vectashield Hardset Mounting Medium (Vector Labs).

Table 2.3: Details of all primary antibodies used in the study.

Primary antibody	Species	Dilution	Supplier	RRID number
KI67	Mouse monoclonal	1/150	Dako, Ely, UK.	AB_2142378
TBR1	Rabbit polyclonal	1/1000	Abcam, Cambridge, UK.	AB_2200219
TBR2	Rabbit polyclonal	1/200	Abcam	AB_778267
PAX6	Rabbit polyclonal	1/500	Cambridge Bioscience, Cambridge, UK.	AB_2565003
NKX2.1	Mouse monoclonal	1/150	Dako	Not available
SOX6	Rabbit polyclonal	1/3000	Abcam	AB_1143033
COUP-TFI	Mouse monoclonal	1/1500	Abcam	AB_742210
COUPT-FII	Mouse monoclonal	1/500	R&D Systems, Abingdon, UK.	AB_2155627
OLIG2	Rabbit polyclonal	1/1000	Merck Millipore, Watford, UK.	AB_10141047
CalR	Mouse monoclonal	1/2000	Swant, Marly, Switzerland.	Not available
Calbindin	Rabbit polyclonal	1/1000	Swant	AB_10000340
GAD67	Mouse polyclonal	1/1000	Merck Millipore.	AB_2278725
SP8	Goat polyclonal	1/500	Santa Cruz, Heidelberg, Germany.	AB_2194626
GABA	Rabbit polyclonal	1/400	Sigma-Aldrich, Poole, UK.	AB_477652
GFAP	Rabbit polyclonal	1/500	Abcam	AB_305808
β-tubulin III	Mouse monoclonal	1/300	Sigma-Aldrich	AB_477590
β-tubulin III	Rabbit polyclonal	1/300	Abcam	AB_444319
OLIG2	Rabbit polyclonal	1/1000	Merck Millipore	

2.5 RNA Sequencing

Apart from the data analysis and interpretation reported in this thesis, this work was carried out as part of another study (Lindsay *et al.*, 2016) (http://www.hdbr.org/expression/). The processes involved and the people who undertook the work are briefly outlined below.

2.5.1 Brain dissections (HDBR staff, Dr Lauren Harkin and Dr Nadhim Bayatti)

Whole fetal brains were isolated from the skull and the meninges were removed. The hemispheres were separated, and the choroid plexus and subcortical structures removed. One or both hemispheres (each hemisphere represented an independent sample) was then divided into 6 blocks. The temporal lobe, including lateral and medial walls was removed and labelled block 6. The remaining cortex was divided into 5 blocks of equal width from the anterior (A) to the posterior (P) pole of the cortex including lateral and medial cortical walls (labelled 1–5). Sections 1, 3, 5, and 6 were used for RNA extraction and corresponded to anterior, central (C), posterior and temporal (T) regions (Figure 2.1). In this study, the quantitative RNAseq analysis only included samples from two developmental time points, 34 at 9–10 PCW and 67 at 11–12 PCW (Table 2.4).

Table 2.4: The number of samples of fetal cortex included at each age and location for RNAseq analysis in this thesis.

Age (PCW)	**Number of samples**				
	Anterior	**Central**	**Posterior**	**Temporal**	**Total**
9-10	9	4	10	11	34
11-12	20	5	24	18	67
					101

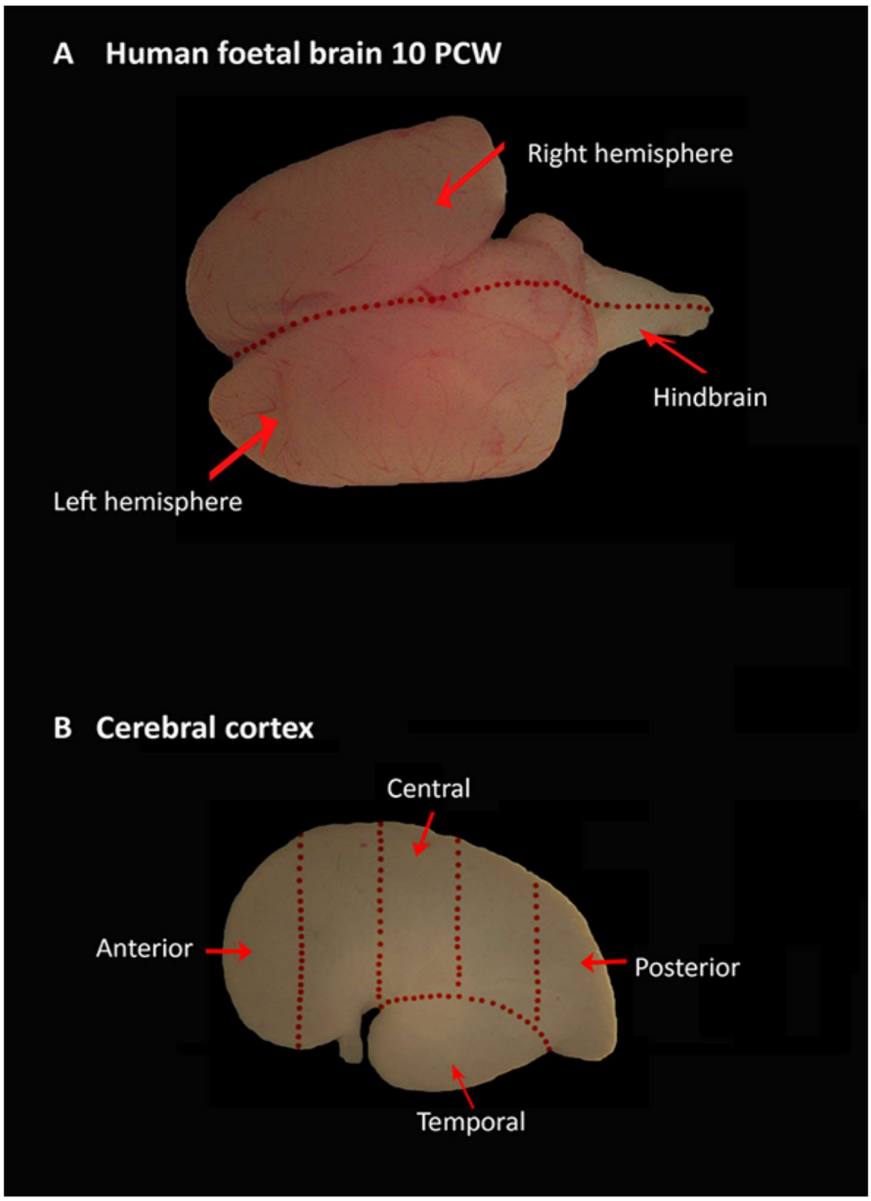

Figure 2.1: Human brain dissection for RNA sequencing. Brains were isolated from the head (A); meninges, blood vessels, and sub-cortical structures were removed, cortex which was then divided into six blocks including the anterior, central, posterior and temporal regions (B). Adapted from (Harkin, 2017).

2.5.2 From RNA extraction to RNAseq data mapping and quality control.

RNA extraction, library preparation and sequencing performed by AROS Applied Biotechnology (Aarhus, Denmark). Poly A RNA was prepared from frozen tissue samples following the company's protocols and quality assurance procedures.

The RNA libraries were prepared following the guidelines produced for the TruSeq Stranded mRNA LT sample prep kit (Illumina part # 15031047 Rev. E) and were quality controlled with respect to concentration and size profile. The libraries contain approximately 120 nucleotides of adapter sequence and the remaining size of the library is derived from the input RNA. The average size of the inserts was 80 bp and the data output corresponded to an average of 90 M reads and a minimum of 63M reads.

Quality control and genomic mapping of RNAseq data were performed by Dr Yaobo Xu at the Newcastle University Bio-informatics unit. Only reads that were at least 20bp in length after trimming were kept. These high-quality reads were then mapped to the human reference genome hg19 with Tophat2 (Kim et al., 2013). Number of reads mapped to genes were counted using HTSeq-count (Anders *et al.*, 2014). RPKMs (Reads per kilo base per million) of genes were calculated and normalized using conditional quantile normalization following the methods set out by Hansen *et al.* (2012).

2.6 Isolation, Expansion, and Differentiation of NSCs from human fetal brain

2.6.1 General Reagents used in the protocol

All reagents were reconstituted and prepared following the supplier instructions:

- NeuroCult NSC Basal Medium (Human)
  Stem Cell Technologies (Catalog #: 05750)

- NeuroCult™ NS-A Proliferation supplements (Human)
  Stem Cell Technologies (Catalog number: 05751)

- NeuroCult™ NS-A Differentiation supplements (Human)
  Stem Cell Technologies (Catalog number: 05752)

- Basic Fibroblast Growth Factor, human (hbFGF)
  Sigma-Aldrich (Catalog number: F0291): A 25 µg/vial of rh bFGF was diluted in 2.5 ml sterile PBS containing 0.1% BSA

- Epidermal Growth Factor, human (hEGF)Sigma, 200 µg
  Sigma-Aldrich (Catalog number: E9644-.2MG): A 200 µg/vial of rh EGF was dissolved in 0.1 ml sterile 10 mM acetic acid containing at least 0.1% BSA

- 0.2% Heparin Sodium Salt in PBS
  Stem Cell Technologies (Catalog number: 07980)

- Recombinant Human Sonic Hedgehog/Shh
  R&D Systems (Catalog number: 1845-SH-100): A 100 µg/vial of rh Shh was dissolved in 1ml sterile PBS containing at least 0.1% BSA

- Bovine serum albumin (BSA)
  Thermo Fisher Scientific, Invitrogen (Catalog number: 15561-020)

- Recovery™ Cell Culture Freezing Medium
  Life Technologies, Gibco (Catalog number: 12648010)

- Penicillin-Streptomycin (5,000 U/mL)
  Thermo fisher (Catalog number 15070-06300

- % 0.05 trypsin-EDTA
  Life Technologies, Gibco (Catalog number 15400-054)

- Soybean trypsin inhibitor
  Sigma-Aldrich (Catalog number: T6522-100mg)

- Distilled Water

  Thermo fisher (Catalog number: 1097703)

- DPBS no calcium, no magnesium

  Life Technologies, Gibco (Catalogue number: 14190-094)

- Hank's balanced salt solution no calcium, no magnesium, no phenol red (HBSS 1x)
  Life Technologies, Gibco (Catalogue number: 14175-053)

2.6.2 Preparation of complete proliferation and differentiation media

The proliferation and differentiation media were prepared according to supplier instructions:

- Preparation of 10 ml complete proliferation media (with cytokines)

1 mL of NeuroCult™ NS-A Proliferation Supplement was added to each 9mL NeuroCult™ NS-A Basal Medium (1: 9 ratio), cytokines were then added as follow:

- 20 µL of 10 µg/mL rh EGF (to give a final concentration of 20 ng/mL rh EGF)
- 10 µL of 10 µg/mL rh bFGF (to give a final concentration of 10 ng/mL rh bFGF)
- 10 µL of 0.2% Heparin (to give a final concentration of 2 µg/mL)

- Preparation of 10 ml complete differentiation media

1 mL NeuroCult™ NS-A Differentiation Supplement was added to each 9 mL NeuroCult™ NS-A Basal Medium (1:9 ratio).

2.6.3 Tissue dissociation and initial plating of primary fetal NSCs in neurosphere cultures.

The isolation and expansion of NSCs were carried out using the neurosphere culturing method (Reynolds and Weiss, 1992; Azari *et al.*, 2011; Siebzehnrubl *et al.*, 2011). In this method, the majority of differentiating and differentiated cells die in 2-3 days, while the neural stem cells continue to proliferate to form neurospheres in the presence of epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF) (Reynolds and Weiss, 1992; Siebzehnrubl *et al.*, 2011).

Human fetal brains (n =7, 9-11 PCW; Table 2.1) were dissected in a sterile 100mm petri dish containing cold Dulbecco's phosphate-buffered saline (DPBS). Using fine forceps, brains were isolated; meninges and blood vessels were removed (Figure 2.2A-C). The GE, anterior cortex, and posterior cortex, from either one or two hemispheres, were detached (Figure 2.2D-F) and minced into tiny pieces before incubation in 0.05% trypsin-EDTA (Thermo Fisher Scientific, Paisley, UK) for 30 minutes in a 37°C water bath for chemical dissociation. Trypsin activity was terminated by adding the same amount of soybean trypsin inhibitor (Sigma-Aldrich). Tissue was pelleted at 110 g for 5 minutes, re-suspended in 5ml basal media (NeuroCult NSC Basal Medium, Stem Cell Technologies, UK) and gently pipetted up and down (15-20 times) until single cell suspension is achieved. The suspension was then filtered through a 70-µm-pore cell strainer (Becton Dickinson). Cells were plated at $2x10^5$ cells /cm^2 into T-25cm^2 flasks in serum-free proliferation media (NeuroCult™ NS-A Proliferation Supplements-Human, Stem Cell Technologies) and supplemented with 20 ng/mL rh EGF (Sigma-Aldrich), 10 ng/mL rh bFGF (Sigma-Aldrich), and 2 µg/mL heparin (Stem Cell Technologies) at 37°C with 95% O_2/ 5% CO_2.

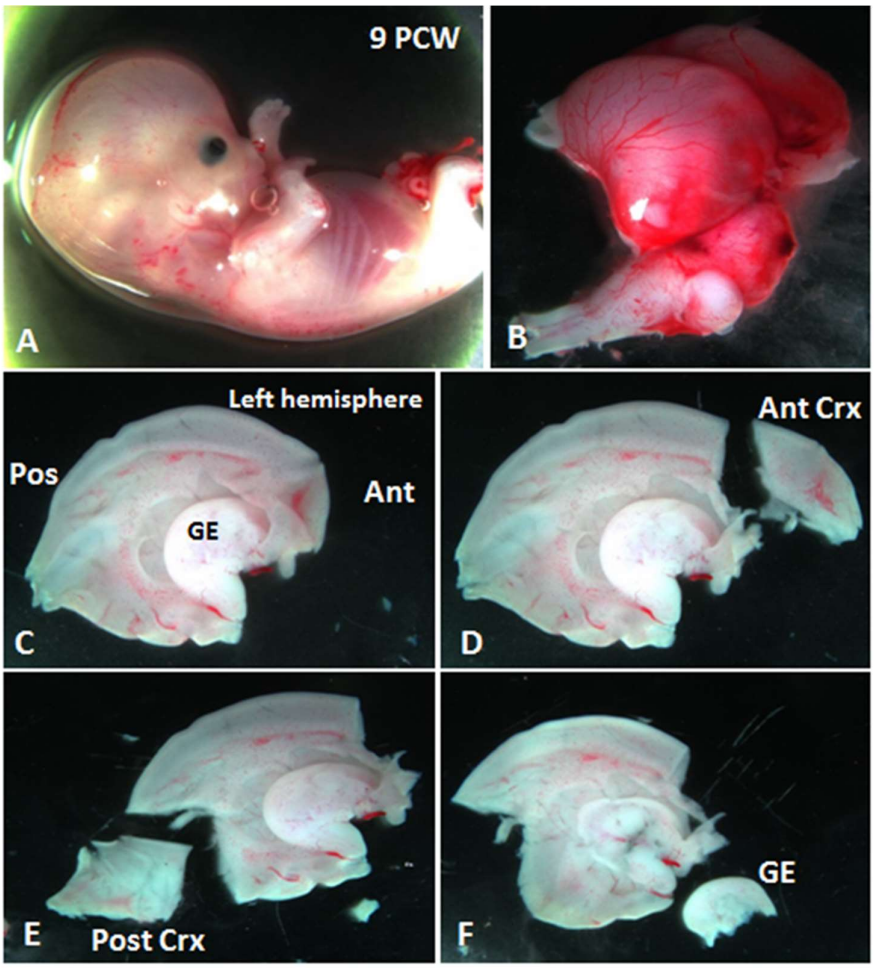

Figure 2.2: Human brain dissection for cell cultures. Brains were isolated from the head (A, B); meninges and blood vessels were removed (C). Anterior (Ant Crx, D), posterior (Pos Crx, D), and ganglionic eminences (GE, B) were detached. A-F from brain No: 7 (Table 2.1).

2.6.4 Plating of the NSCs for differentiation

The medium was replenished every two days for 10-15 days until the neurospheres reach approximately 100-150 μm in diameter. The condition of the cultures was monitored daily, viable neurospheres generally appear semi-transparent and phase bright, with many of the cells on the outer surface displaying microspikes (Reynolds and Weiss, 1992; Azari *et al.*, 2011; Siebzehnrubl *et al.*, 2011). Neurospheres were mechanically dissociated into single cells for passaging and/or differentiation.

For cell differentiation, cells were plated at $2x10^5$ cells /cm^2 onto poly-L-lysine coated glass coverslips, in 12-well culture dishes, in differentiation media (NeuroCult™ NS-A Differentiation Supplements-Human, Stem Cell Technologies) for 8 days at 37°C with 5% CO_2. A half-medium change was carried out every two days, cells were then fixed with cold 4% paraformaldehyde (Sigma-Aldrich) in PBS for 20 minutes and subjected to immunocytochemistry.

2.6.5 Treatment of cell cultures with rh SHH

Two group of cell cultures were treated every two days with two concentrations of recombinant human SHH (100 ng/ml and 200 ng/ml, R&D Systems). The first group was treated for 12 days in differentiation media, the second group was treated for 14 days in proliferation media and 12 days in differentiation media. The counting and images displayed in this thesis are from the group of cells treated with 200 ng/ml concentration in differentiation media.

2.7 Immunocytochemistry

After fixation, cells were washed three times with PBS and blocked with 10% fetal calf serum in 0.3% Triton X-100/PBS for 1 hour at room temperature. Primary antibodies diluted in 10% fetal calf serum in 0.3% Triton X-100/PBS were applied overnight at 4°C. The list of antibodies used in immunocytochemistry and their dilutions are found in Table 2.3. On the second day, cells were washed with PBS and incubated with Texas Red conjugated goat anti-rabbit and fluorescein conjugated horse anti-mouse secondary antibodies (Vector Laboratories) at 1:200

dilution in PBS for 2 hours at room temperature. Cells were counterstained and mounted with anti-fade mounting medium containing DAPI (Vector laboratories).

2.8 Image Acquisition

2.8.1 Light microscopy and slide scanning

Images of all immunoperoxidase staining presented in this study were captured using either a Zeiss Axioplan 2 microscope or Leica SCN400 Slide Scanner (Newcastle Biomedicine Biobank Imaging facility). Processing of images, which included only adjustment of brightness and sharpness, was achieved using the Adobe Photoshop CS6 software.

2.8.2 Fluorescent microscopy

The double immunofluorescent figures (sections and cells) were obtained with a Zeiss Axioimager Z2 apotome using DAPI, GFP, and Texas Red filters. Triple immunofluorescent images were obtained with a Nikon A1R confocal microscope using DAPI, GFP, Texas Red, and CY5 filters. The excitation and emission values for the used fluorophores are shown in Table 2.5. Processing of images, which included only adjustment of brightness and sharpness, was achieved using the Adobe Photoshop CS6 software.

Table 2.5: The Excitation/ emission values (nm) for DAPI and fluorophore used in double and triple labelling.

Fluorophore	**Excitation/Emission (nm)**
DAPI	358⁄461
Fluorescein	494/517
CY3	550/570
Texas Red	550/570
CY5	649/665

2.9 Cell Quantifications

2.9.1 Cell counts in proliferative zones of 12 PCW fetus sections

Nine sections from one 12 PCW brain were selected at intervals along the anterior–posterior axis and immunoperoxidase stained for NKX2.1, OLIG2, or COUPTFII (see chapter 3). For each section, using images obtained with the slide scanner, a counting box 100 micron-wide and approx. 500-750 micron-deep (the exact dimensions were recorded and used in calculations) was placed over the ventricular and subventricular zones (VZ and SVZ; delineated by the expression of PAX6 and KI67), with the 100 micron edge parallel to the ventricular surface, at three equally spaced locations within the following regions of the section (if present); lateral, dorsal, medial, or ventral cortex, MGE, LGE, or ventral CGE, or sub-cortical septum. The number of immunopositive cells within these counting boxes was recorded manually and the area of the box measured (Figure 2.3). From these counts, the average density of immunopositive cells in the VZ/SVZ of each anatomical region was recorded.

In a 3D reconstruction of a 12–13 PCW fetal brain made from MRI scans (Figure 2.4, available at http://database.hudsen.eu); using Image J software (https://imagej.nih.gov/ij/), we calculated the volume of the proliferative zones (VZ/SVZ) of each of the brain regions that we had counted cells in (Table 2.6) and then multiplied the volume of the brain region by the average density of immunopositive cells in that region to give an estimate of the total number of immunopositive cells in that brain region (see Table 3.1). The percentage of cells in each compartment from the total number of cells (in VZ/SVZ) with the standard error were also calculated. In this way, we took into account that although the cortex contained a low density of some cell types, the much larger volume of the cortical regions might contain a relatively large number of cells.

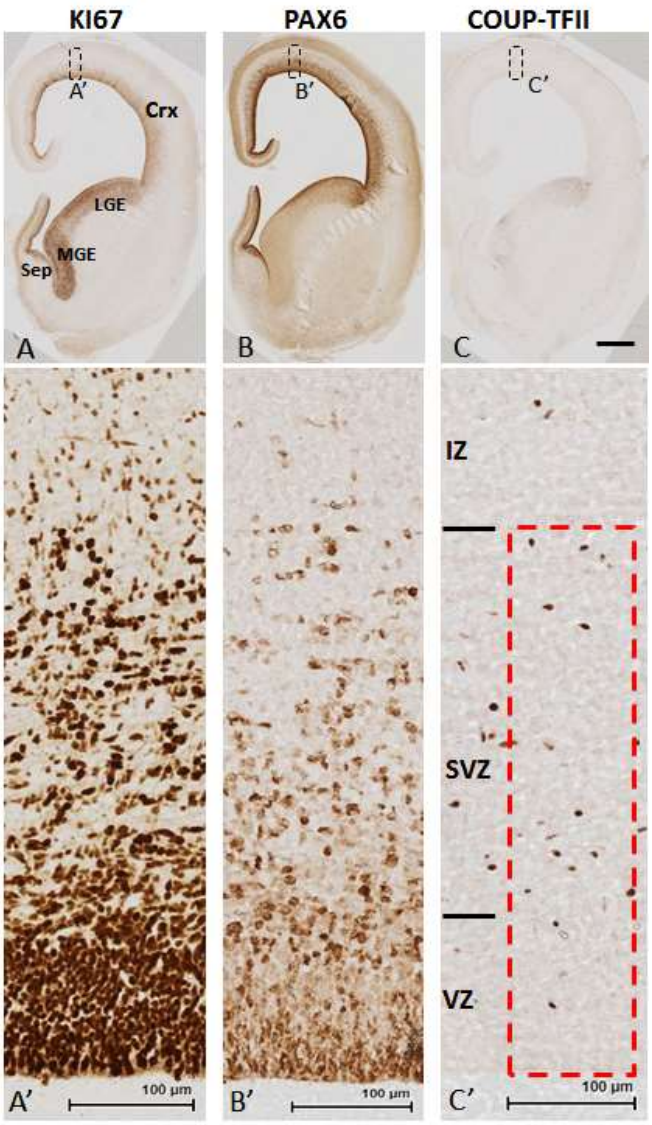

Figure 2.3: Example of cell counts boxes in proliferative zones of 12 PCW fetus sections. (A, B) The proliferative zones of different compartments were delineated by the expression of cell division marker KI67 and/or the radial glia cell marker PAX6. (B) Coronal section stained for COUPTFII. (A', B') KI67 and PAX6 delineated the VZ and SVZ in the cortical wall. (C') Example of 100 micron-wide and approximately 500 micron-deep counting box (red boxed area) for COUP-TFII+ cells in the proliferative zone of dorsal cortex. Boxed areas in A, B, and C show where images (A'-C') were taken. A-C' from brain No: 18. Scale bar: 1 mm in C (and for A and B). 100 µm in A'-C'.

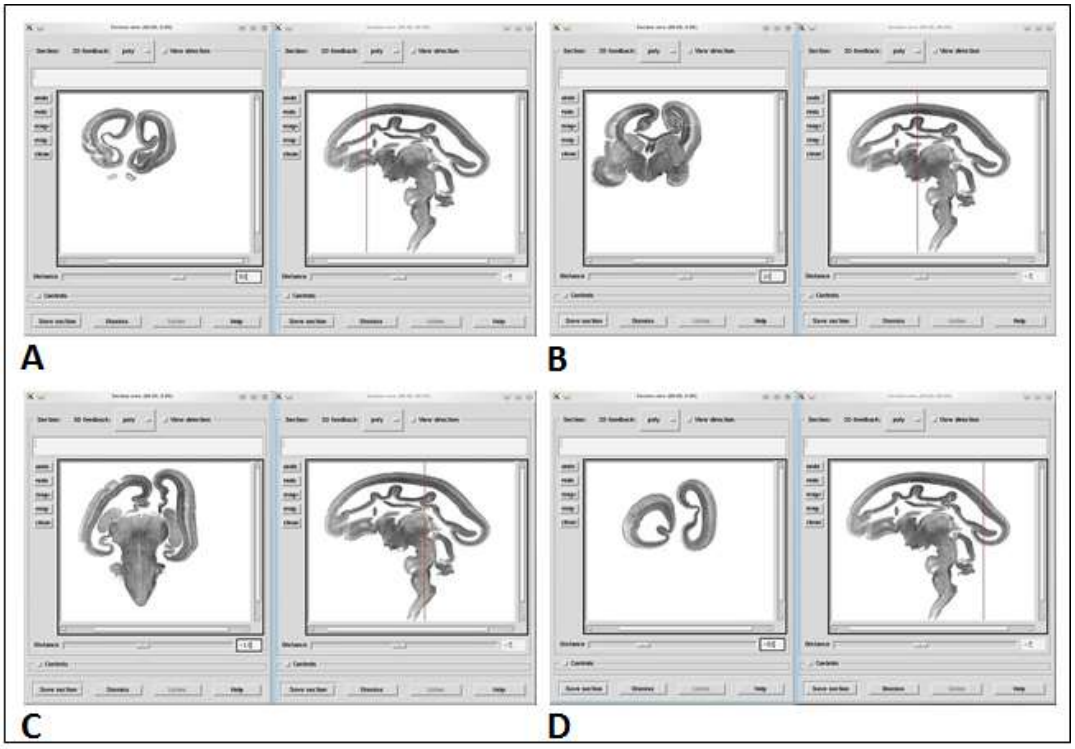

Figure 2.4: Orientation using a 3D MRI model of 12 PCW human fetal brain. The panels have images from the 3D model visualised using MAPaint software (ADD URL). In each panel the left hand image shows the coronal section while the right hand image has a sagittal section of the model. The red line on the sagittal section indicates the position of the coronal section in the left hand image. **(**A) Anterior coronal sections, only anterior cortex can be seen at this level. (B) Section at the level of the rostral thalamus showing central cortex, MGE, LGE, and septum. (C) Section at the level of the caudal thalamus showing central cortex and CGE. (D) Posterior coronal sections, only posterior cortex can be seen at this level. (Available at http://database.hudsen.eu).

Table 2.6: The volume (mm^3) of different brain regions of 3D reconstruction of a 12–13 PCW fetal brain made from MRI scans.

Brain Region	Volume (mm3)
Cortical Proliferative Zone	2196.6
MGE	155.9
LGE	380.3
Ventral CGE	161.6
sub-cortical septum	46.8

2.9.2 Cell counts in double fluorescent 8 and 12 PCW immunostained sections

The counting was performed on sections labeled with two markers each along with the nuclear staining (DAPI). Cells were manually counted from 5 sections from each fetal sample (8 PCW, n = 2 and 12 PCW, n = 2). Sections were observed under medium magnification (10x), rectangular counting boxes of approx. 300-500 μm width were placed over the ventricular/subventricular zones (VZ/SVZ) and intermediate zone/CP (IZ/CP) delineated by the nuclear staining (DAPI) on intact parts of the anterior and posterior cortex (Figure 2.5). Mean values with the standard error were calculated. Experimental groups were compared using a 2-tailed t-test.

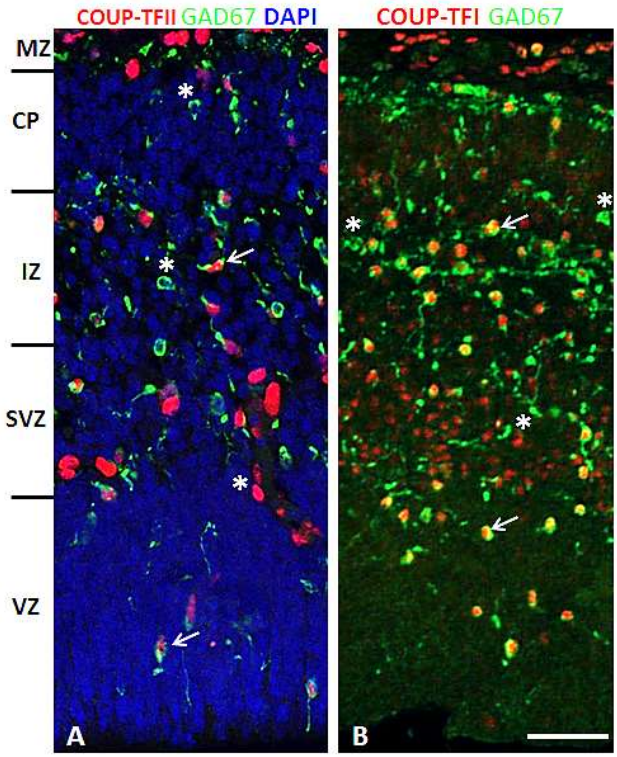

Figure 2.5: Example of cell counts in double fluorescent immunostained sections. (A, B) Double labelling for COUP-TFII and COUPTFII (red) with GAD67 (green) in the cortical wall of 8 PCW fetus, the nuclear staining (DAPI) was used to delineate the different compartments of cortical wall (VZ,SVZ,IZ,CP, and MZ). Arrows indicate examples of double labelled cells; asterisks indicate examples of single labelled cells. A and B from brain No: 1. Scale bar: 100 μm in B (and for A).

2.9.3 Cell counts of dissociated cell cultures

For quantification in dissociated culture, cells from 3 different fetal brains were cultured in 12-well plates were fixed after culture and immunocytochemistry carried out with antibodies to various markers and the nuclear staining (DAPI), each staining combination was made in duplicate (see chapter 5, Figure 5.4 and Figure 5.6). Photomicrographs (20x) were captured from 3 random fields of view from each well from which manual cell counts were made (therefore n = 18). Mean values with the standard error were calculated. Experimental groups were compared using a 2-tailed t-test.

Chapter 3: Expression of the Transcription Factors NKX2.1, OLIG2 and COUP-TFII in Early Fetal Human Telencephalon

3.1 Summary

The extent of similarities and differences between cortical GABAergic interneuron generation in rodent and primate telencephalon remains contentious. We examined expression of three interneuron precursor transcription factors, alongside other markers, using immunohistochemistry on 8–12 post-conceptional weeks (PCW) human telencephalon sections. NKX2.1, OLIG2, and COUP-TFII expression occupied distinct (although overlapping) neurogenic domains which extended into the cortex and revealed three CGE compartments: lateral, medial, and ventral. NKX2.1 expression was very largely confined to the MGE, medial CGE, and ventral septum confirming that, at this developmental stage, interneuron generation from NKX2.1+ precursors closely resembles the process observed in rodents. OLIG2 immunoreactivity was observed in GABAergic cells of the proliferative zones of the MGE and septum, but not necessarily co-expressed with NKX2.1, and OLIG2 expression was also extensively seen in the LGE, CGE, and cortex. At 8 PCW, OLIG2+ cells were only present in the medial and anterior cortical wall suggesting a migratory pathway for interneuron precursors via the septum into the medial cortex. By 12 PCW, OLIG2+ cells were present throughout the cortex and many were actively dividing but without co-expressing cortical progenitor markers. Dividing COUP-TFII+/PAX6+ progenitor cells were localized to ventral CGE and gave rise to calretinin expressing interneurons in the CGE as previously described, which not only migrated posteriorly into the cortex from ventral CGE but also anteriorly via the LGE. COUP-TFII expressing progenitors were also numerous in adjacent ventral cortex co-expressing PAX6 in proliferative zone and TBR1 in the post-mitotic zone mostly likely giving rise to glutamatergic pyramidal cells in the ventral cortex. However, small numbers of COUP-TFII cells were also found undergoing division in anterior and dorsal regions of the cortex. These did not co-express PAX6 suggesting they are most likely interneuron progenitors. Whether they migrated to the cortex, retaining the capacity to divide, or were born in the dorsal telencephalon, is still unclear.

3.2 Introduction

Humans have considerably expanded cognitive abilities compared to all other species which may be dependent on the evolution of a greater interconnectedness of a larger number of functional modules (DeFelipe, 2011; Buckner and Krienen, 2013). This not only depends on the physical presence of neurons, axon pathways, and synapses, but also on synchronicity of neural activity between cortical areas binding together outputs of all neurons within a spatially distributed functional network (Singer and Gray, 1995; Fries, 2009). The synchronicity essential to higher order processing is dependent on the activity of GABAergic interneurons (Whittington *et al.*, 2011; Buzsáki and Wang, 2012), and we might predict a more sophisticated functional repertoire for interneurons in higher species (Yáñez *et al.*, 2005; Molnár *et al.*, 2008; DeFelipe, 2011; Povysheva *et al.*, 2013; Clowry, 2015). Is this expanded repertoire of functional types matched by an evolution of their developmental origins? It is well established in rodents that GABAergic interneurons are born almost entirely outside the neocortex in the ganglionic eminences and associated structures (such as the preoptic area) from which they migrate tangentially into the cortex (De Carlos *et al.*, 1996; Parnavelas, 2000; Marín and Rubenstein, 2001; Welagen and Anderson, 2011).

Whether or not the cortical proliferative zones are a source of interneurogenesis, to what extent and significance, is a contentious issue (Molnár and Butt, 2013; Clowry, 2015). Some researchers have proposed that primates generate significant numbers of interneurons in the proliferative zones of the dorsal telencephalon (Letinic *et al.*, 2002; Petanjek *et al.*, 2009a; Zecevic *et al.*, 2011; Radonjić *et al.*, 2014a; Al-Jaberi *et al.*, 2015a) as well as in the ganglionic eminences. Other groups have convincingly argued that interneuronogenesis is essentially the same in primates as in rodent models (Hansen *et al.*, 2013b; Ma *et al.*, 2013; Arshad *et al.*, 2015). As there is growing evidence that conditions such as autism, schizophrenia, and congenital epilepsy, may have developmental origins in the failure of interneuron production and migration (DeFelipe, 1999; Lewis *et al.*, 2005; Uhlhaas and Singer, 2010; Marín, 2012), it is important that we understand fully the similarities and differences between human development and that in our animal models. Therefore, a detailed study of expression of three transcription factors expressed by interneuron progenitors, NKX2.1, OLIG2, and COUP-TFII was carried out between the ages of 8–12 post-conceptional weeks (PCW) which have been a relatively neglected period of development in the previous studies of interneurogenesis.

3.3 Aim of study

The present chapter firstly aimed to map and quantify the expression of three transcription factors expressed by interneuron progenitors NKX2.1, OLIG2, and COUP-TFII between the ages of 8–12 PCW, an important stage of development prior to the arrival of thalamic innervation. Secondly, reveal the complex organization for the CGE and septum into distinct neurogenic domains. Thirdly, demonstrate the distinct migration pathways of GABAergic interneuron from the ventral telencephalon into the cortex. Finally, explore the potential expanded origin GABAergic interneuron in human fetal brain.

3.3 Results

Examination of our immunoperoxidase labelled sections for various markers at low magnification revealed details of the characteristics of the CGE and septum in human not fully reported on before in detail. Therefore, the results section begins by describing these regions before moving on to describe the level of expression of each GABAergic interneuron precursor transcription factor in different parts of the telencephalon, aided by a more detailed knowledge of CGE and septal sub-compartments.

3.3.1 The anatomical position of the caudal ganglionic eminence (CGE)

The position of the CGE can be determined with respect to other subcortical landmarks in H&E stained sections (Figure 3.1). For example, in the horizontal plane, at the level of the internal capsule, the MGE and LGE appeared as prominent bulges into the lateral ventricles, and in an anterior position relative to the internal capsule. The CGE can be seen as the part of the GE positioned caudally to the internal capsule immediately adjacent to the ventral/temporal cortex (Figure 3.1A). In sagittal sections, the most dorsal part of the CGE appeared as well-defined protrusion into the lateral ventricle, close to the hippocampus, the central part lies next to the narrow ventral extension of the lateral ventricles, while the most ventral part of CGE is located

immediately adjacent to the ventral /temporal cortex. Anteriorly, and continuous with CGE, either the LGE or MGE can be seen in lateral and medial cut parasagittal sections, respectively (Figure 3.1B,C). In a coronal section plane, cut anterior to the thalamus, only MGE and LGE can be delineated but not CGE (Figure 3.1D); in a section at the level of the anterior half of thalamus, parts of MGE and LGE can be seen dorsal to the internal capsule and only the most ventral part of the CGE was observed ventral to the internal capsule and close to the ventral/ temporal cortex (Figure 3.1E,F). At the level of the caudal half of thalamus and caudal to the internal capsule, the CGE was present but not the MGE and LGE (Figure 3.1G).

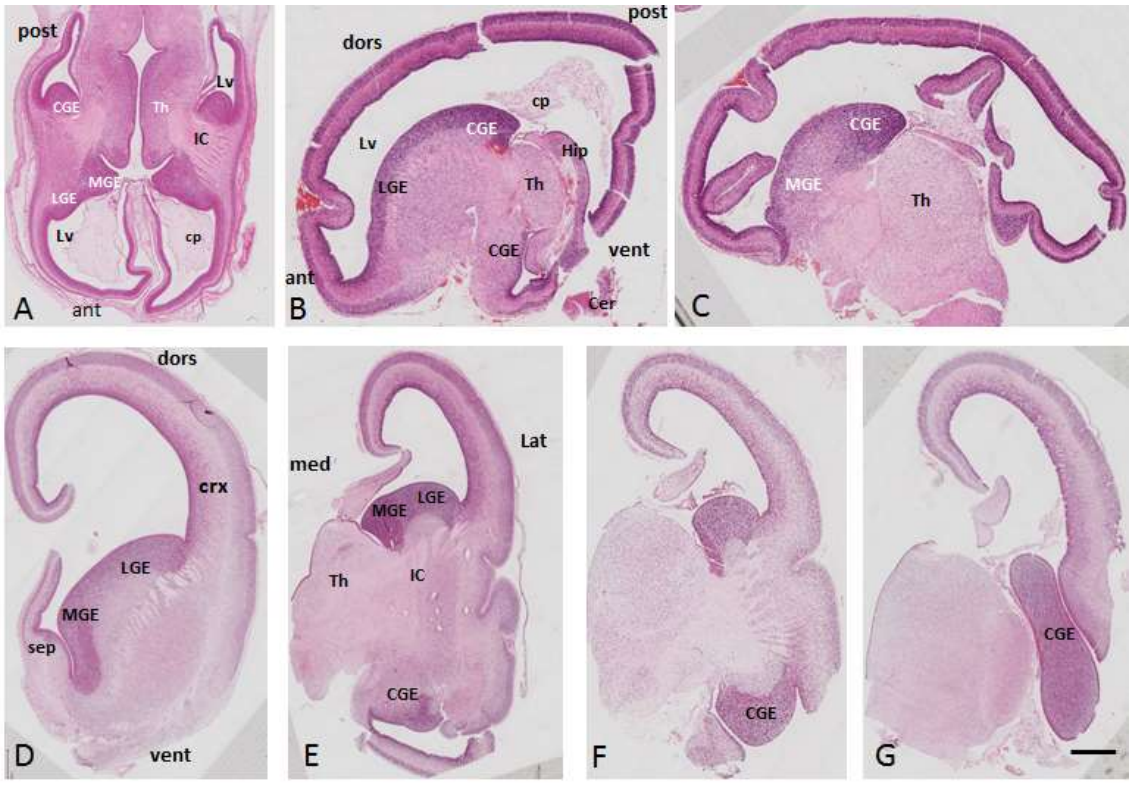

Figure 3.1: The anatomical position of the subdivisions of GE in human fetal forebrain. (A) Horizontal section at 8 PCW. (B) Lateral parasagittal section at 12 PCW. (C) Medial parasagittal section at 12 PCW. (D) Coronal section anterior to the thalamus at 12 PCW. (E) Coronal section at the level of the anterior half of thalamus at 12 PCW. (F,G) Coronal section at the level of the caudal half of thalamus and caudal to the internal capsule at 12 PCW. cp: choroid plexus, Lv: lateral ventricle, Hip: hippocampus, Th: thalamus, Cer: cerebellum, crx: cortex, IC: internal capsule. A from brain No. 4; B and C from brain No. 17; D-G from brain No. 18 (Table 2.1). Scale bar: 1 mm in G (and for A-F).

3.3.2 The molecular subdivisions of the caudal ganglionic eminence (CGE)

The molecular definition of the CGE as a distinct neurogenic domain different from those found in the MGE and LGE is still unclear, mainly because no genetic mutant model has been known to affect specifically CGE. Anatomically, as mentioned in the previous section, the CGE is considered as a caudal extension of both MGE and LGE principally because the CGE shares molecular features with these two regions. In rodents, the most lateral part expresses Gsh2 and ER81 which are required for LGE patterning (Corbin *et al.*, 2003; Stenman *et al.*, 2003) whereas, the medial part expresses NKX2.1, characteristic of the MGE (Sussel *et al.*, 1999; Corbin *et al.*, 2003). Flames *et al.* (2007) have shown that CGE does not contain any specific pools of neural progenitors different from those found in the LGE and MGE in mice. Despite these findings, fate mapping studies reported that specific interneuronal subtypes that express calretinin or/and vasoactive intestinal peptide are exclusively derived from the CGE but not from MGE and LGE (Nery *et al.*, 2002; Butt *et al.*, 2005). In addition, the GABAergic interneuron precursor marker COUP-TFII is preferentially expressed in CGE, where it is required to drive CGE-derived interneuron migration into the cortex (Kanatani *et al.*, 2008).

In order to further identify the specific neurogenic domains that exist in the CGE of human fetal brain, the expression of several transcription factors expressed by interneuron progenitors was studied, and revealed that the CGE can be subdivided into three major compartments, medial, lateral and ventral (Figure 3.2; Figure 3.3). PAX6 was expressed in a gradient with higher expression in the cortical proliferative zones to lower expression in the LGE. In addition to this gradient, a well-defined cortical/subcortical boundary was also revealed by an abrupt change in the expression pattern of PAX6 located ventral to the physical sulcus between the cortex and the bulge of LGE. Whereas in the cortex PAX6 expression is confined to easily recognisable ventricular, subventricular, and intermediate zones (VZ, SVZ and IZ) in the LGE this organization was not well defined, with a more diffuse cell population in the subcortical SVZ (Figure 3.2A,C). A complementary expression pattern of PAX6 and NKX2.1 was seen across the GE, as previously described at 7-8 PCW (Pauly *et al.*, 2014). While PAX6 was expressed in the LGE, decreasing in expression from the lateral boundary with the cortex to the boundary with the MGE, NKX2.1 was almost exclusively expressed in the MGE (Figure 3.2A-D). A marked boundary between PAX6 and NKX2.1 expression was located at the level of the intereminential sulcus between LGE and MGE. This division also extended continuously

and caudally into the CGE. PAX6 expression extended to the VZ of the most dorsal and lateral part of the CGE which protruded into the lateral ventricle, whereas NKX2.1 expression extended to the medial part of the CGE which lay close to the ventral extension of the lateral ventricles (Figure 3.2C,D; Figure 3.3A,A',B,B'). Previous studies in rodents defined these two parts of the CGE as caudal extensions of the LGE and MGE, respectively (Corbin *et al.*, 2003; Flames *et al.*, 2007). Accordingly, these two domains can be anatomically defined as lateral CGE (LCGE) and medial CGE (MCGE). However, no molecular domains are found in these two regions distinct from those found the LGE and MGE, respectively.

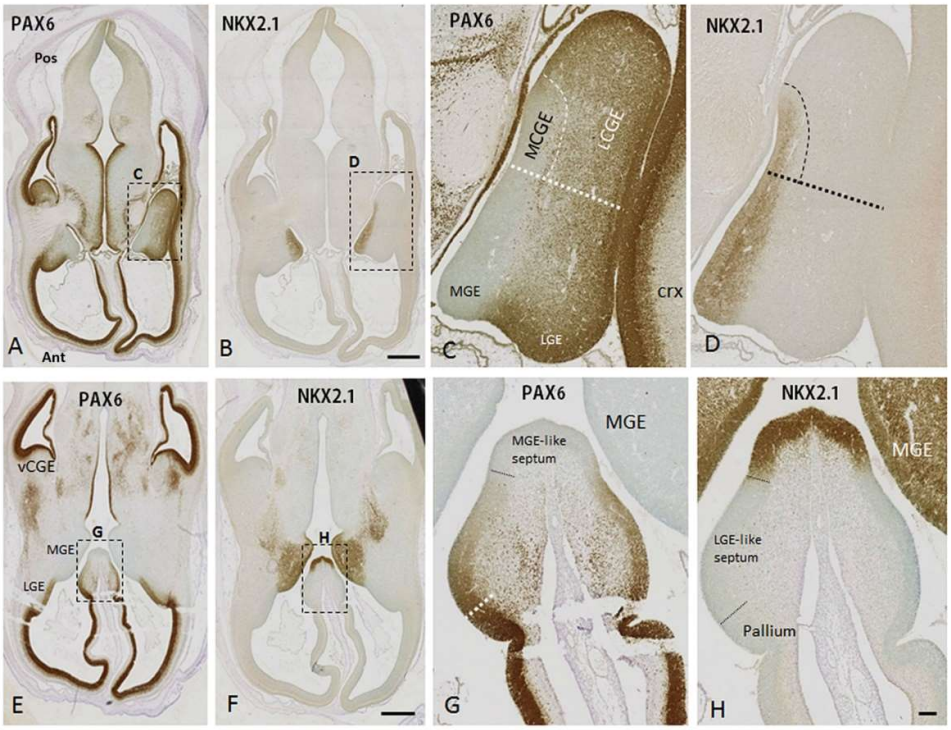

Figure 3.2: Complementary expression of PAX6 and NKX2.1 in the ganglionic eminences and septum of human fetal forebrain at 8 PCW. All sections are in the horizontal plane. (A) PAX6 was expressed in a gradient with higher expression in the proliferative zone of the cortex to lower expression in the LGE and its caudal extension (LCGE). (B) NKX2.1 expression was mainly confined to the MGE and its caudal extension (MCGE). (C,D) Higher magnification of boxed areas in A and B. (E,F) Ventral sections cut at the level of the septum; PAX6 was densely expressed in the proliferative zone of VCGE, but no NKX2.1 expression was found in VCGE. (G,H) Higher magnification of boxed areas in E and F. Similar to the ganglionic eminences, PAX6 was expressed in a gradient from the cortex part to dorsal part of the septum (LGE-like septum) and NKX2.1 was exclusively expressed in the most ventral part of the septum (MGE like septum). A-H from brain No. 4. Scale bars 1 mm in F (and for A, B, and E); 100 μm in H (and for C, D, and G).

Interestingly, these two domains could not be distinguished in the most ventral part of CGE, which was located immediately adjacent to the ventral/temporal cortex (Figure 3.2E,F; Figure 3.3A'',B''). Thus, we propose there is a third compartment to the CGE in human, "ventral CGE" (VCGE) which was characterized by strong immunoreactivity for PAX6, but not NKX2.1. Despite the high PAX6 expression in the VCGE, there was still a distinct boundary between it and the adjacent cortex, characterized by a thicker cortical VZ compared with more condensed PAX6+ cells in the SVZ of the VCGE (Figure 3.3A'',B''). As the CGE is recognised as the birth place of calretinin (CalR) expressing interneurons in rodents (Nery *et al.*, 2002; Butt *et al.*, 2005) we studied the expression of CalR in the three defined compartments of the CGE. CalR was preferentially expressed in cells of the VZ and SVZ of the LCGE and VCGE. Only scattered CalR+ cells were observed in MCGE (Figure 3.3C-C"). In addition, as will be shown later in this chapter, COUP-TFII progenitor cells which give rise mainly to calretinin-expressing interneurons were exclusively found in VCGE but not LCGE and MCGE (see below). All these findings suggest that only the dorsal part of CGE (LCGE and MCGE) shares the molecular features of the LGE and MGE; however, distinct neurogenic domains can be identified ventrally (vCGE).

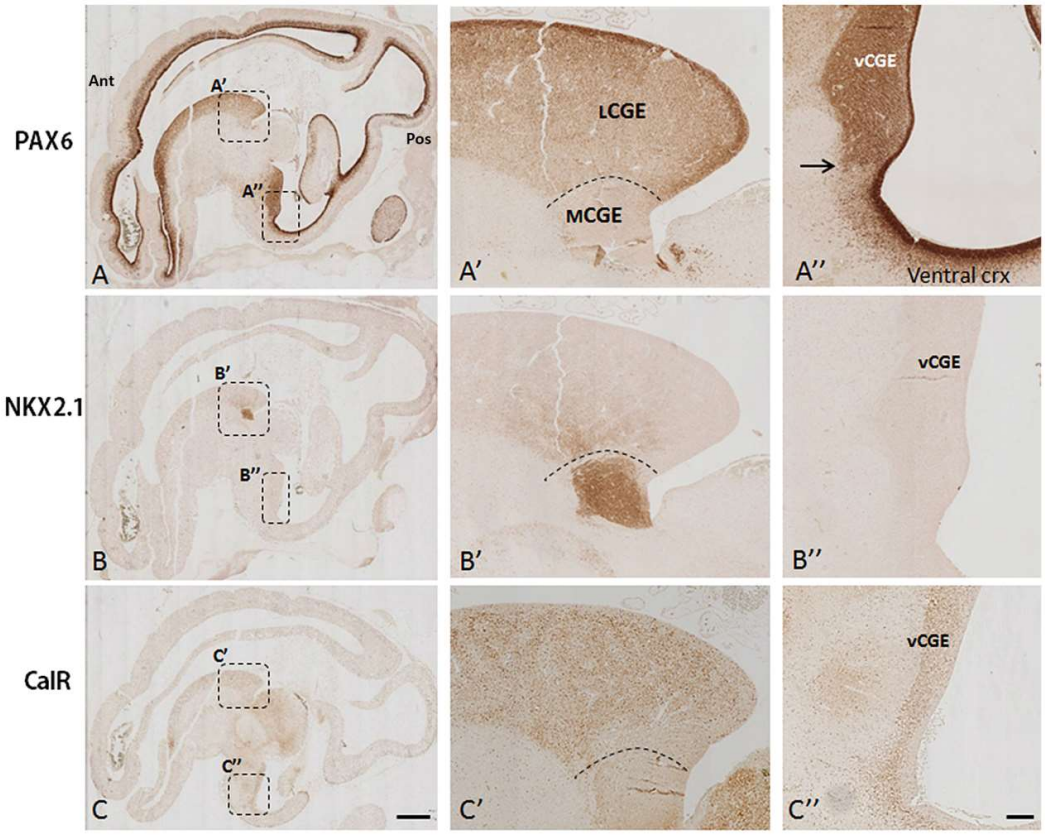

Figure 3.3: The subdivisions of the CGE in sagittal sections at 12 PCW. (A, A', A'') PAX6 was expressed in the proliferative zone of LCGE but not MCGE, PAX6 was also expressed in the VCGE with a distinct cortical/subcortical boundary (arrow in A''). (B, B', B'') NKX2.1 was confined to the caudal extension of MGE (MCGE). (C,C',C'') Calretinin (CalR) is preferentially expressed in ventricular and subventricular zones of the LCGE and only scattered cells have been observed in MCGE, consistent with PAX6. Ant (anterior); Pos (posterior). A-C'' from brain No. 17. Scale bars: 1mm in C (and for A, B); 100 μm in C'' (and for A', A'', B', B'', B').

3.3.3 The subdivisions of the septum

The septum is a largely subcortical structure in mouse that is also known as a contributor of cortical GABAergic interneurons (Wonders and Anderson, 2006). However, far too little attention has been paid to the molecular features of this structure either in human or in rodents. The transcriptional morphology showed that the septum shares common molecular features of the ganglionic eminences along the dorsoventral axis (Figure 3.2; Figure 3.4). Complementary expression was exhibited for PAX6 and NKX2.1; the most ventral part of septum could be

defined as MGE-like septum characterized by strong immunoreactivity for NKX2.1 but not PAX6 expression. More dorsally we found LGE-like septum, which was characterized by moderate expression of PAX6 but not NKX2.1. The most dorsal part of septum had a cortical rather than sub-cortical identity, manifested by higher PAX6 expression in the VZ and SVZ and expression of TBR1 by post-mitotic cells in the SVZ, IZ and cortical plate (Figure 3.2E-H; Figure 3.4). Similarly, the expression patterns of OLIG2 and SP8 in MGE-like and LGE-like septum (as shown later in this chapter and chapter 4) shared their patterns of expression with the MGE and LGE, respectively. In addition, a new migration pathway of septum derived cells, not described previously, was also described in this chapter (see below).

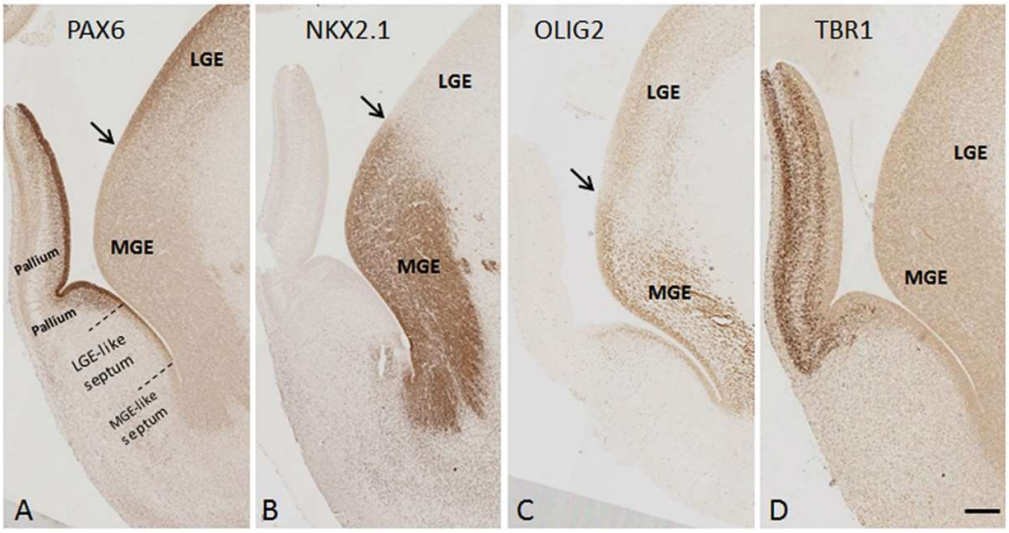

Figure 3.4: Distinct expression patterns of PAX6, NKX2.1, OLIG2 and TBR1 show three subdivisions of the septum. Pallial septum was characterised by strong expression of PAX6 (A) and TBR1 (D) the presence of some OLIG2+ cells (C) and an absence of NKX2.1 (B). LGE-like septum was characterised by a dorsal to ventral gradient of PAX6 expression (A) OLIG2 expression (C) and an absence of NKX2.1 expression (B). MGE-like septum exhibited NKX2.1 (B) and OLIG2 (B) expression only. An arrow marks the border between the LGE and MGE. A-D from brain No. 18. Scale bar: 100 μm in D (and for A-C).

3.3.4 Subcortical neurogenic domain of NKX2.1 progenitors

In the ventral telencephalon, NKX2.1 expression was almost entirely confined to the MGE including its caudal extension (the MCGE) and the ventral part of the septum (MGE-like septum) (Figure 2B,D,F,H; Figure 3.4B).

Double immunofluorescence labelling for NKX2.1 and GAD65/67 (enzyme catalysing synthesis of the inhibitory neurotransmitter gamma-aminobutyric acid, GABA) of 8 PCW human sections revealed that they co-localized in the majority of cells in the SVZ of the MGE; GAD65/67 was also expressed in migrating cells in the cortex; however, no NKX2.1+ cells were found in the cortex at 8 PCW (Figure 3.5A) in agreement with the previous findings in rodents and human that NKX2.1 is downregulated in GABAergic interneurons migrating out of MGE (Marín and Rubenstein, 2001; Letinic *et al.*, 2002; Hansen *et al.*, 2013b).

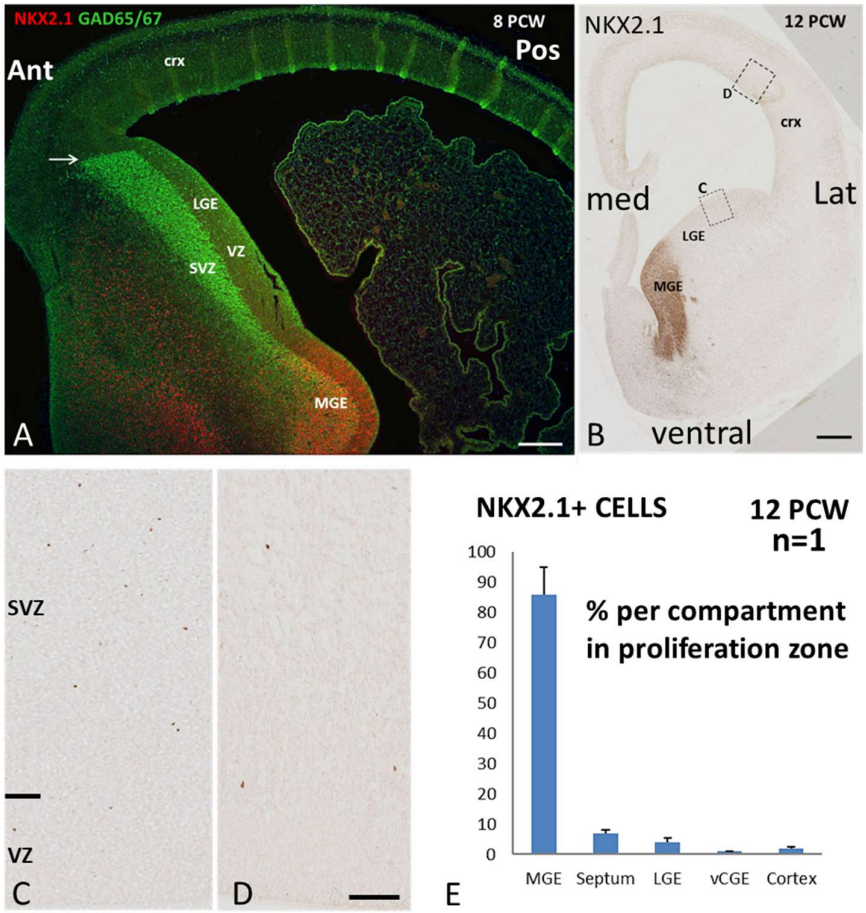

Figure 3.5: The expression of NKX2.1 in the human fetal forebrain. (A) Double labelling for GAD65/67 (green) and NKX2.1 (red) in coronal section at 8 PCW. Co-localization detected as orange signal. GAD65/67 was expressed mainly in the subventricular zone (SVZ) of the MGE and LGE. In LGE, GAD65/67 immunoreactivity also showed a clear cortical/subcortical boundary anteriorly (arrow). NKX2.1 was expressed in the ventricular zone (VZ) and subventricular zone (SVZ) of the MGE, and in cells probably migrating through the non-proliferative mantle zone of LGE toward the cortex (crx). No NKX2.1+ cells were found in the cortex at 8 PCW. (B, C, D) At 12 PCW, NKX2.1 was expressed in the majority of cells in the MGE; scattered NKX2.1+ cells were found in the proliferative zones of the LGE and cortex. (E) The distribution of NKX2.1+ cells in the proliferative zones of different regions of human fetal forebrain at 12 PCW (n=1, see Table 3.1 for more details). A from brain No. 5; B-D from brain No.18. Scale bars: 1 mm in A and B; 100 μm in D (and for C).

However, at 12 PCW, dispersed NKX2.1 positive (NKX2.1+) cells were found in the cortical VZ and SVZ sometimes far removed from the ganglionic eminences and septum (Figure 3.5B-D) but no NKX2.1+ cells were observed to co-express KI67 (not shown) a marker for active cell division (Scholzen and Gerdes, 2000). Quantification of the average density of NKX2.1+ in the proliferative zones of the MGE, septum**,** LGE, VCGE, and the cortex of a 12PCW brain cut in the coronal plane (Figure 3.5E; Table 3.1) estimated that 93% of NKX2.1 cells in proliferative layers were found in the MGE and ventral septum, 4.6 % in the LGE and VCGE, and only 2.4 % in the cortex. Although only a very small percentage, the presence of NKX2.1+ cells in the cortex of 12 PCW brain suggests that these cells could be generated in the proliferative zone of the dorsal telencephalon, and their incidence in the cortex gradually increases with age. However, since no evidence for dividing NKX2.1+ cells in the cortex, these cells could be also generated in the MGE (or septum) but continued expressing NKX2.1 while migrating.

Table 3.1: Cell counts in Proliferative zones of 12 PCW fetus.

Compart-ment	Volume (mm^3)	NKX2.1			OLIG2			COUPTF-II		
		Density cells/mm^3 $x10^3$	Number $X10^6$	%	Density cells/mm^3 $x10^3$	Number $X10^6$	%	Density cells/mm^3 $x10^3$	Number $X10^6$	%
MGE	155.9	593.4	92.5	86.5	450.7	70.3	35.9	43.3	6.7	1.5
LGE	380.3	10.6	4.0	3.8	176.6	67.2	34.3	329.8	125.4	27.2
vCGE	161.6	5.5	0.9	0.8	191.1	30.9	15.8	1118.3	180.7	39.2
Septum	46.8	147.4	6.9	6.5	81.1	3.8	1.9	15.2	0.7	0.2
Cortex	2196.6	1.2	2.6	2.4	10.8	23.7	12.1	67.4	148	32.1

The volume of each compartment, which refers only to the SVZ and VZ, was estimated from a 3D reconstruction of post-mortem MRI scans. Cell counts were made in randomly placed counting frames, a density calculated and the value extrapolated to represent the whole compartment. The percentage of cells refers to the proportion in the compartment of the total number of cells expressing each transcription factor in the proliferative zones.

3.3.5 Expression of OLIG2 in human fetal ventral telencephalon

Distinct patterns of OLIG2 immunoreactivity were seen in MGE, LGE, septum and CGE compartments of human fetal brain. At both 8 and 12 PCW, OLIG2 was strongly expressed in cells of the VZ and SVZ of the MGE, but weaker expression was observed in the VZ and SVZ of the LGE**;** we observed aggregations of OLIG2+ cells amongst OLIG2- cells throughout the SVZ of the MGE (Figure. 3.6A,B; Figure 3.7A-C). We estimated that OLIG2+ cells in the MGE are largely dedicated to neurogenesis at these ages because there was no expression for markers of oligodendrocytes precursors like NKX2.2 (this study, data not shown) and SOX10 (Hansen *et al.,* 2013). In CGE compartments, both the level and the pattern of expression of OLIG2 in the MGE and LGE was extended caudally to the MCGE and LCGE, respectively (Figure 3.7A-E); similar to NKX2.1, the level of OLIG2 expression in the vCGE was only confined to very few cells (Fig. 4k) confirming that this compartment of the CGE contains neurogenic domain distinct from those located dorsally (MCGE and LCGE) which are more likely considered as caudal extension of the MGE and LGE, respectively.

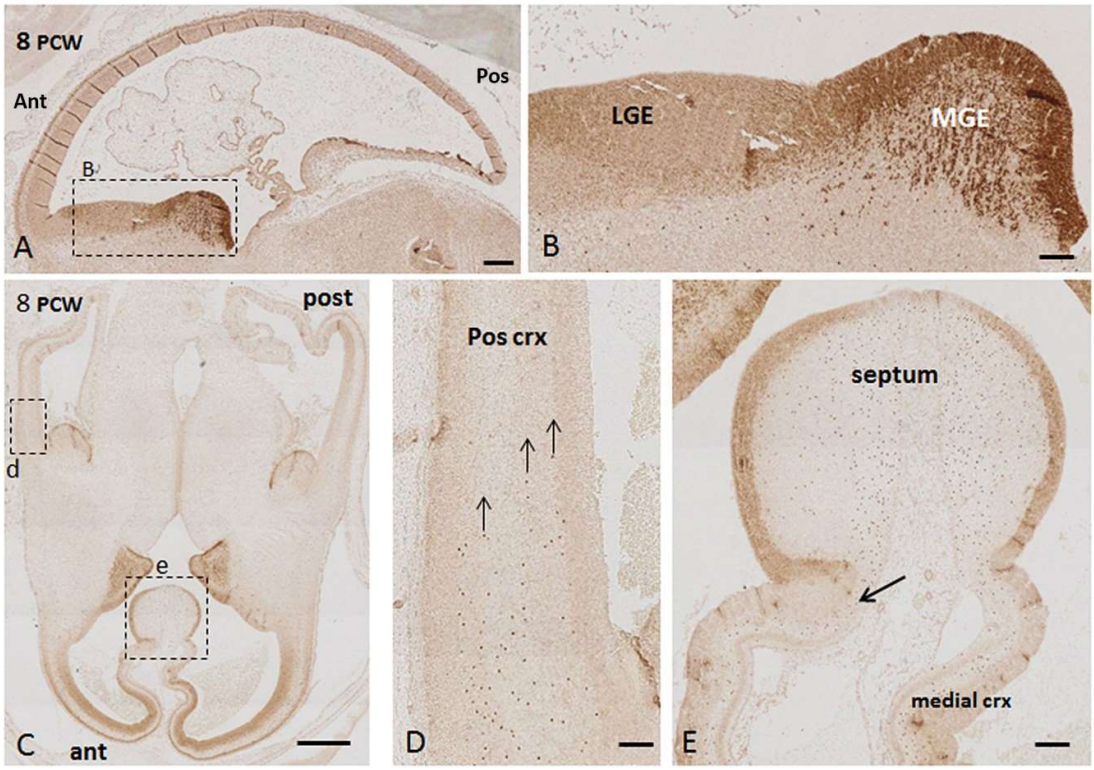

Figure 3.6: The expression pattern of OLIG2 in human fetal forebrain at 8 PCW. (A) OLIG2 was strongly expressed in the proliferative zones of MGE. Weaker expression was observed in LGE. (B) Higher magnification of boxed area in A. (C) The anterior (ant) cortex was heavily populated with OLIG2+ cells whereas no OLIG2+ cells were found in the most posterior cortex. (D) Higher magnification of boxed area in C, OLIG2+ cells from the GE appeared to be only starting to invade the posterior cortex (arrows). (E) OLIG2 was expressed in the proliferative zone of septum, with a stream of OLIG2+ cells appearing to migrate (arrow) into the medial cortex (crx). A and B from brain No. 5; C-E from brain No. 2; H from brain No. 4. Scale bars: 1 mm in A, C; 100 μm in B, D, E.

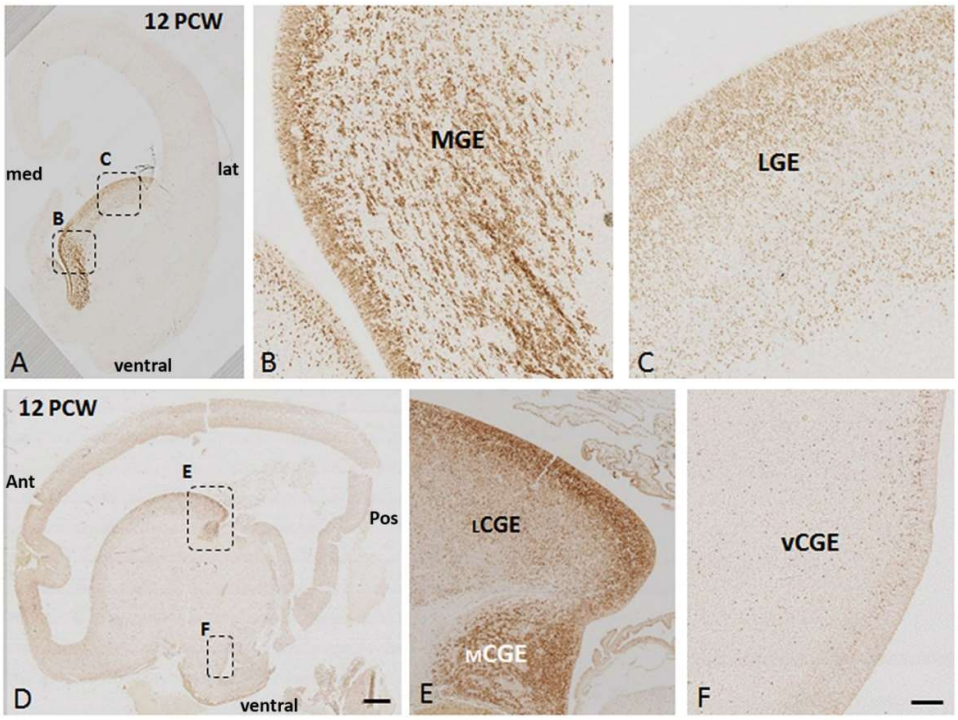

Figure 3.7: The expression pattern of OLIG2 in human fetal forebrain at 12 PCW. (A, B) Similar to 8 PCW, OLIG2 was strongly expressed in the proliferative zone of MGE at 12 PCW, with aggregations of OLIG+ cells amongst OLIG2- cells. (C) Relatively weaker expression was observed in LGE. (D, E) The expression pattern of OLIG2 in the MGE and LGE extended to the MCGE and LCGE, respectively. (F) Scattered OLIG2+ cells were observed in the VCGE. A, B and C- coronal section plane. D, E and F- sagittal section plane. A-C from brain No. 18; D-F from brain No. 17. Scale bars: 1 mm in D (and for A); 100 µm in F (and for B,C,E).

Although OLIG2 was highly expressed in the NKX2.1-expressing neurogenic domain, showing overlapped expression in the MGE and ventral septum; cellular co-localization of these two markers revealed the presence of three separate population of progenitor cells in the MGE, NKX2.1-/OLIG2+, NKX2.1+/OLIG2-, and NKX2.1+/OLIG2+ (Figure 3.8A) which could contribute to the neuronal diversity generated in this domain. To confirm that OLIG2+ cells at this stage of human forebrain development (8-12 PCW) were GABAergic interneuron precursors, OLIG2 and GAD65/67 double labelling was performed it was found that most OLIG2+ cells in the SVZ of the MGE co-expressed GAD65/67 (Figure 3.8B,C). Furthermore, a proportion of cells in the MGE were triple labelled with OLIG2, NKX2.1, and GAD65/67 (Figure 3.8D-G). However, although both OLIG2 and calretinin were expressed in LCGE and MCGE, no double labelling for these two markers was detected (data not shown) suggesting that calretinin expressing cells (interneurons) are not generated from OLIG2 and NKX2.1 expressing progenitors.

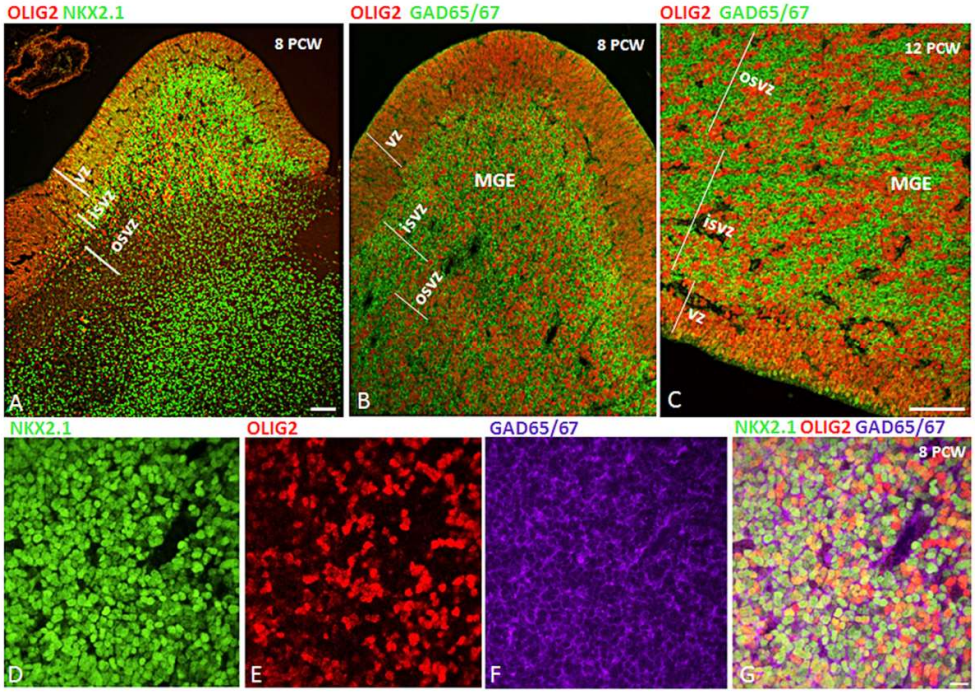

Figure 3.8: OLIG2 and NKX2.1 co-localization in the MGE of human fetal forebrain at 8 and 12 PCW. (A) Double labelling for NKX2.1 (green) and OLIG2 (red) in the MGE at 8 PCW showed three population of cells located in the MGE: the first expressed only OLIG2 (red), the second expressed only NKX2.1 (green), and a third population co-localized these two markers (yellow). (B, C) Double labelling for OLIG2 (red) and GAD65/67 (green) in the MGE at 8 and 12 PCW showed that most of nuclear OLIG2+ cells were double labelled with GAD65/67 in the cytoplasm. (D, E, F, G) Triple labelling for NKX2.1 (green), OLIG2 (red), and GAD65/67 (purple) in the MGE at 8 PCW showed many cells coexpressed the transcription factors NKX2.1 and OLIG2 (nuclear staining, yellow) and GAD65/67 (cytoplasmic, purple). A,B, D-E from brain No. 5; C from brain No. 19. Scale bars: 100 µm in A; 50 µm in B, C; 20 µm in G (and for D, E, F).

3.3.6 Expression of OLIG2 in human fetal dorsal telencephalon

At 8 PCW a quite different pattern of OLIG2 expression was observed in the posterior and anterior cortex. A stream of OLIG2+ cells from the GE appeared to be starting to invade the posterior cortex, however no OLIG2+ cells were observed in the most posterior cortex at this stage (Figure 3.6C,D). In contrast, the anterior cortex was heavily populated with strongly OLIG2 immunoreactive cells and there was a moderate immunostaining throughout the cortical wall including the cortical plate (Figure 3.6C), as was previously observed at 7.5 PCW (Al-Jaberi et al, 2015) suggesting a role for OLIG2 in cortical arealization. In addition to the OLIG2+ cells seen entering the cortex from the LGE, a stream of OLIG2+ cells also appearing to migrate from the septum into the medial wall of the anterior cortex (Figure 3.6C,E) revealing a new migration pathway (medial pathway) of septum- derived cells into the cortex, which is in conflict with previous findings in rodents where cells from the septum only migrate into the cortex via the lateral pathway through the MGE and LGE (Pleasure *et al.*, 2000; Wonders and Anderson, 2006; Morozov *et al.*, 2009; Faux *et al.*, 2012). One other explanation for the increasing density of OLIG2+ cells in the anterior cortical wall is that considerable number of OLIG2+ cells in the anterior cortex were double labelled with KI67 showing that these cells were dividing (Figure 3.9A) which suggested either a dorsal origin for these cells or that they retained proliferative capacity after migrating into the cortex.

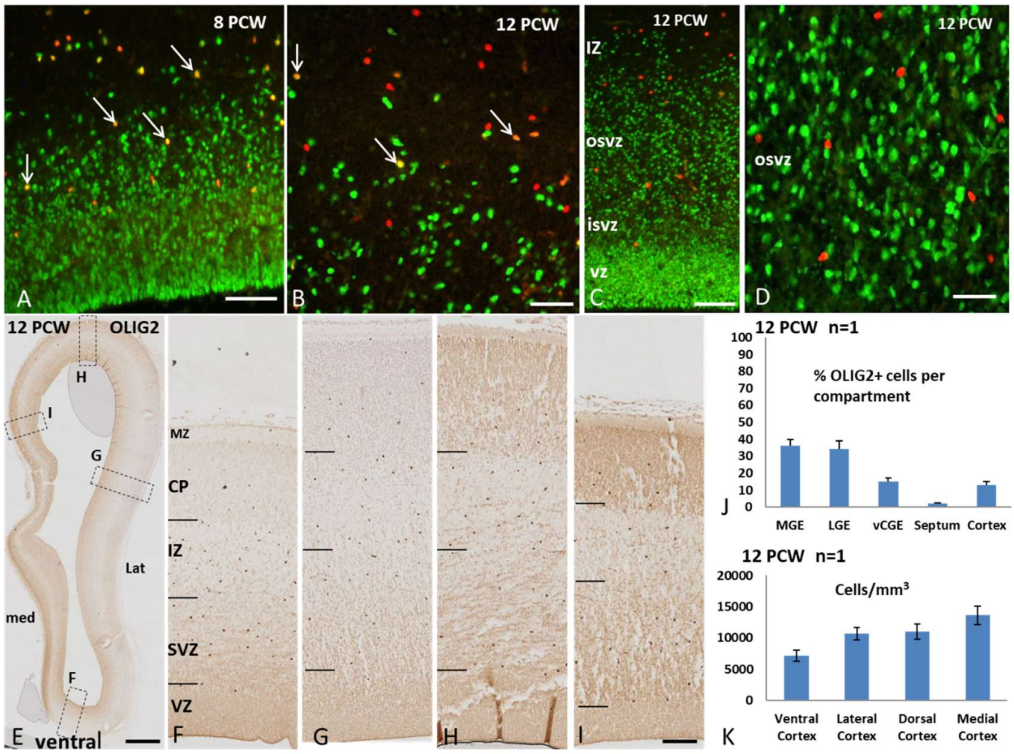

Figure 3.9: Double labelling for OLIG2 (red) with KI67, PAX6, and TBR2 (green) in human fetal forebrain at 8 and 12 PCW. (A) Many OLIG2+ cells (red) co-expressed the cell division marker KI67 (green) at 8 PCW (arrows). (B) Many OLIG2+ cells co-expressed KI67 at 12 PCW (arrows). (C) OLIG2+ cells (red) did not co-express the radial glia cell marker PAX6 (green). (D) OLIG2+ cells (red) did not also co-express the intermediate progenitor cell marker TBR2 (green). (E) OLIG2 expression in the cortex of coronal section 12 PCW. (F) OLIG2 expression in ventral cortex. (G) OLIG2 expression in lateral cortex. (H) OLIG2 expression in the dorsal cortex. (I) OLIG2 expression in the medial cortex. (J) The distribution of OLIG2+ cells in the proliferative zones between different regions of human fetal forebrain at 12 PCW (n=1). (K) The average density of OLIG2+ cells in the ventral cortex, lateral cortex, dorsal cortex, and medial cortex of 12 PCW human fetal brain (n=1). Images A-D were taken from the anterior cortical wall. Boxed areas in E show where images (F-I) were taken. A from brain No. 2; C-I from brain No. 18. Scale bars: 50µm in A; 20µm in B; 50 µm in C; 20 µm in D; 1 mm in E; 100 µm in I (and for G, H).

At 12 PCW, OLIG2+ cells populated the whole cortex and were mainly seen in the SVZ and IZ, nevertheless scattered positive cells were sometimes observed in the VZ and the cortical plate (CP; Figure 3.9E-I). A proportion of OLIG2+ cells in the cortex were also found to co-express KI67 (Figure 3.9B); However OLIG2+ cells were not double-labelled with either PAX6 or TBR2 (Figure 3.9C,D) showing that OLIG2 is not expressed by typical cortical radial glial progenitors or intermediate progenitors (Bayatti *et al.*, 2008a; Lui *et al.*, 2011). Finally, the average density of OLIG2+ cells has been also quantified in the proliferative zones of different regions of the ventral and dorsal telencephalon (Table 3.1). Overall, OLIG2 expression was far less confined to the MGE than NKX2.1, with approximately 38% of all OLIG2+ cells in proliferative layers found in the MGE and the adjacent ventral septum, 50% in the LGE and VCGE, and 12% in the cortex (Figure 3.9J; Table 3.1). When comparing the average density between four different cortical regions, a higher density was found in the medial cortex with a decreasing gradient to the latero-ventral regions (Figure 3.9K) as demonstrated above, the medial migration of OLIG2 + cells from the septum could be the reason for the raised density in the medial cortex.

3.3.7 The vCGE and MGE/LGE boundary are exclusive source of COUP-TFII progenitors in the ventral telencephalon

In the ganglionic eminences, COUP-TFII expression was very specific at 8 PCW; it was largely confined to the CGE compartments and MGE/LGE boundary although dispersed cells were also observed within the MGE and LGE (Figure 3.10A-C). At 12 PCW, COUP-TFII was still highly expressed in the proliferative zones of the CGE, with a decreasing gradient of COUP-TFII+ cells toward the LGE and anterior cortex, and only a few cells occupied the MGE; however, strong immunoreactivity was still observed at the MGE/LGE boundary (Figure 3.11A-C; Figure 3.12A-D). COUP-TFII immunoreactivity also revealed a clear cortical/subcortical boundary between the LGE and the lateral cortex (boundary located ventral to the physical sulcus between the cortex and the bulge of LGE (Figure 3.11D). Although COUP-TFII is expressed either side of this boundary, there is markedly higher expression in the LGE. Similarly, Pauly *et al.* (2014) reported an abrupt transition from high to low DLX2 expression (subcortical marker, a transcription factor expressed upstream of COUP-TFII) going from the LGE to cortex in human at 7–8 PCW, even though PAX6 was expressed on either side of the boundary (Figure 3.2A). However, no similar boundary was observed by

COUP-TFII expression between the vCGE and the adjacent ventral/temporal cortex, where strong COUP-TFII expression appeared to be continuous across these two regions (Figure 3.12A).

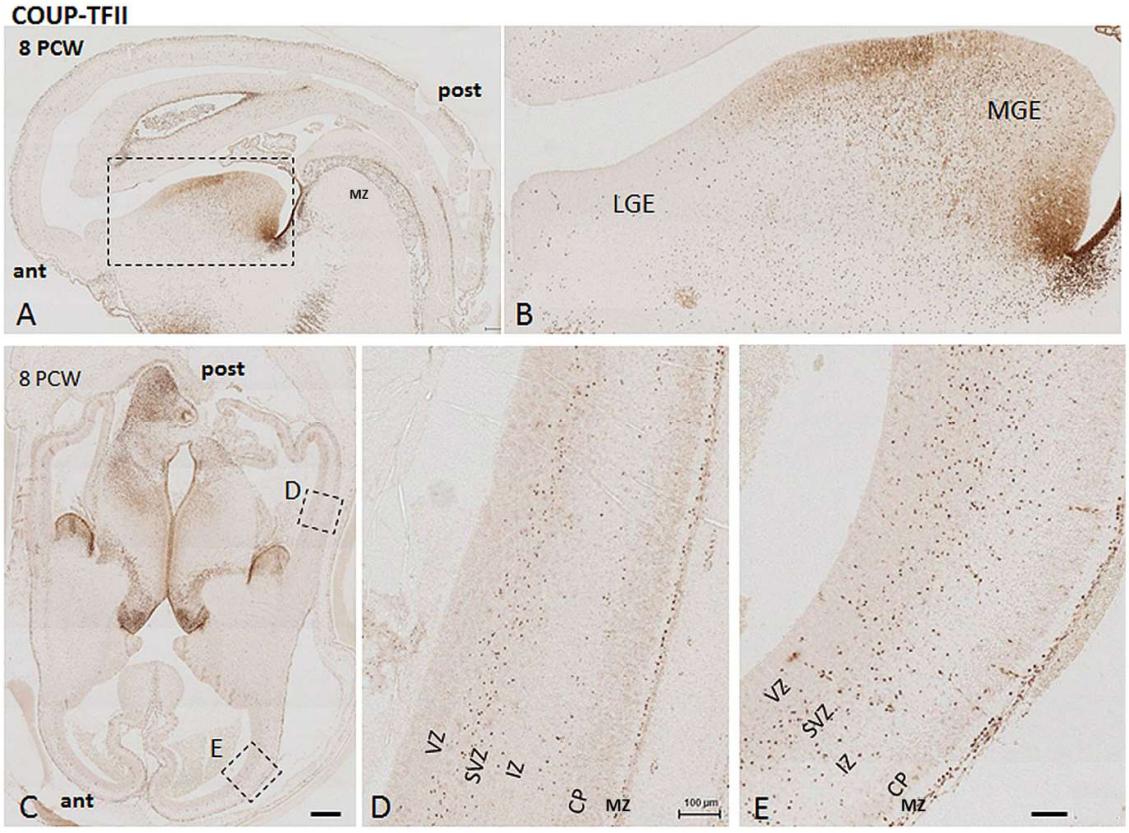

Figure 3.10: The expression pattern of COUP-TFII in human fetal forebrain at 8 PCW. (A, B) COUP-TFII expression was mainly expressed in the CGE and at the boundary between MGE and LGE. Scattered cells were also observed in MGE and LGE. (C) The expression pattern of COUP-TFII in the anterior (ant) and posterior (pos) cortex at 8 PCW. (D) Higher magnification of boxed area in the posterior cortex in C, COUP-TFII expression appeared to be restricted to two migratory streams, one in the subventricular zone (SVZ) and one at the border between the intermediate zone (IZ) and the cortical plate (CP). (E) Higher magnification of boxed area in the anterior cortex in C, COUP-TFII+ cells were found in all layers of the cortex. A and B from brain No. 1; C-E from brain No. 2. Scale bars: 1 mm in C (and for A); 100 µm in E (and for B,D).

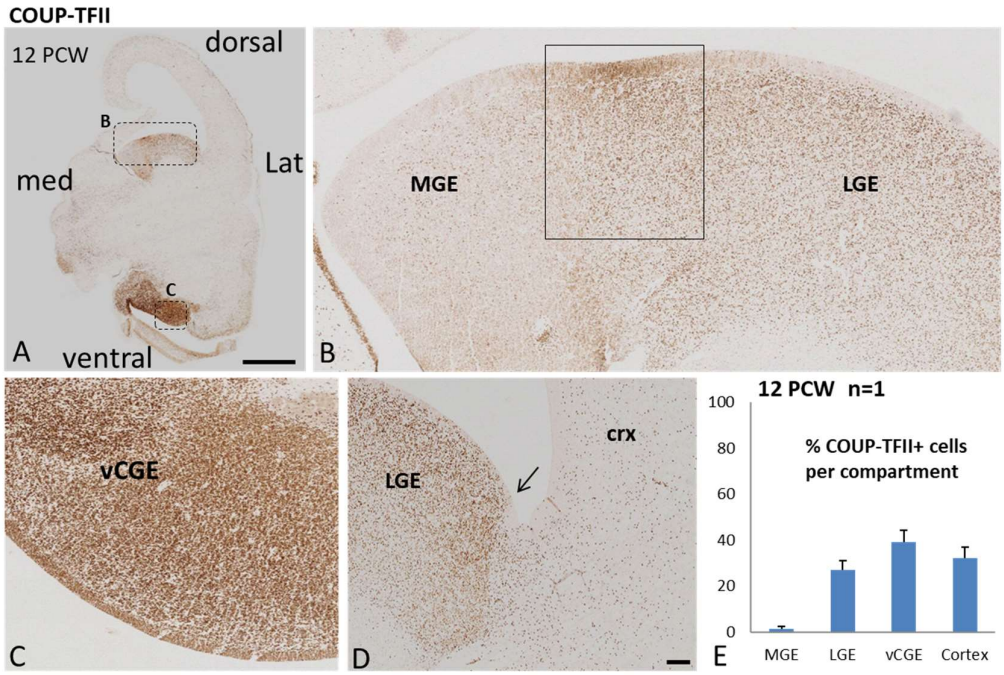

Figure 3.11: The expression pattern of COUP-TFII in human fetal forebrain at 12 PCW. (A, B,C) COUP-TFII was strongly expressed in VCGE, moderately expressed in LGE, with scattered COUP-TFII+ cells found in the MGE. Strong expression was observed in the VZ/SVZ at the boundary between MGE and LGE (boxed area in (B). (D) COUP-TFII expression showed a distinct cortical/subcortical boundary (arrow) between LGE and cortex (crx). (E) The distribution of COUP-TFII+ cells in the proliferative zones between different regions of human fetal forebrain at 12 PCW (n=1). A-D from brain No. 18. Scale bars: 1 mm in A; 100 μm in D (and for B,C).

Quantification of the average density of COUP-TFII+ cells in the proliferative zones of MGE, LGE, and VCGE (and cortex) in a coronally cut brain at 12 PCW showed that the proportion of all COUP-TFII+ cells located in the vCGE (~39%) was considerably higher than in the much larger LGE (27%) with only a very small proportion found in the MGE and ventral septum (<2%) (Figure 3.11E; Table 3.1). Examination of the cellular co-localization of COUP-TFII with KI67 in the GE subdivisions was performed to further investigate the origin of these COUP-TFII expressing cells. Notably, COUP-TFII/KI67 co-localization was only observed in the ventral compartment of the CGE (vCGE) and MGE/LGE boundary. Although the most dorsal part of the CGE (LCGE) showed high expression of COUP-TFII there was no evidence of dividing (KI67 expressing) COUP-TFII+ cells even in the proliferative zones (Figure 3.12A,B; figure 3.13A-C). Similarly, the MCGE showed relatively lower expression of COUP-

TFII and in post-mitotic cells only (Figure 3.12A,B). These findings are in agreement with Hansen *et al.* (2013b) who found a gradient of KI67 positive COUP-TFII cells between the most ventral part of the CGE and the more dorsal and anterior regions. Thus the vCGE is the main birth place for all COUP-TFII+ precursors in the ganglionic eminences but surprisingly most of these COUP-TFII+ cells precursors co-expressed PAX6, a marker for dorsal radial glial progenitor cells (Figure 3.13F).

The CGE is the major source of cortical CalR expressing interneurons in rodents (Miyoshi *et al.*, 2010) and COUP-TFII is required for the migration of CGE cell in the caudal migratory stream (CMS) into the posterior cortex (Tripodi *et al.*, 2004; Kanatani *et al.*, 2008). In human, the decreasing gradient of COUP-TFII+/CalR+ cells from the CGE compartments toward the LGE and anterior cortex (Figure 3.14) supported by the fact that vCGE not LGE (see above, Figure 3.13) is the source of most COUP-TFII+ cells, suggests the presence of additional anterior migratory stream of COUP-TFII/CalR expressing cells into the anterior cortex. It is worth mentioning that substantial number of cells in different regions in the GE and cortex expressed these two markers separately (Figure 13.14) suggesting that there is a distinct population of CalR-expressing GABAergic interneurons which are COUP-TFII independent.

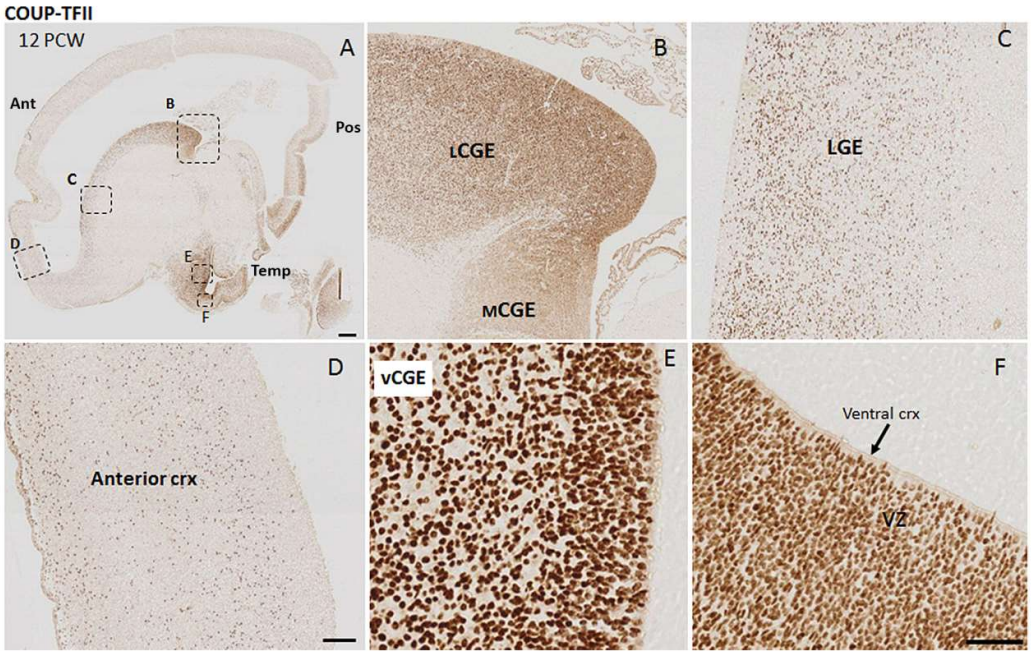

Figure 3.12: COUP-TFII was predominantly expressed in VCGE but spans sub-cortical/cortical domains in human fetal forebrain. (A) COUP-TFII expression in a sagittal section at 12 PCW. (B,C,D) COUP-TFII was highly expressed in the proliferative zone of LCGE with lower expression in the LGE and anterior cortex (ant). (E) The strongest expression was observed in the proliferative zone of vCGE where COUP-TFII+ cells were not organized radially. (F) Strong expression of COUP-TFII in the VZ of ventral/temporal cortex with radial nuclear morphology of COUP-TFII+ cells. Ant: Anterior, Pos: posterior; Temp: temporal, Crx: cortex. A-F from brain No. 17. Scale bars: 1mm in A; 100µm in D (and for B, C); 100µm in F (and for E).

3.3.8 COUP-TFII Expression in the dorsal telencephalon

A distinct distribution of COUP-TFII+ cells between the anterior and posterior cortex was found at 8 PCW. In the anterior cortex, COUP-TFII protein was localized to all layers. Although most COUP-TFII+ cells were located in the SVZ and IZ, a considerable number of cells were also observed in the VZ and CP (Figure 3.10C,E). A different distribution of COUP-TFII+ cells was observed in the posterior cortex, where cells were restricted to what appeared to be two migratory streams; a major one in the SVZ, and a less defined one in the nascent pre-subplate at the border between the IZ and the CP. few, if any, COUP-TFII+ cells were found in the VZ (Figure 3.10C,D). COUP-TFII was also expressed in cells in the outer layer of MZ

which are most likely Cajal–Retzius cells (Figure 3.10D,E; Meyer et al., 2000; Meyer et al., 2002; Zecevic et al., 2011).

By 12 PCW, about 32% of all COUP-TFII+ cells in proliferative zones of the telencephalon were located in the cortex (Table 3.1) and particularly dense immunoreactivity for COUP-TFII in the VZ and SVZ of the ventral parts of the frontal and temporal cortex was observed (with decreasing gradients from the medial wall to the lateral wall) located close to the vCGE (Figure 3.15A,B). Most of COUP-TFII+ cells in the VZ of ventral cortex showed a radial morphology whereas cells in the VZ of the VCGE showed disorganized morphology (Figure 3.12E,F). Similar to the vCGE, most of COUP-TFII+ cells in the VZ of ventral/temporal cortex were double labelled with KI67 (Figure 3.13D). Furthermore, we also found double labelling of COUP-TFII cells with the radial glial progenitor cell marker PAX6 (Figure 3.13F) and the post-mitotic glutamatergic neuron marker TBR1 (Figure 3.13H). In the anterior and dorsal cortical regions, a substantial number of COUP-TFII+ cells was observed in the VZ and SVZ and were not double labelled with PAX6 or TBR1 (Figure 3.13G,I) suggesting they are most likely GABAergic interneurons; interestingly, many of these cells were also dividing (co-expressing KI67; Figure 3.13E). Although it is still controversial that interneuron precursors can retain the proliferative capacity after migrating to the cortex (Hansen *et al.*, 2013b; Ma *et al.*, 2013; Radonjić *et al.*, 2014a) the presence of these dividing cells far distant from the vCGE (the origin of COUP-TFII+ interneuron precursors) may indicate that these cells are generated locally in the cortex.

Finally, we quantified the average density of COUP-TFII+ cells in the proliferative zones across the cortex (Figure 3.15A-F) and found a decreasing gradient of density from higher gradient in the ventral cortex to a lower gradient more dorsally. In a recent study, Reinchisi *et al.* (2012) reported that COUP-TFII+ cells are more abundant in the temporal/caudal cortex of human fetal brain, which was attributed to a caudal migratory stream from the CGE. However, our results (see above) suggest that, in humans, the neurogenic domain of COUP-TFII expressing progenitor cells is not confined to the CGE, but extends to the ventral cortex (Figure 3.13A) and includes radial glial progenitor cells that generate glutamatergic neurons.

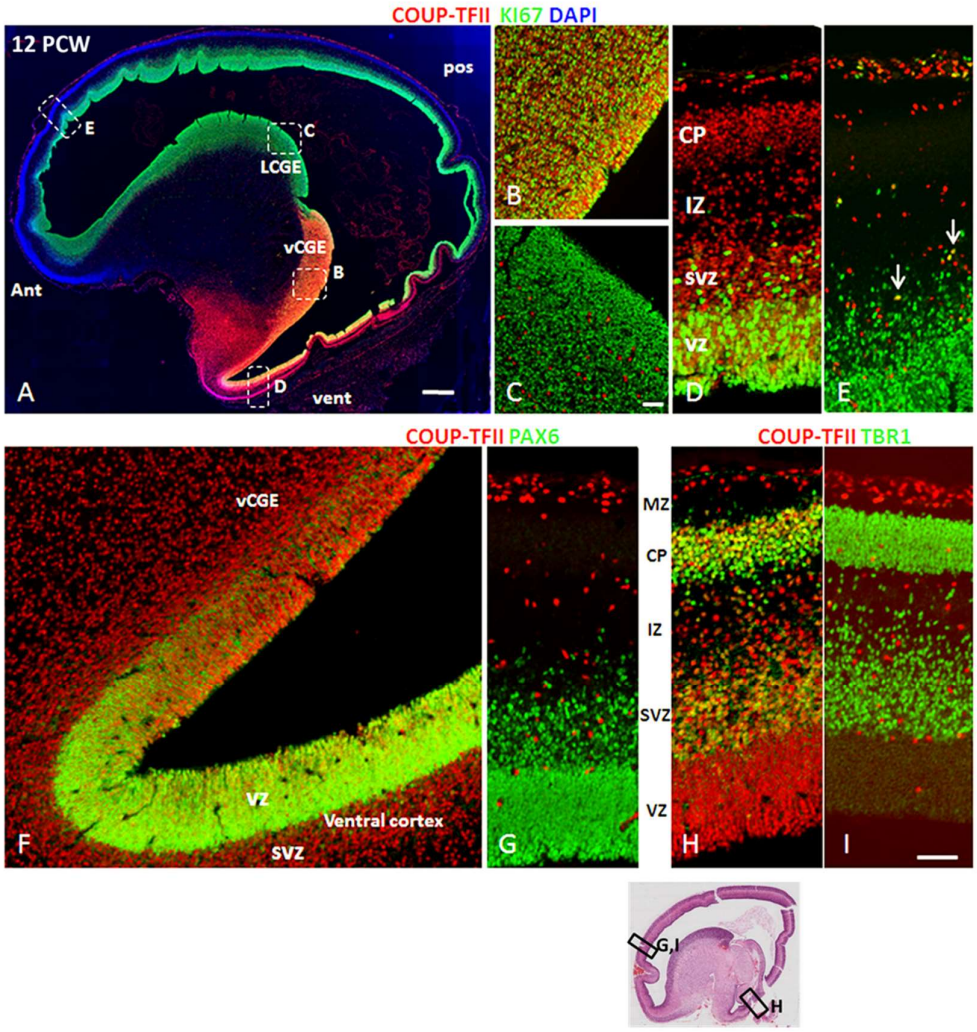

Figure 3.13: Double labelling for COUP-TFII (red) with KI67, PAX6, and TBR1 (green). (A) Double labelling for COUP-TFII and KI67 in sagittal section of 12 PCW human fetal brain (A). In the GE, COUP-TFII and KI67 co-localization was mainly observed in vCGE (yellow, B) but not in LCGE (C). In the cortex, Most of COUP-TFII+ cells in the proliferative zone in the ventral cortex also showed double labelling with KI67 (D) with only few double labelled cells seen in dorsal cortex (arrows, E). (F,G) COUPT-TFII+ cells in the proliferative zone of vCGE and ventral cortex co-expressed PAX6 (yellow, F) but not cells in the dorsal cortex (G). (H,I) A proportion of COUP-TFII + cells were double labelled with TBR1 in the ventral cortex (yellow, H), no double labelling was observed in the dorsal cortex (I). The inset is drawing of sagittal sections with boxed areas where images (G-I) were taken. A-I from brain No. 20. Scale bars: 1mm in A, 100 μm in C (and for B); 100 μm in I (and for D-H).

In conclusion, it appears that the neurogenic domain for COUPT-FII precursors occupies subcortical and cortical domains in early human fetal brain (Figure 3.15 G) expanding from the vCGE into the cortical wall of the ventral/temporal cortex where COUP-TFII might have a role in cortical arealization. However, dividing COUPTFII+ cells were also present in the dorsal and anterior cortical walls which might be interneuron precursors generated locally in the cortex; furthermore, an additional anterior migratory stream of COUP-TFII/CalR expressing cells from the vCGE via LGE into the anterior cortex was observed. Altogether, these findings could provide a reasonable explanation for the significantly higher incidence of CalR expressing interneurons in primate (Hladnik *et al.*, 2014).

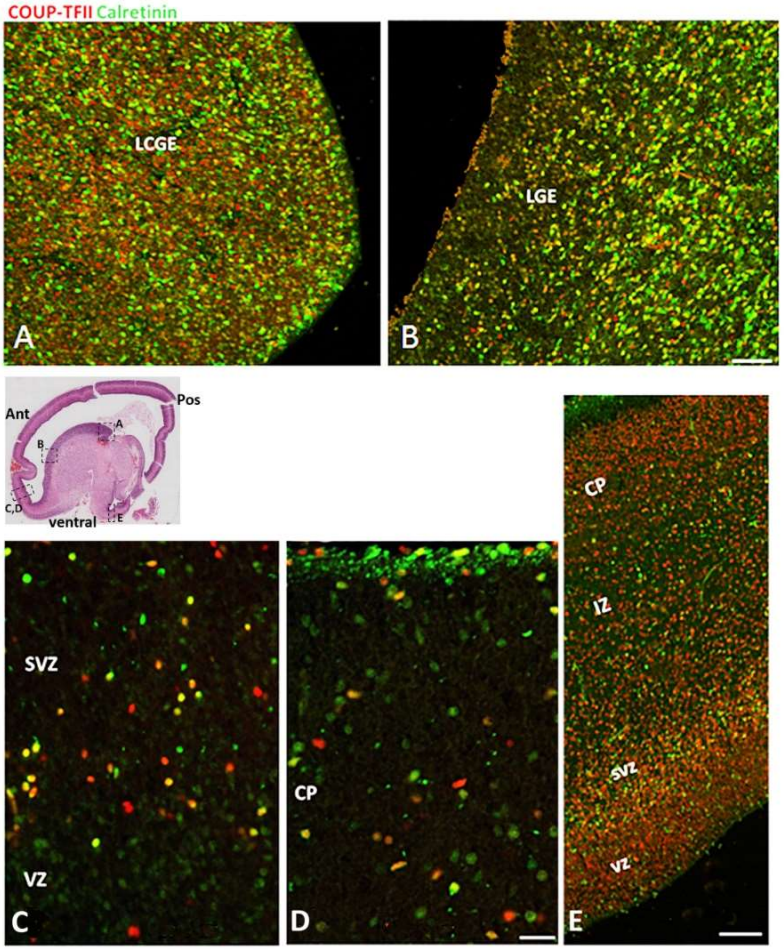

Figure 3.14: Double labelling for COUP-TFII (red) and calretinin (CalR, green) in a sagittal section at 12 PCW. Decreasing gradient of COUP-TFII /CalR double labelled cells (yellow) was observed from LCGE (A) LGE (B) and anterior cortical wall (C,D). Many COUP-TFII+ cells in the ventral cortex also co-expressed CalR (E). The inset is drawing of sagittal sections with boxed areas where images (A-E) were taken. A-E from brain No. 17. Scale bars: 50 μm in A, B; 20 μm in D (and for E); 100 μm in E.

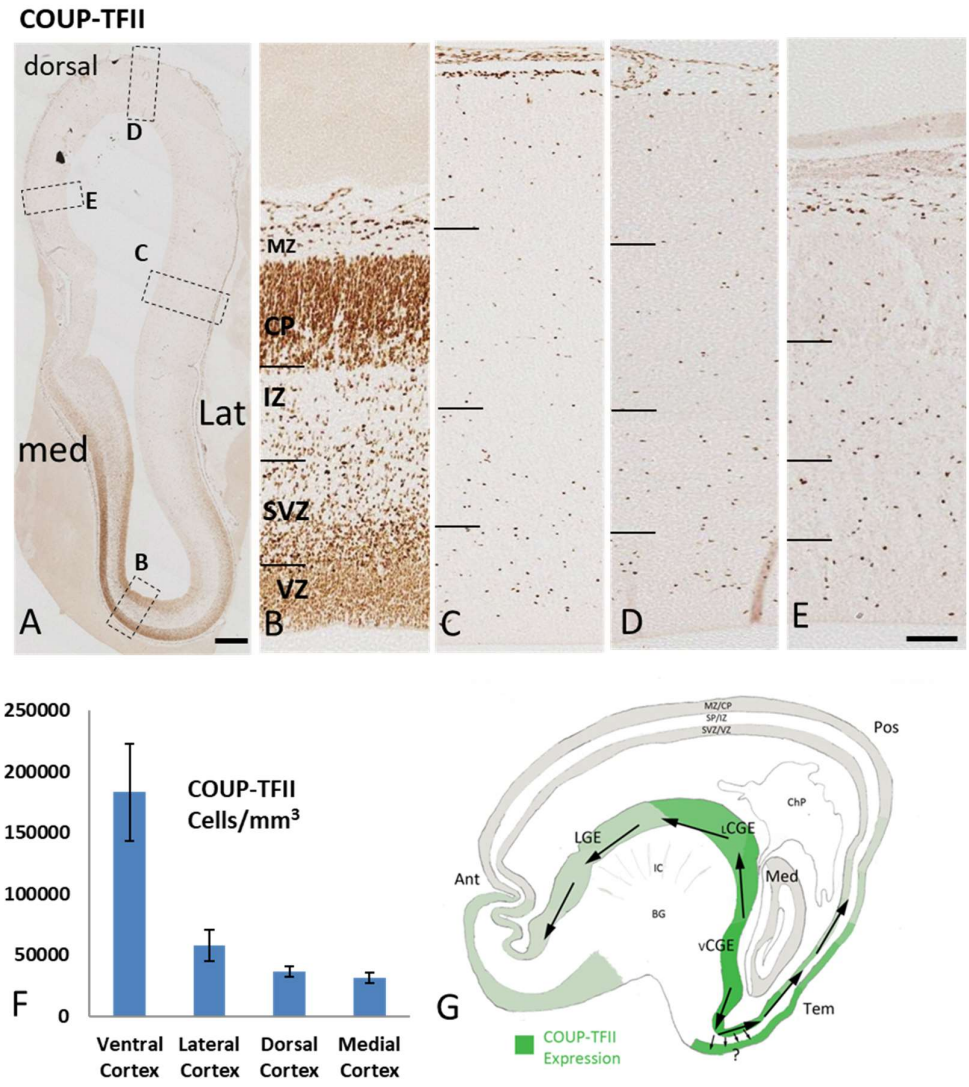

Figure 3.15: The distribution of COUP-TFII+ cells in different compartment of 12 PCW human fetal cortex. (A) COUP-TFII expression in frontal cortex (coronal section, 12 PCW). (B) COUP-TFII expression in ventral cortex (C) lateral cortex (D) dorsal cortex and (E) the medial cortex. (F) The average density of COUP-TFII+ cells in the ventral, lateral, dorsal and medial cortex. (G) Schematic diagram showing the distribution of COUP-TFII+ cells in the telencephalon and proposed migratory paths from the VCGE to the anterior and posterior cortex (large arrows). COUP-TFII progenitors also undergo division in the ventral cortex but the migratory paths and phenotype of the cells remains unclear (small arrows). A-E from brain No. 19. Scale bars: 1 mm in A; 100 μm in E (and for B-D). Ant, anterior cortex; Pos, posterior cortex; Tem, temporal cortex; Med, medial cortex; BG, basal ganglia; ChP, choroid plexus; MZ/CP, marginal zone/ cortical plate; SP/IZ, presubplate/intermediate zone; SVZ/VZ, subventricular zone/ventricular zone.

3.4 Discussion

We have described the expression patterns of three transcription factors important to the generation of cortical interneurons in the early fetal human telencephalon and demonstrated that they occupy distinct (although overlapping) neurogenic domains which can extend into the cortex. NKX2.1 was very largely confined to the MGE, MCGE and ventral septum, and at this stage of development these observations support previous studies suggesting interneuron generation from NKX2.1 positive cells may be identical in nature with the process occurring in rodents (Hansen *et al.*, 2013b; Ma *et al.*, 2013; Arshad *et al.*, 2015). OLIG2 was expressed by cells in the proliferative zones of the MGE, MCGE and septum, co-expressed with GAD65/67, but was not necessarily co-expressed with NKX2.1, and was also extensively expressed in the LGE**,** LCGE and in dividing cells in the cortex; observations previously unreported at this key stage of development. Within the ganglionic eminences dividing COUP-TFII precursors were localised to the VCGE as previously described (Hansen *et al.*, 2013b) but were also numerous in adjacent regions of ventral cortex. From careful examination of multiple expression patterns, we have been able to more accurately compartmentalise the CGE than has previously been attempted, and describe additional ventral to dorsal migratory streams for interneuron precursors not previously reported in rodents, as will be discussed in more detail below. Further evidence for interneuron generation in the human dorsal telencephalon has been presented.

3.4.1 Anatomical and molecular subdivisions of the CGE

In rodents the CGE has been identified as a source of specific GABAergic interneuronal subtypes different from those generated from the MGE (Miyoshi *et al.*, 2010; Rudy *et al.*, 2011). Some researchers have concluded that the CGE comprises caudal extensions of LGE and MGE, respectively by virtue of its gene expression patterns (Corbin *et al.*, 2003; Flames *et al.*, 2007). However as additional transcription factors such as COUP-TFI and COUP-TFII are enriched in CGE, it is possible that the CGE has evolved as a distinct neurogenic domain separate from the MGE and LGE (Kanatani *et al.*, 2008). The present study has revealed that in the developing human brain, the lateral and medial portions of the CGE share the expression patterns of PAX6, OLIG2, and NKX2.1 of the LGE and MGE, respectively. However, in addition to these lateral and medial portions of the CGE, the extension of the CGE along the

lateral ventricle into the greatly enlarged temporal lobe has produced a third compartment distinguishable by its characteristic co-localisation of intense COUP-TFII and PAX6 expression in the proliferative layers. Dividing COUP-TFII+ cells were confirmed as being confined to this ventral region of the CGE (Hansen *et al.*, 2013b). In addition, unlike the dorsally located lateral and medial portions, almost no NKX2.1+ cells were found in the VCGE. These findings suggest that the anatomical and molecular boundaries of the CGE should be defined carefully and separately, with the dorsal region formed from caudal extensions of the LGE and MGE, albeit with a higher density of post-mitotic COUP-TFII and CalR positive cells, and the ventral region having its own molecular signature including COUP-TFII positive progenitor cells.

3.4.2 Are anterior and medial migratory streams prominent in the human telencephalon?

Quantitative PCR, microarray, in situ hybridisation and immunohistochemical studies between 8-12 PCW have previously identified an anterior to posterior gradient of expression of multiple genes identified with GABAergic interneurons and GABAergic neurotransmission including transcription factors characteristic of interneuron precursors, isoforms of GAD, GABA receptor sub-units and calcium binding proteins (Bayatti *et al.*, 2008a; Ip *et al.*, 2010; Al-Jaberi *et al.*, 2015a) seemingly at odds with the accepted lateral (MGE derived) and posterior (CGE derived) pathways of migration for interneuron precursors from ventral to dorsal telencephalon (Wonders and Anderson, 2006). This led to speculation that the anterior cortex in particular may be a novel site for generation of interneurons in the primate telencephalon, perhaps to populate the enlarged prefrontal lobes of the primate brain (Al-Jaberi *et al.*, 2015a; Clowry, 2015). The present study offers up the alternative explanation that migrating interneurons may more rapidly invade the anterior than the posterior cortex, even from apparently caudal structures such as the vCGE. We saw evidence of an anterior migratory stream of COUP-TFII and CalR expressing cells from the vCGE, where COUPTFII expressing progenitors exclusively underwent division, to the anterior cortex via the LCGE, LGE and ventral pallium (Figure 3.12A-D, Figure 3.13A). Such cells were more numerous in the anterior than posterior cortex, as previously described for CalR+ neurons (Bayatti *et al.*, 2008a). Examination of our 3D reconstructions of the 12 PCW fetal brain confirmed that this path length is similar or even shorter than that from the VCGE to the dorso-posterior cortex via the temporal lobe (Figure 3.15G). Recently this migration route has been also described in rodents (Touzot *et al.*, 2016)

but may be more prominent in human which has expanded frontal associative area (Hladnik *et al.*, 2014).

In addition, we have observed increased expression of OLIG2 in the anterior compared to posterior cortex in agreement with previous studies (Ip *et al.*, 2010; Al-Jaberi *et al.*, 2015a) particularly at 8 PCW where there was also a distinct medial to lateral gradient of OLIG2 expression. In this case the migratory stream appeared to derive from progenitor cells in the MGE and sub-cortical septum, and enter the cortex via the medial wall. This is in direct contradiction to what has been reported in rodents where interneurons populating medial wall derived structures such as the hippocampus are described as deriving from the MGE and CGE via lateral migration (Pleasure *et al.*, 2000; Wonders and Anderson, 2006; Morozov *et al.*, 2009; Faux *et al.*, 2012). In our preparations we found evidence that OLIG2+ and NKX2.1+ progenitors reside in the septum and OLIG2+ cells, at least, migrate medially to the cortex. Again this is in disagreement with findings in rodents, where septum derived cells were reported not to enter the cortex at all (Rubin *et al.*, 2010). Thus we propose that the human or primate brain possesses an additional medial migratory pathway (Figure 3.6) for GABAergic interneurons populating frontal and medial areas of the cerebral cortex. The much larger human cortex may require additional migratory pathways compared to smaller mammalian brains. However, it is worth noting that a medial migratory pathway for NKX2.1 positive precursors from the MGE to the medial pallium has recently been reported in the shark (Quintana-Urzainqui *et al.*, 2015) therefore such a pathway cannot be proposed as evolutionarily novel to the human brain. Instead we might speculate that this is missing or relatively small and overlooked in rodent compared to other vertebrate species.

3.4.3 Potential dorsal telencephalic origin of GABAergic interneurons

Based on studies conducted principally around mid-gestation, Radonjić *et al.* (2014a) proposed that three mechanisms exist for the production of cortical interneurons in primates; generation in the ventral telencephalon followed by migration to the cortex, precursors arriving in the cortex from the ventral telencephalon and undergoing further division intra-cortically, and cortically derived progenitors giving rise to interneurons. The last two proposals are controversial, being firmly rejected by recent influential and persuasive studies (Hansen *et al.*,

2013b; Ma *et al.*, 2013; Arshad *et al.*, 2015). However, our present study found clear evidence for the second mechanism. OLIG2+ precursors appeared to follow migratory paths into the cortex, however OLIG2+ cells were also shown to be undergoing proliferation and these OLIG2+ cells did not co-express any markers of cortically derived progenitors such as PAX6 or TBR2 (although such double-labelling has been reported at later stages of human development (Jakovcevski and Zecevic, 2005)). This firmly suggests that OLIG2 is not immediately downregulated in cells entering the cortex from subcortical structures, unlike NKX2.1, and that these cells may retain the ability to divide within the cortex, preferentially within anterior and medial locations, where the highest density of such cells was found. However, there also remains the possibility that OLIG2+/TBR2- intermediate progenitor cells are generated by cortical radial glial progenitor cells which go on to produce GABAergic interneurons.

It is also clear that in the more ventral areas of the anterior and temporal cortex there is high expression of COUP-TFII expressing progenitor cells and post-mitotic neurons. That these cells co-express either PAX6 or TBR2, and that post-mitotic cells co-expressing TBR1 and COUPTFII were also observed, demonstrates that in the cortex dividing COUP-TFII+ progenitors give rise to glutamatergic neurons. Although there are also COUP-TFII+/CalR+ presumptive interneurons present, it is impossible to judge whether these have migrated in from the adjacent CGE, or been generated intra-cortically. However, small numbers of COUP-TFII cells were also found undergoing division in anterior and dorsal regions of the cortex did not co-express PAX6 suggesting they are most likely interneuron progenitors. Whether they migrated to the cortex, retaining the capacity to divide, or were born in the dorsal telencephalon, is still open to debate. However, a neuronal progenitor marker GSX2, expressed upstream of COUP-TFII, which localises to the LGE and CGE in rodent (Hsieh-Li *et al.*, 1995; Wang *et al.*, 2013) has been found to be expressed in cells undergoing division in the VZ/SVZ of the human fetal cortex (Radonjic *et al.*, 2014) making intra-cortical generation a possibility.

Whether or not proliferative NKX2.1+ progenitor cells are present in the cortex is contentious. Our observation at 12 PCW of NKX2.1+ cells throughout the latero-medial extent of the cortical wall, making up about 2.4% of all NKX2.1+ cells in the proliferative zones of the telencephalon at this time, whivh is in conflict with Hansen *et al.* (2013b) who reported nearly no NKX2.1+ cells in the cortex and only close to LGE/lateral cortex border. However, our

findings are in partial agreement with Radonjić *et al.* (2014a) who found NKX2.1+ cells in the cortical wall of human and macaque monkey fetal forebrains (at later stages of development, 15-22 PCW for human) undergoing active division, as did Arshad *et al.* (2015) in human between 16-28 PCW although in very small numbers. As no NKX2.1+ cells were seen in the cortex at 8PCW in agreement with previous studies (Hansen *et al.*, 2013b; Pauly *et al.*, 2014) we propose that with age the incidence of NKX2.1+ cells in the cortex gradually increases, along with the capacity to undergo proliferation. Whether these cells are generated in the cortex or have migrated there from the ventral telencephalon without downregulating NKX2.1 remains a question for further investigation.

3.4.4 OLIG2 and COUP-TFII as regulators of cortical arealisation

The division of the cerebral cortex into functional areas (the cortical map) differs little between individuals in any given species (Rakic *et al.*, 2009). Previous work on rodent development has identified certain transcription factors (e.g. PAX6, SP8, EMX2, COUP-TFI) expressed in gradients across the neocortex that appear to control regional expression of cell adhesion molecules and organization of area specific thalamocortical afferent projections (López-Bendito and Molnár, 2003; O'Leary *et al.*, 2007; Rakic *et al.*, 2009). There may be common mechanisms between species, as the developing human neocortex displays counter-gradients of PAX6 and EMX2 at early stages of cortical development (Bayatti *et al.*, 2008b). However the human cerebral cortex is composed of different and more complex local area identities and so might be specified by a wider range of transcription factor gradients; for instance an anterior to posterior gradient of CTIP2 expression has been observed in human early fetal cortex (Ip *et al.*, 2011). In the present study, a prominent anterior to posterior gradient of OLIG2 expression, and a ventral to dorsal gradient of COUP-TFII expression were observed. In both cases the transcription factors are also expressed at moderate levels in the cortical plate as well as the proliferative zones, suggesting that areal specification mechanisms in cells extend into the post-mitotic period. The extent to which these gradients interact with interneuron precursors is not known, but we might speculate that OLIG2 or COUP-TFII control expression of cell adhesion molecules locally that attract migrating cells expressing the same transcription factors, setting up the migratory pathways into the cortex for interneurons arriving medially via the septum (OLIG2+) or laterally via ventral anterior or temporal cortex (COUP- TFII+).

3.5 Conclusion

Evidence continues to accumulate that cortical GABAergic interneuron production in primates differs in certain details from what has been learnt from our rodent models. A higher proportion of interneurons arise from the CGE in primates and we provide a description of the compartmentalisation of the CGE. This chapter presents further evidence that interneuron precursor cells may undergo division in the cortex, although it remains to be proven whether they are originally generated in the dorsal telencephalon. Finally, whereas in rodents interneuron precursors are believed to enter the cortex from the ganglionic eminences exclusively via lateral and posterior routes, in human we provide evidence of pathways via the anterior and medial cortex.

Note: text, data and figures in this chapter have been taken from a recently published original article (Alzu'bi *et al.*, 2017) published under a creative commons licence (See appendices):

- Alzu'bi, A., Lindsay, S., Kerwin, J., Looi, S.J., Khalil, F. and Clowry, G.J. (2017) **'Distinct cortical and sub-cortical neurogenic domains for GABAergic interneuron precursor transcription factors NKX2. 1, OLIG2 and COUP-TFII in early fetal human telencephalon'**, Brain Structure and Function, 222(5), pp. 2309-2328.

Chapter 4: Arealisation and GABAergic Interneuron Specification in the Early Human Fetal Telencephalon.

4.1 Summary

Studies in rodents show roles for the transcription factors COUP-TFI, COUP-TFII and SP8 in telencephalic patterning and neuron migration. In human dorsal telencephalon at 8-12 post-conceptional weeks, RNAseq and immunohistochemistry revealed cortical COUP-TFI expression in a high ventro-posterior to low anterior gradient except for raised immunoreactivity in the anterior ventral pallium. SP8, on the other hand exhibited a distinct counter gradient of expression from high anterior to low ventro-posterior. However, unlike in mouse, COUP-TFI and SP8 were extensively co-expressed in dorsal sensory neocortex and dorsal hippocampus. On the other hand COUPTFI/COUPTFII co-expression defined ventral temporal cortex and ventral hippocampus. In the ganglionic eminences COUP-TFI immunoreactivity demarcated the proliferative zones of CGE, dorsal MGE, MGE/LGE boundary, and ventral LGE whereas COUP-TFII was limited to ventral CGE and the MGE/LGE boundary. SP8 was expressed in the SVZ of the LGE, ventral CGE, and LGE-like septum. Co-labelling with GABAergic interneuron markers revealed that COUP-TFI was expressed in subpopulations of either MGE-derived (SOX6+) or CGE-derived (calretinin+) cortical interneurons. COUP-TFII expression was mainly confined to CGE-derived interneurons. Twice as many GAD67+ cortical cells co-labelled for COUP-TFI than for COUP-TFII and a fifth of COUP-TFI+ cortical cells also co-expressed COUP-TFII. SP8 was expressed in a population of CGE- derived COUP-TFI/II positive interneurons; however, the LGE could be also a source of cortical interneurons that expressed SP8 solely. In conclusion, the expression pattern of these three and other related TFs delineates the subdivisions of the developing human GE and several routes of cell migration from the GE compartments into the cortex (anterio-laterally and posteriorly), basal telencephalon, and olfactory bulb.

4.2 Introduction

COUP-TFI and COUP-TFII are related transcription factors that show very high degrees of homology in their C-terminal ligand binding domain (97%) and central DNA binding domain (80%) but far less in their N-terminal region (45%) which includes an activation function domain (Wang *et al.*, 1989; Ladias and Karathanasis, 1991; Li *et al.*, 2003). This suggests that although they may control transcription in a similar way, each COUP-TF may respond to quite different sets of activators or repressors. Therefore, it is perhaps not surprising that the COUP-TFs are expressed in overlapping but still distinct patterns of expression in developing mouse forebrain. COUP-TFI is expressed in a high caudal to low rostral gradient across the whole cerebral cortex from Embryonic day (E) 9.5 whereas COUP-TFII is only highly expressed in more restricted regions, either nested within the COUP-TFI expressing domain but limited to the caudal-most region of the neocortex, or expressed in the caudo-medial wall where COUP-TFI expression is low (Qiu *et al.*, 1994; Takiguchi-Hayashi *et al.*, 2004; Tripodi *et al.*, 2004; Flore *et al.*, 2016). In the ganglionic eminences expression of both COUP-TFs is considered characteristic of the CGE (Tripodi *et al.*, 2004). However, COUP-TFI is actually expressed throughout the mouse ganglionic eminences from E10.5 to E12.5 before becoming restricted to the corticostriatal boundary, dorsal MGE, and CGE, by E13.5 (Lodato *et al.*, 2011).

In mouse, the high expression of COUP-TFI caudally plays an important role in cortical arealisation by suppressing frontal cortex associated gene expression (Armentano *et al.*, 2007; Faedo *et al.*, 2008; Borello *et al.*, 2013; Alfano *et al.*, 2014a). COUP-TFI is known to promote the caudal identity of cortical region mainly by opposing the function Fgf signalling (released from the ANR and known to promote rostral cortical development) by repressing the downstream effector of Fgf 8 and 17, MAPK/ERK signalling, (Faedo *et al.*, 2008) and promoting the expression of negative regulators of Fgf8, sprouty 1 and 2 (Faedo *et al.*, 2010). Unlike other transcription factors that are involved in the cortical patterning in mouse (PAX6, EMX2, and SP8) COUP-TFI regulates the areal patterning not only in the neural progenitors, but also in the post-mitotic cells (Liu *et al.*, 2000; Alfano *et al.*, 2014a) although EMX2 has been shown to be expressed in post-mitotic cells early in human cortical development (Bayatti et al, 2008b). Inactivation of COUP-TFI function in post-mitotic neurons resulted in expansion of the motor area at the expense of the sensory area (Alfano *et al.*, 2014a). COUP-TFI has been also shown to have a number of roles in defining the functional identity of cortical neurons.

For instance, it suppresses the differentiation of corticospinal motor neurons in the caudal somatosensory cortex, allowing for their correct specification in frontal cortex (Tomassy *et al.*, 2010).

COUP-TFI promotes faster radial migration of newborn glutamatergic neurons towards the cortical plate (Alfano *et al.*, 2011) ensuring that concomitant axon outgrowth from these caudal/temporal cortical neurons happens in time to meet developmental deadlines such as when callosal axons are permitted to cross the midline. On the other hand, the role and/or mechanism by which COUP-TFII could contribute to the cortical map is not currently well understood; in mouse COUPTFII expression is limited to the most caudoventral telencephalon with a medial high to lateral low gradient (Tang *et al.*, 2012). In human, we have previously shown (See chapter 3) that COUP-TFII is highly expressed in the progenitor and post-mitotic zones of the ventro-temporal region, mainly in cells that give rise to glutamatergic neurons, suggesting that COUP-TFII also has a potential role in cortical arealization.

Similarly, COUP-TFI and COUPT-TFII showed distinct expression patterns in mouse GE suggesting a role in specifying GABAergic forebrain neuron phenotype. Conditional loss of COUP-TFI function in the intermediate progenitor and post-mitotic interneurons alters the balance between CGE and MGE derived cortical interneurons without reducing their total number. CGE- derived interneurons were significantly decreased, whereas the number of MGE-derived interneurons increased (Lodato *et al.*, 2011). It is proposed that before E13.5, COUP-TFI expression in the MGE inhibits division of progenitor cells, and at later stages promotes production of CGE-derived interneurons. COUP-TFII expression is largely restricted to CGE-derived interneurons (Kanatani *et al.*, 2008; Miyoshi *et al.*, 2010). Both transcription factors control rate and direction of cell migration (Tripodi *et al.*, 2004) by regulating expression of molecules crucially involved in cell migration such as neuropilins (Tang *et al.*, 2012) and the chemokine CXCL12 and its receptor CXCR4 (Boudot *et al.*, 2014) important in controlling tangential migration of interneurons and Cajal-Retzius cells (Stumm *et al.*, 2003; Borrell and Marín, 2006). COUP-TFI and COUP-TFII are expressed in different populations of cells in the GE; COUP-TFI in cells following dorsal (to cortex) and ventro-caudal (to diencephalon) migratory pathways (Tripodi *et al.*, 2004) and COUP-TFII in caudally migrating cells from the CGE to the most posterior part of the telencephalon (Yozu *et al.*, 2005; Kanatani *et al.*, 2008; Faux *et al.*, 2012) however, conditional knockdown of COUP-TFII has no effect

upon caudal migration of cortical interneurons which appear to maintain expression of COUP-TFI instead (Tang *et al.*, 2012). In human, to our knowledge, the expression of COUP-TFI in human ventral telencephalon and in a population of cortical GABAergic interneurons has not been identified up to until now. Whereas, consistent with previous findings (Hansen *et al.*, 2013b), we have shown in the previous chapter that vCGE and MGE/LGE boundary are the main source of COUP-TFII+ cells in the GE of human fetal brain; in addition, a proportion of CalR+ cells found in the cortex also co-localized COUPTFII.

Another transcription factor that is widely expressed in ventral and dorsal telencephalon and known to have essential roles in the cortical arealization and generation of GABAergic interneurons is the zinc finger transcription factor SP8 (Waclaw *et al.*, 2006; Sahara *et al.*, 2007; Waclaw *et al.*, 2010; Ma *et al.*, 2012; Borello *et al.*, 2013). In dorsal telencephalon, SP8 is a downstream effector of FGF8 which is required to promote rostral cortical identity (Sahara *et al.*, 2007). SP8 is expressed in a complementary pattern to COUP-TFI with high rostral to low caudal gradient (Sahara *et al.*, 2007); while COUP-TFI represses FGF signalling (Faedo *et al.*, 2008; Faedo *et al.*, 2010) SP8 maintains *Fgf8* transcription in the ANR (Sahara *et al.*, 2007). The opposite expression of these two transcription factors regulates the balance of cortical patterning between frontal/motor and caudal/sensory areas (Armentano *et al.*, 2007; Borello *et al.*, 2013). In ventral telencephalon, SP8 plays an essential role in the differentiation of mouse LGE-derived interneurons that populate the amygdala and the olfactory bulb via the rostral migratory stream (Waclaw *et al.*, 2006; Waclaw *et al.*, 2010). However, in human and mouse, SP8 is also expressed in a proportion of cortical interneurons that are most likely derived from the dorsal lateral/ caudal GE (Ma *et al.*, 2012; Ma *et al.*, 2013).

4.3 Aim of Study

The present chapter firstly aimed to explore the extent to which expression of COUP-TFs and SP8 the in the human forebrain mirrors that in the rodent models. Secondly, to investigate the potential role for COUP-TFs and SP8 in interneuron specification, and which migratory pathways interneurons expressing these TFs follow. Finally, to reveal the complex subdivisions of the human ganglionic eminences based on the expression of COUP-TFs, SP8**,** and other related transcription factors.

4.4 Results

4.4.1 Gradients of COUP-TFI, COUP-TFII and SP8 mRNA expression across the developing cerebral cortex.

Thirty four samples of RNA were taken at 9-10 PCW and sixty seven at 11-12 PCW from four different regions of the cerebral cortex (Figure 4.1A) and subjected to quantitative RNA seq analysis. Expression of *COUP-TFI, COUP-TFII* and *SP8* was compared with two other genes, *FGFR3 and ROBO1* that are predicted to show gradients of expression from previous animal experiments and human studies (Iwata and Hevner, 2009; Ip *et al.*, 2010; Ip *et al.*, 2011; Miller *et al.*, 2014). Both *COUP-TFI* and *COUP-TFII* were expressed across the cortex between 9 and 12 PCW (Figure 4.1B,C) however *COUP-TFI* was more highly expressed than *COUP-TFII* in all regions and in both age groups. Significantly greater *COUP-TFI* expression was observed in the temporal and posterior, compared to central and anterior cortex, at 9-10 PCW ($p<0.05$; Figure 4.1B). Although a decrease in *COUP-TFI* expression was observed in the temporal and posterior cortex at 11-12 PCW, the levels still remained significantly higher in these regions (Figure 4.1B). *COUP-TFII* showed a consistent but more confined expression pattern with significantly higher expression observed in the temporal lobe compared to all other cortical regions. No significant difference was observed between anterior, central and posterior regions at 9-10 PCW, although there were differences at 11-12 PCW. *COUP-TFII* expression also decreased in the temporal regions with age but remained significantly higher than in the rest of the cortex (Figure 4.1C). *SP8* mRNA showed a complementary expression pattern with

COUP-TFI and *COUPTFII* mRNA; a higher level expression was observed in anterior and central regions compared to posterior and temporal regions at 9-10 PCW. At 11-12 PCW, the overall expression considerably decreased; however, the expression level in anterior and central regions were significantly higher than in other cortical regions (Figure 4.1D).

We tested our RNAseq data for the expression of other genes like *ROBO1* and *FGFR,* two genes that have known gradient expression patterns in the developing human cortex (Ip *et al*., 2010; Ip *et al.,* 2011; Miller *et al.,* 2014), and confirmed that graded expression can occur in the human cortex in both directions at these developmental stages and that the gradients seen for *COUP-TFs and SP8* were not an artefact of the experimental procedure. *ROBO1* showed decreasing anterior to posterior gradients as expected (Figure 4.1E) (Ip *et al.*, 2010; Ip *et al.*, 2011), whereas *FGFR3* showed a distinct counter gradient, particularly at the earlier ages, similar to *COUP-TFI* (Figure 4.1F) (Ip *et al.*, 2010; Miller *et al.*, 2014).

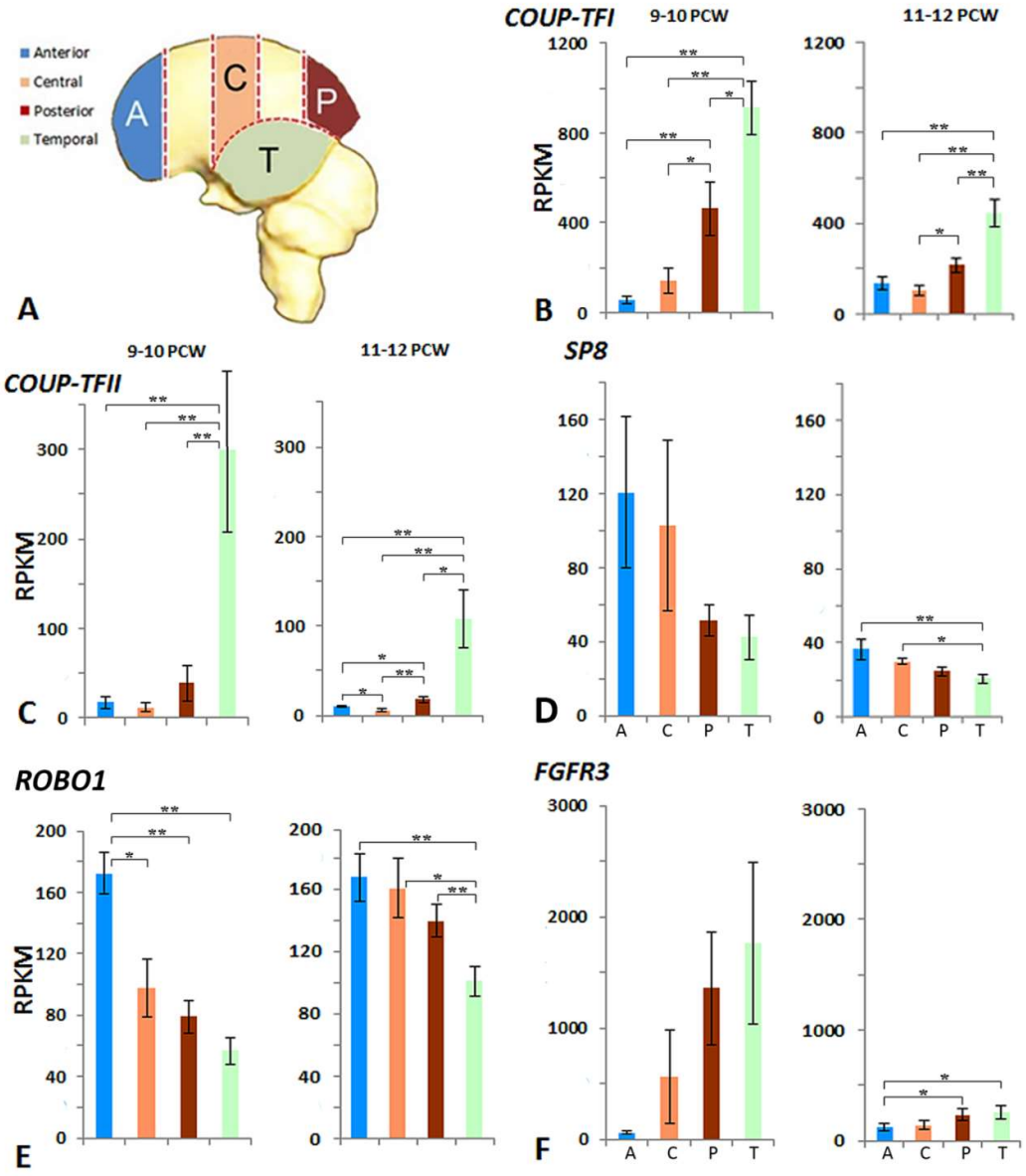

Figure 4.1: Gradients of *COUP-TFI, COUP-TFII, SP8, ROBO1*, and *FGFR3* expression across the cortex by RNAseq. (A) The location of four cortical sampling cortical regions; anterior (A), central (C), posterior (P), and temporal (T). (B) *COUP-TFI* expression is significantly higher in temporal and posterior regions at all ages. (C) Highest expression of COUP-TFII in the temporal regions, with no significant difference in the expression between anterior, central, and posterior regions at 9–10 PCW, although there were differences at 11–12 PCW. (D) SP8 was expressed in decreasing anterior posterior gradient at 9-10 PCW, the overall level of expression decreased at 11-12 PCW. (E) ROBO1 at both 9-10 and 11-12PCW is expressed in decreasing anterior posterior gradients (F) FGFR3 expressed in increasing anterior posterior gradient at both timepoints. Asterisk(s) represent statistically significant differences between regions (1-way ANOVA, Tukey's post hoc comparison, *P < 0.05, **P < 0.01). Error bars represent the standard error of the mean.

4.4.2 Characterization of COUP-TFI expression across the cortex and in relation to COUP-TFII and SP8

In sagittal sections from across the medio-lateral axis of 8 PCW human telencephalon, COUP-TFI immunoreactivity was seen to increase along an anterior to posterior gradient (Figure 4.2A-A''; Figure 4.3A) consistent with the previous reports in rodents (Armentano *et al.*, 2007; Borello *et al.*, 2013; Alfano *et al.*, 2014a). This gradient was not confined to the proliferative ventricular (VZ) and subventricular (SVZ) zones of the cortical wall, but was also observed in the post-mitotic intermediate zone (IZ) and cortical plate (CP; Figure 4.3A-D). Generally, cellular COUP-TFI immuno-labelling was found in all layers of the posterior cortical wall, however, relatively stronger expression was observed in proliferative compared to the post-mitotic zones. A gradual decrease in COUP-TFI immunoreactivity was seen through the central to the anterior cortex (Figure 4.3B-D). COUP-TFI expression in the VZ of the anterior cortex was restricted to only a few scattered cells, however weak to moderate immunoreactivity was observed in many cells of the SVZ/IZ/CP, indicating a more important role for this transcription factor in post-mitotic neurons of the anterior cortex (Figure 4.3B). Interestingly, in the ventral pallium (VP; the part of the developing cortex immediately adjacent to the LGE) there was strong expression in both the proliferative and post-mitotic zones even in more anterior regions (Figure 4.2A'). COUP-TFI was also expressed in the cortical hem, choroid plexus, and pia matter but with no apparent gradients across these structures (Figure 4.2A-A''; Figure 4.3A).

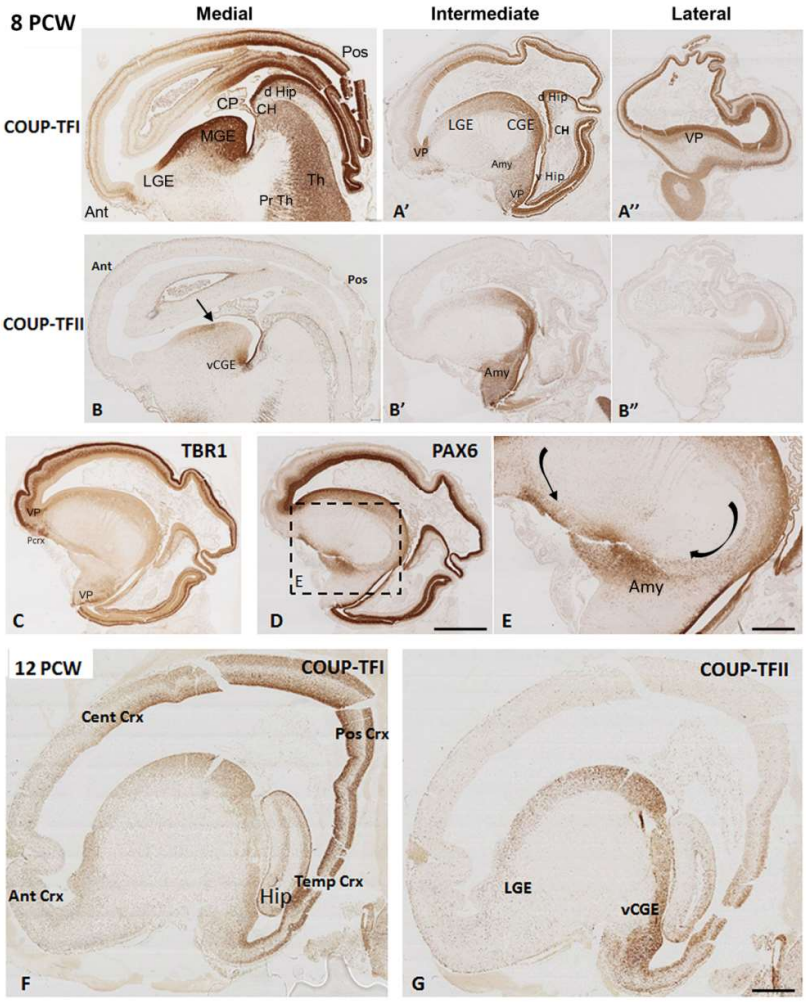

Figure 4.2: Gradients of COUP-TFI and COUP-TFII immunoreactivity across the telencephalon. (A-A'') Parasagittal sections from medial to lateral parts of the 8 PCW fetal brain; COUP-TFI was highly expressed in the majority of the GE except the dLGE, strong expression was also observed in the proliferative zones and cortical plate of posterior, lateral and temporal cortex but also anterior ventral pallium. (B-B'') COUPT-FII was mainly expressed in the vCGE and at the MGE/LGE boundary (arrow, B) and temporal ventral pallium (VP, B', B'') and amygdala (Amy, B') with dispersed scattered cells throughout the rest of the GE. (C) TBR1 expression shows location of anterior and temporal ventral pallium and piriform cortex (Pcrx). D and E show that two streams of PAX6 positive cells appear to migrate (arrows) from the LGE and CGE towards the amygdala and accumulate there. (F, G) COUP-TFI was expressed in high ventro-posterior to low dorso-anterior gradient across the cortex; COUP-TFII expression was high in the ventral/temporal cortex and low in the dorsal cortex. Ant: anterior (= rostral), pos: posterior (=caudal), cent: central, Crx: cortex, Pcrx: piriform cortex, CP: choroid plexus, d and v Hip: dorsal and ventral hippocampus, CH: cortical hem, LGE: lateral ganglionic eminence, CGE: caudal ganglionic eminence, vCGE: ventral caudal ganglionic eminence, VP: ventral pallium, Th: thalamus, Pr Th: pre thalamus, Amy: amygdala. A-E were taken from brain No. 1; F and G were taken from brain No. 17 (Table 2.1). Scale bars: 2mm in D (and for A-C); 500 µm in E, 2mm in G (and for F).

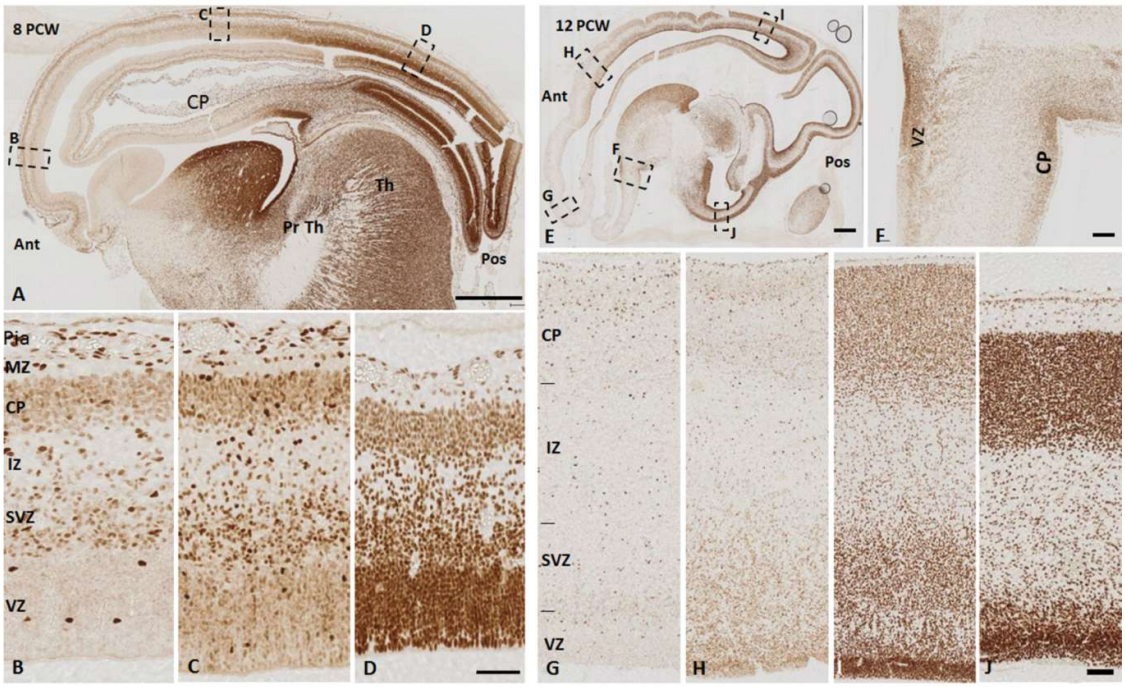

Figure 4.3: COUP-TFI immunoreactivity in the cortical wall of early human fetal brain. (A) Distinct high posterior to low anterior gradient in COUP-TFI expression across cortex in sagittal section 8 PCW. (B) Only a few scattered COUP-TFI+ cells observed in anterior cortex VZ, moderate to weak immunoreactivity in the SVZ/IZ/CP. (C) Moderate immunoreactivity across cortical wall of central cortex. (D) Strongest immunoreactivity in posterior cortex, relatively stronger in proliferative than in postmitotic zones. (E) Similar expression gradient at 12 PCW. (F) Strong COUP-TFI expression in proliferative and postmitotic zones of ventral pallium (VP). (G) COUP-TFI+ cells broadly distributed to all cortical layers of anterior cortex at 12 PCW. (H–J) COUP-TFI expression increased gradually in central, posterior, and temporal (ventral) regions, respectively. Boxed areas in A and E show where images (B–D and F–J) were taken. Ant, anterior; pos, posterior; VZ, ventricular zone; SVZ, subventricular zone; MZ, marginal zone; cp, choroid plexus; Pia, pia matter; VP, ventral pallium; Th, thalamus; Pr Th, pre-thalamus. A-D were taken from brain No. 1; E-J were taken from brain No. 20. Scale bars: 500 μm in A, E; 100 μm in D (and for B, C); 100 μm in F; 100 μm in J (and for G, H, I).

This gradient was maintained at later stages (10 and 12 PCW) and there was still strong expression in the VP (Figs. 4.2F; Figure 4.3E-J; Figure 4.4G). However, a change in the expression pattern in the cortical wall of the anterior cortex was observed by 12 PCW, with scattered COUP-TFI+ cells localized to all layers, including occasionally in the VZ (Figure 4.3G). In general, COUP-TFI protein expression consistently reflected the mRNA expression level seen by RNA seq analysis. Furthermore coronal sections through the anterior- posterior axis of the telencephalon revealed, in addition to a posterior/anterior gradient, a pronounced

decreasing ventral to dorsal gradient of COUP-TFI expression in both the proliferative and post-mitotic zones (Figure 4.5A,B).

COUP-TFI positive (+) cells showed a distinct pattern of co-localization with three cortical cell type-specific markers PAX6 (radial glia) TBR2 (intermediate progenitors) and TBR1 (post-mitotic pyramidal neurons) (Bayatti *et al.*, 2008a; Lui *et al.*, 2011) in different cortical areas (Figure 4.6). In posterior cortex, most COUP-TFI+ cells double-labelled with PAX6, TBR2 or TBR1, however a small proportion expressed COUP-TFI alone (Figure 4.6A'-C'). In contrast, the majority of COUP-TFI+ cells in the anterior cortex did not co-localise with cortical markers (Figure 4.6A-C). Double immunofluorescence with the cell division marker KI67 (Scholzen and Gerdes, 2000) revealed that most actively proliferating COUP-TFI+ cells were located in the posterior cortex; however a few were seen in the anterior cortex (Figure 4.7A-C). Thus in posterior/temporal cortex COUP-TFI is expressed by both progenitor cells and post-mitotic pyramidal neurons. In the anterior cortex, COUP-TFI is almost entirely confined to a few post-mitotic cells likely to be of sub-cortical origin.

Finally, as was demonstrated in chapter 3 (section 3.3.8) COUP-TFII immunoreactivity was expressed within the ventro-temporal region nested within the larger COUP-TFI expressing domain (Figure 4. 2B',B'',G; Figure 4.5C). COUP-TFI showed a complementary expression pattern to SP8 immunoreactivity which was expressed in a counter gradient from high anterior to low posterior as previously described in rodents (Sahara *et al.*, 2007; Zembrzycki *et al.*, 2007) (Figure 4.8A,F; Figure 4.9A). Interestingly, whereas there was extensive overlap of expression of COUP-TFI and SP8 in parietal, dorso-posterior, dorso-temporal and dorsal hippocampus (Figure 4.9A), SP8 and COUP-TFII expressions formed sharp boundaries such that SP8 was excluded from the ventral posterior and temporal cortex including the ventral hippocampus (Figure 4.9C). Unlike COUP-TFI and COUP-TFII, SP8 was downregulated in post-mitotic neurons in the cortical plate (Figure 4.9A,C). These observations are in accord with the RNA seq data (see above) and previous observations by immunohistochemistry (Ma *et al.*, 2013) but the double labelling experiments presented in this study throw light on possible interactions between the three transcription factors in human cortical development.

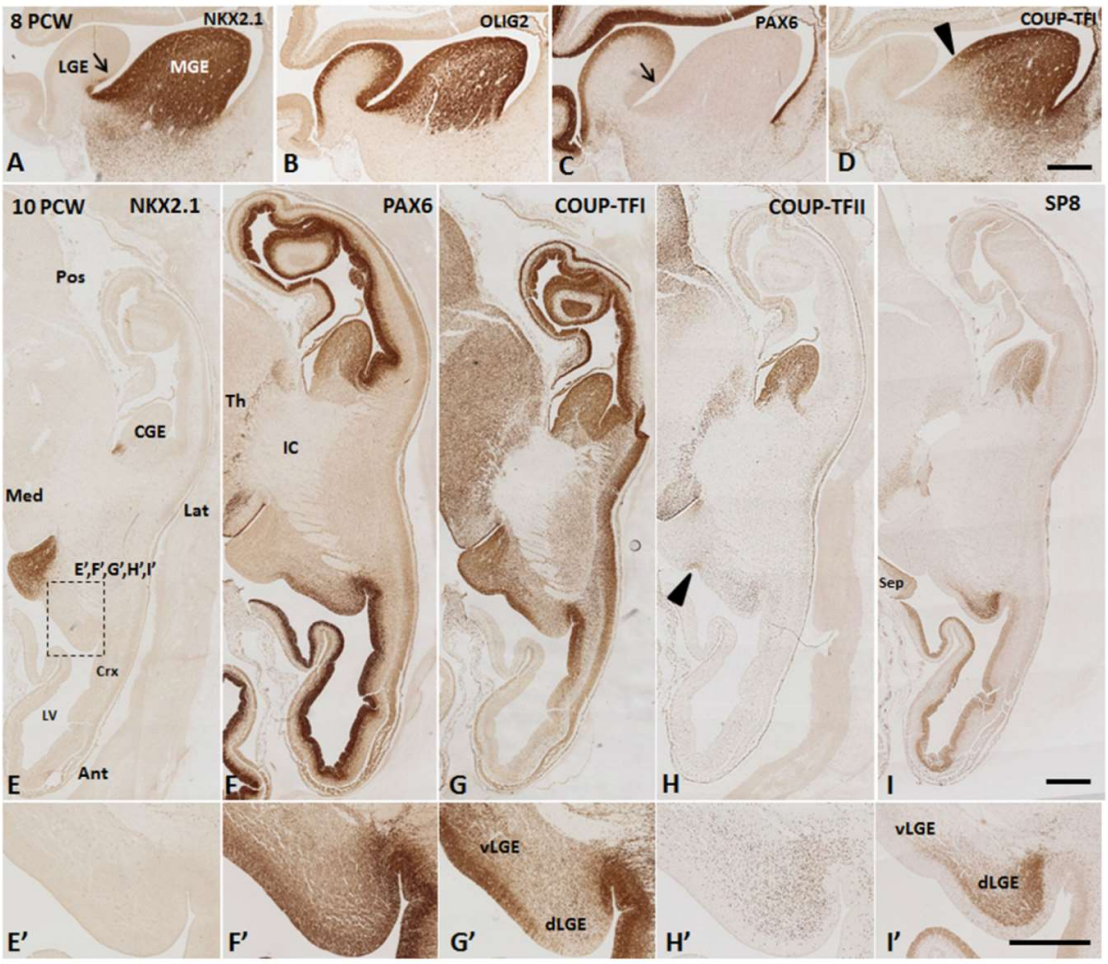

Figure 4.4: Multiple progenitor domains in the GE of early human fetal brain. (A-D) Medial parasagittal sections of 8 PCW human fetal brain. NKX2.1 expression was mainly confined to the MGE (A). OLIG2 was highly expressed in the MGE and LGE (B). PAX6 expressed in a gradient with higher expression in the proliferative zone of the cortex to lower expression in the LGE(C), arrows in A and C indicate to the boundary between MGE and LGE. COUP-TFI showed partial overlapped expression with NKX2.1 and OLIG2 in the MGE, arrow head marks the boundary between two distinct domains in the MGE (D). (E-I) Horizontal sections of 10 PCW human fetal brain; NKX2.1 expression was confined to the MGE and its caudal extension (E,E'). PAX6 was complementary expressed with NKX2.1 in the GE (F,F'). COUP-TFI was expressed in the VZ/SVZ of the MGE, CGE, vLGE, and only in migrating cells in the dLGE (G,G'). COUPT-FII highly expressed in CGE, the MGE/LGE boundary (arrowhead), and in stream of cells entering the cortex from dLGE (H,H'). SP8 was expressed in the SVZ of the CGE, and in increasing gradient from the vLGE to dLGE (I, I'). Boxed areas in E Shows where images (E'-I') were taken. Ant, anterior; pos, posterior; Lat, lateral, Med, medial; Crx, cortex; IC, internal capsule; Sep, septum; v and d LGE, ventral and dorsal LGE; Th, thalamus. A-D were taken from brain No. 1; E-I' were taken from brain No. 10. Scale bars: 200 μm in D (and for A–C); 1 mm in I (and for E-H), 200 μm in I' (and for E'–H').

4.4.3 COUP-TFI differentially expressed in the subdivisions of the ganglionic eminences

The expression of COUP-TFI immunoreactivity was mapped to the sub-divisions of the GE revealed by the expression patterns of three transcription factors PAX6, NKX2.1, and OLIG2; (Chapter 3; Figure 4.4) (Pauly *et al.*, 2014). When correlated with NKX2.1 and OLIG2 expression, COUP-TFI immunoreactivity revealed two distinct neurogenic domains in the MGE; one large dorsal domain characterized by overlapped and intense cellular expression of COUP-TFI, NKX2.1 and OLIG2 in the proliferative zone (dMGE) and a smaller ventral domain (vMGE) characterized by strong expression of NKX2.1 and OLIG2 only (Figure 4.4A-D; Figure 4,5D,E). In rodents NKX2.1 was expressed throughout the MGE and is required for the specification of all MGE-derived neurons (Flames *et al.*, 2007); we observed that a proportion of COUP-TFI+ cells in the dMGE co-expressed NKX2.1 (Figure 4.10A, B). Similar co-expression was also observed with OLIG2 (Figure 4.10C).

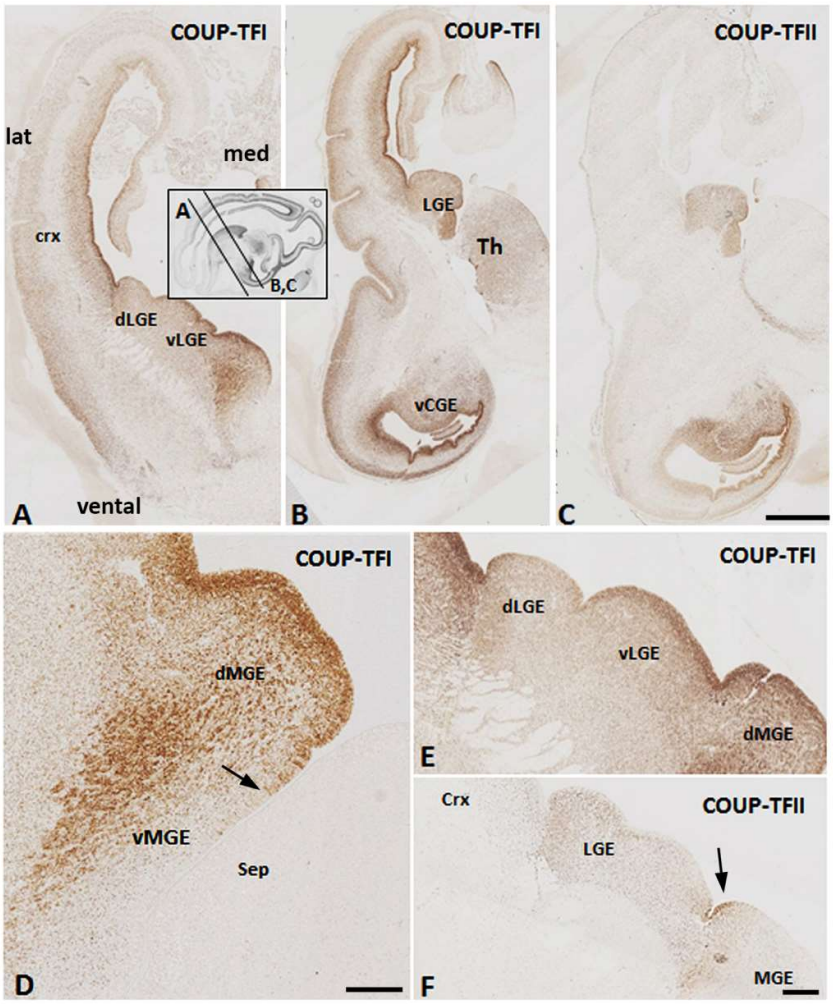

Figure 4.5: COUP-TFI and COUP-TFII expression in coronal sections of 12 PCW human fetal brain. (A, B) Two sections cut at different levels; in addition to a posterior-anterior gradient, COUP-TFI was also expressed in decreasing ventro-dorsal gradient. (C) COUP-TFII was highly expressed in vCGE and the adjacent ventral/temporal cortex with lower expression in the dorsal cortex. (D) COUP-TFI was highly expressed in MGE, but not in VZ of most ventral part of MGE and the adjacent septum, where COUP-TFI immunoreactivity was confined to dispersed cells. (E) COUP-TFI also strongly expressed in VZ of vLGE but not dLGE. (F) COUP-TFII highly expressed at MGE/LGE boundary; moderate expression in LGE and only scattered cells in MGE. The inset is drawing of 12 PCW sagittal sections shows the levels at which sections A and B were cut. Sections D and E are higher magnifications of section A while section F is a higher magnification of a section at the same level as A. Crx: cortex, Sep: septum, v and d LGE: ventral and dorsal LGE, v and d MGE: ventral and dorsal MGE, Th: thalamus. A-F were taken from brain No. 19. Scale bars: 2mm in C (for A, B, and C); 500 µm in D; and 500 µm F (for E and F).

Intense COUP-TFI expression marked the proliferative zones at the MGE/LGE boundary and the ventral part of the LGE (vLGE; Figure 4.4G,G'; Figure 4.5D,E). In the dorsal LGE (dLGE) COUP-TFI expression was limited to a few scattered cells in the post-mitotic mantle at 8 PCW (Figure 4.4D); however, more cells appeared here at 10 and 12 PCW, possibly cells migrating into the cortex (Figure 4.4G'; Figure 4.5E). In the CGE, COUP-TFI+ cells were present throughout the proliferative zones of d and vCGE (Figure 4.3E; Figure 4.4G; Figure 4.5B). Co-localization of COUP-TFI with KI67 in the GE similarly showed that proliferating COUP-TFI+ cells were only found in the dMGE, CGE and vLGE, but not in the dLGE (Figure 4.7).

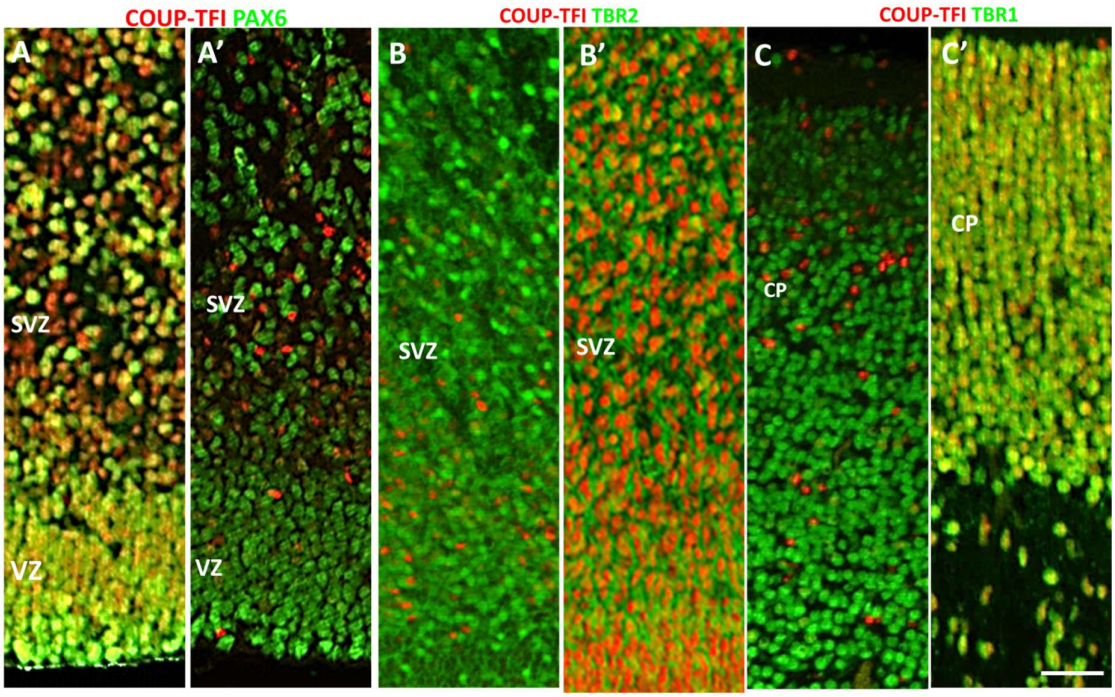

Figure 4.6: Double labelling for COUP-TFI (red) and PAX6, TBR2, and TBR1 (green). (A, A') Double labelling for: COUP-TFI and the radial glia marker PAX6 in anterior (A) and posterior cortex (A'). (B, B') COUP-TFI and the intermediate progenitor marker TBR2 in anterior (B) and posterior cortex (B'). (C, C') COUP-TFI and the post-mitotic pyramidal neuron marker TBR1 in anterior (C) and posterior cortex (C'). The inset is drawing of 12 PCW sagittal sections with boxed areas where pictures (A-C') were taken. VZ: ventricular zone, SVZ: sub-ventricular zone, IZ: intermediate zone, pSP: pre sub-plate CP: cortical plate. A-C' were taken from brain No. 17. Scale bar = 200 μm.

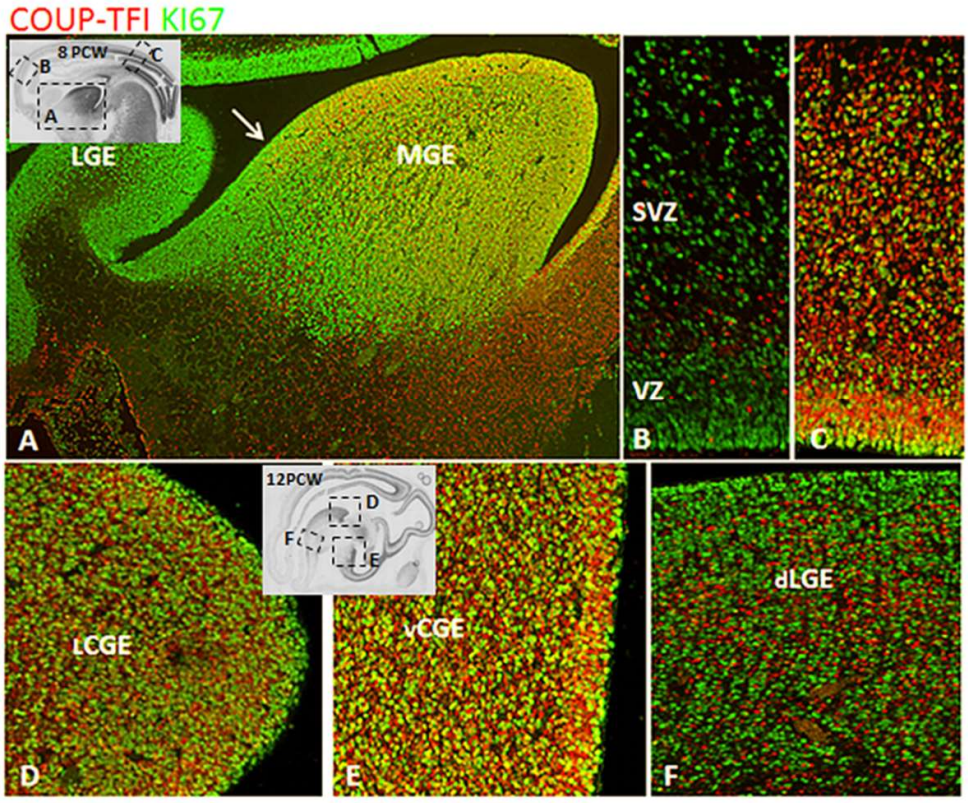

Figure 4.7: The progenitor domains of COUP-TFI at 8 and 12PCW. (A-C) Double labelling for COUP-TFI and the cell division marker KI67 in the VZ/SVZ of the GE (A) anterior cortex (B) and posterior cortex (C) at 8 PCW. (D-F) Double labelling for COUP-TFI and KI67 in the LCGE (D) vCGE (E) but not dLGE (D) of 12 PCW. The insets are drawing of sagittal sections with boxed areas where images (A-C and D-E) were taken. VZ: ventricular zone, SVZ: sub-ventricular zone, dCGE: dorsal CGE, vCGE: ventral CGE. A-C were taken from brain No. 1; D-F were taken from brain No. 20. Scale bars = 500 μm in A; 100 μm in B (and for C); 200 μm in E (and for D, F).

4.4.4 COUP-TFII expressed in both distinct and partially overlapping GE domains with COUP-TFI

We have previously demonstrated (See chapter 3, section 3.3.7) that COUP-TFII expression was not only confined to the sub-cortical territory in the ventral CGE (vCGE) where the majority of dividing COUP-TFII cells are found but also extended into the proliferative and post-mitotic zones of the adjacent ventral region of the temporal cortex (Figure 4.2B',G; Figure 4.5C). However, while COUP-TFII appeared to be continually expressed throughout the vCGE and ventro-temporal cortex, a cortical/sub-cortical boundary was still clearly delineated by the expression of TBR1 and PAX6; TBR1 was exclusively expressed in the post-mitotic zone of the cortex (Figure 4.2C); whereas PAX6 was expressed in a gradient, high in all cortical proliferative zones to progressively lower across the GE proliferative zones from vCGE to LGE (Figure 4.2D). A stream of COUP-TFII+ and PAX6+ cells was observed that appeared to migrate out from the CGE into the posterior part of the mantle zone lateral and ventral to the GE, a region that may anatomically correspond to the medial amygdaloid nuclei. However, the anterior pole of the same region contained only PAX6+ cells (not COUP-TFII+) which were most likely to be derived from the PAX6+/COUP-TFII- dLGE (Figure 4.2B',D, E). COUP-TFII positive progenitor zones of the temporal cortex are likely to contribute neurons to the cortical nuclei of the amygdala complex as well (Tang *et al.*, 2012). There was less COUP-TFI than COUP-TFII immunoreactivity in the mantle zone of the ventro-posterior telencephalon (vCGE and adjacent ventral/temporal cortex) (Figure 4.2A', F). No marked COUP-TFII expression was observed in the COUP-TFI expressing anterior ventral pallium (Figure 4.2B-B').

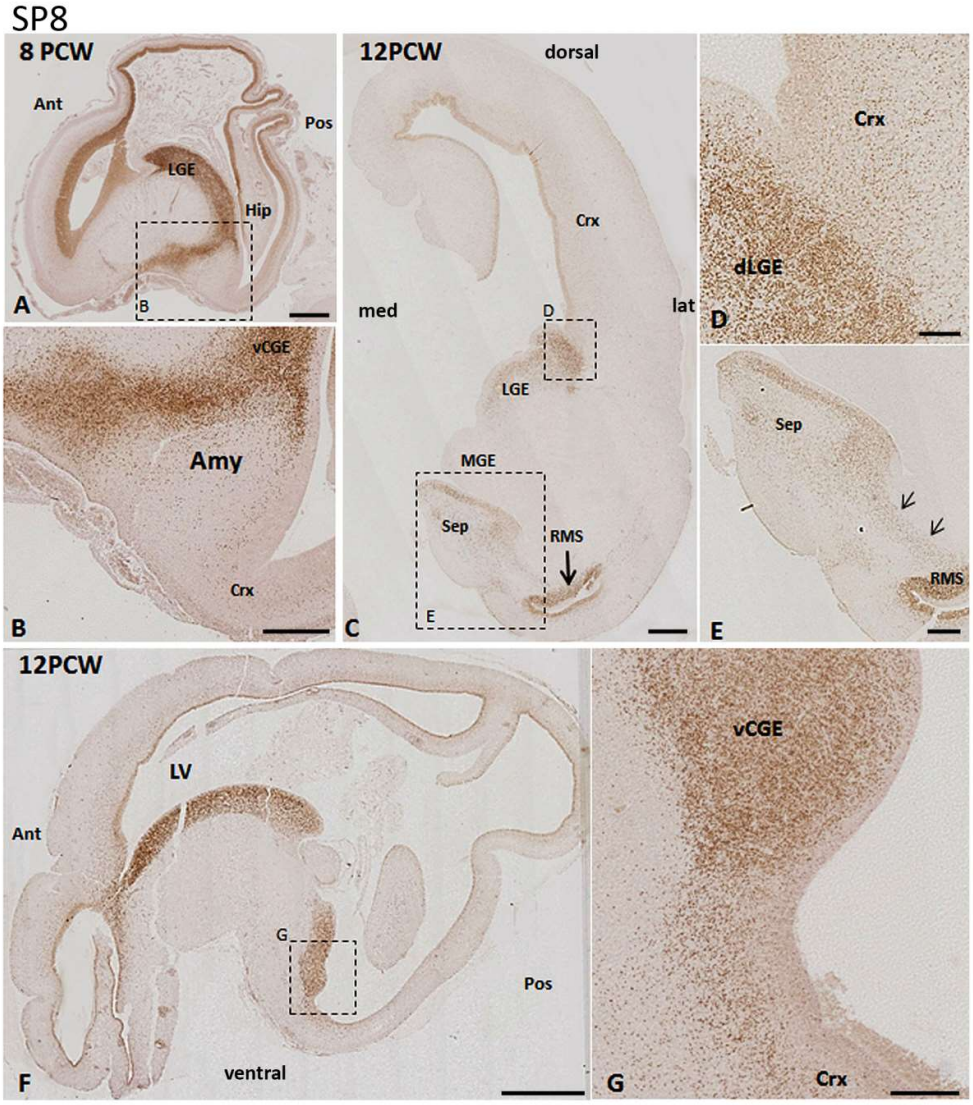

Figure 4.8: The expression pattern of SP8 in the human fetal brain at 8 and 12 PCW. (A, B) SP8 was expressed in high dorso-anterior to low ventro-posterior gradient in cortical VZ, and in SVZ of the LGE and CGE at 8 PCW (A). Broad stream of SP8+ cells appearing to migrate ventrally from vCGE towards the posterior part of the mantle zone lateral and ventral to the GE, with only few SP8+ cells entering ventral-temporal cortex from the vCGE at this stage (B). (C-E) SP8 expression in coronal section of 12 PCW fetal brain (C), stream of SP8+ cells appeared to be entering the cortex from dLGE (D), SP8 was also expressed in septum with stream of cells appearing to migrate ventrally and rostrally into the rostral migratory stream (RMS;E). (F, G) SP8 expression in sagittal section at 12 PCW (F), many SP8+ cells appeared to be entering the ventral-temporal cortex from vCGE at 12 PCW (G). Boxed area in A, C and F show where images (B, D, E and G) were taken. ant: anterior, pos: posterior, Crx: cortex, Hip: hippocampus, Amy: amygdala, MGE: medial ganglionic eminence, dLGE: dorsal LGE, vCGE: ventral CGE, Sep: septum, RMS: rostral migratory stream, LV: lateral ventricle. A and B were taken from brain No. 1; C-E were taken from brain No. 19; F and G were taken from brain No. 20. Scale bars: 500 µm in A, C; 200 µm in B,E,G; 100 µm in D; 2mm in E.

Double immunofluorescence histochemistry revealed co-expression of COUP-TFI and COUP-TFII by cells in both the cortex and GE (Figure 4.11). Throughout both compartments COUP-TFI was more widely expressed than COUP-TFII, however a large proportion of cells in the CGE were immunoreactive for both transcription factors (Figure 4.11A, E, F). Gradients of either single or double-labelled cells suggested two possible migratory pathways out from the CGE: posteriorly into the temporal cortex, and anterio-laterally through the LGE into the anterior and central cortical regions (Figure 4.11B,E-H) (Touzot *et al.*, 2016). At 8 PCW, it appeared that the posterior pathway was predominant (Figure 4.2A', B'; Figure 4.11B) however, the number of the cells that migrated anterio-laterally increased by 10-12 PCW (Figure 4.11G). This suggests that the pathway selected for cell migration out of the CGE is controlled in a temporal manner. The present study also shows that, in addition to the CGE, COUP-TF+ cells could also originate from the MGE (COUP-TFI+; Figure 4.5A,D) and the MGE/LGE boundary (COUP-TFI+, COUP-TFII+, and COUP-TFI+/COUP-TFII+ cells; Figure 4.2A,B). At 8 and 12 PCW respectively, 20 ±3.6 % and 22 ±3 % of all COUP-TFII+ cells in the cortex also expressed COUP-TFI. The highest proportion of double-labelled cells was observed in the ventro-temporal cortex (Figure 4.11 C,D,H-J).

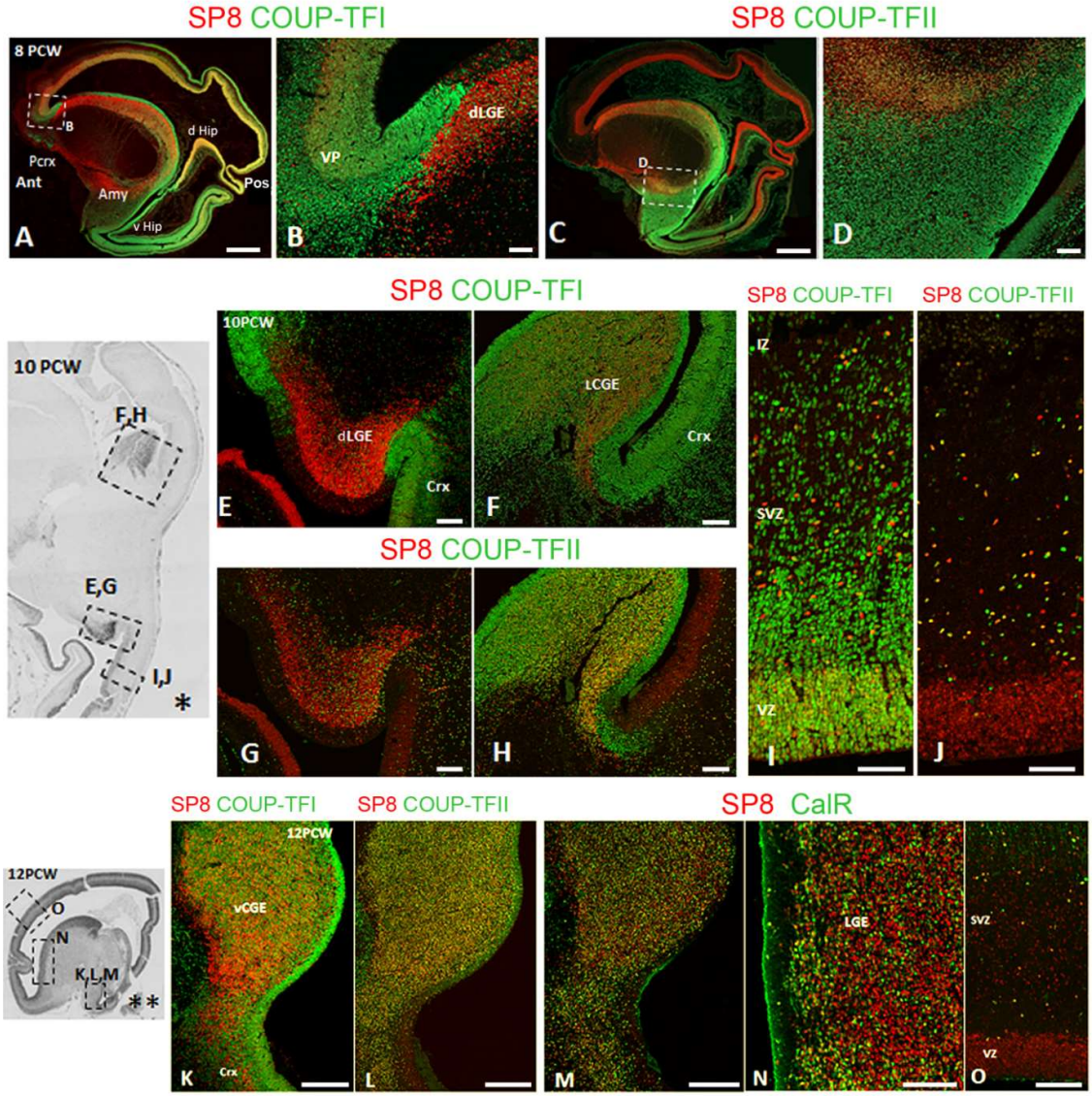

Figure 4.9: Double labelling for SP8 (red) and COUP-TFI, COUP-TFII, and Calretinin (green). (A) Counter gradients of SP8 and COUP-TFI expression in cortical VZ broadly overlapped (yellow) including in posterior cortex (Pos) and the hippocampal primordium dorsal to the cortical hem (dHip). Only anterior cortex (Ant) predominantly expressed SP8, and only ventral temporal cortex including ventral hippocampus (vHip) exclusively expressed COUP-TFI. Likewise, a counter gradient of SP8 expression in SVZ and COUP-TFI in VZ/SVZ was apparent in GE. The medial amygdala (Amy) predominantly expressed SP8, whereas ventral pallium (VP) and piriform cortex (PCrx) expressed COUP-TFI. (B) COUP-TFI expression in the anterior ventral pallium (VP) with SP8 confined to LGE. (C) In the cortex SP8 and COUP-TFII show abrupt expression boundaries; COUP-TFII confined to ventral temporal lobe. (D) Posterior regions of medial amygdala populated by SP8+/COUP-TFII+ cells. (E) the dLGE/cortex boundary was still sharply delineated by SP8 and COUP-TFI expression at 10 PCW. (F) Relatively small numbers of SP8+/COUPTFI- cells migrate from LCGE into the cortex at this stage. (G) Low density of COUP-TFII+ cells in SP8+ dLGE. (H)

High density of SP8 + /COUP-TFII+ cells in SVZ of the LGE-Like CGE. (I and J) SP8+/COUP-TFI+ cells predominantly seen in cortical VZ although double-labelled cells present in SVZ and IZ (I). SP8+/COUP-TFII+ double-labelled cells predominantly observed in SVZ and IZ (J). (K and L) SP8 Co-expressed with both COUP-TFI and COUP-TFII in ventral CGE. (M, N, O) Extensive co-expression of SP8 and CalR in the vCGE (M) but also in the LGE and cortex (N, O). Crx, cortex; d and vHip, dorsal and ventral hippocampus; Amy, amygdala; VP, ventral pallium; dLGE, dorsal LGE; L and vCGE, lateral and ventral CGE; VZ, ventricular zone. A-D were taken from brain No. 1; E-J were taken from brain No. 10; K-O were taken from brain No. 17. Scale bars: 2mm in A, C; 500 μm in K, L, M; 200 μm in E–H, N, O; 100 μm in B, D, I, J.

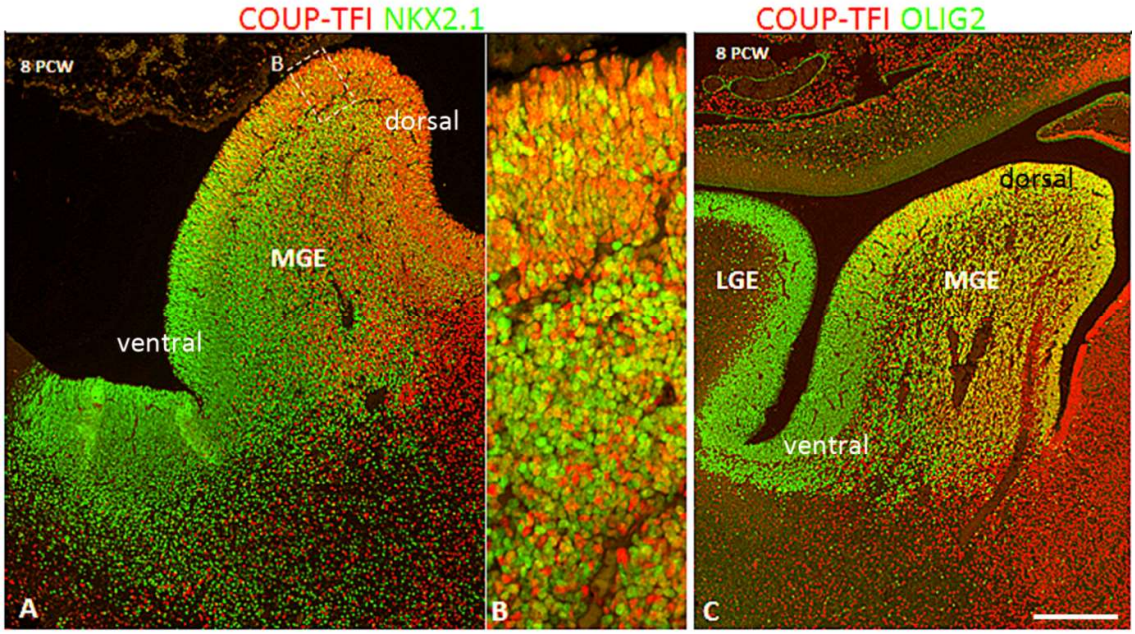

Figure 4.10: Double labelling for COUP-TFI (red) with NKX2.1 and OLIG2 (geen). (A, B) Double labelling for COUP-TFI and NKX2.1 in the VZ/SVZ of the MGE of 8 PCW. Cells in the VZ are largely double-labelled, but the further into the SVZ, the more cells become labelled for either one transcription factor or the other (B). (C) shows double labelling for COUP-TFI and OLIG2 in the VZ/SVZ of the MGE of 8 PCW. In both cases double-labelling is confined to the dorsal MGE. The boxed area in (A) shows where image (B) was taken. MGE: medial ganglionic eminence, LGE: Lateral ganglionic eminence. A-C were taken from brain No. 1. Scale bars: 500 μm in A and C; 20 μm in B.

4.4.5 SP8 expressed in distinct populations of post-mitotic COUP-TF expressing CGE and LGE derived cells.

In mouse the transcription factor SP8 plays a role in the differentiation of LGE-derived interneurons that populate the amygdala and the olfactory bulb via the rostral migratory stream (RMS) (Waclaw *et al.*, 2006). In the human ventral telencephalon, SP8 was expressed in the SVZ of the LGE with an increasing gradient from ventral to dorsal, in the septum and in the RMS (Figure 4.4I, I'; Figure 4.8). Two streams of SP8+ cells were observed migrating ventrally and rostrally into the RMS from both the dLGE and septum (Figure 4.8C,E) suggesting the septum also contributes interneurons to the human olfactory bulb. SP8 was also highly expressed across the SVZ of the vCGE (Figure 4.8A,B) not only in the caudal extension of the LGE (LCGE) as previously described (Ma *et al.*, 2013). Two migratory routes were identified for SP8+ cells in the vCGE, a large number of SP8+ cells formed a migratory stream from the vCGE ventrally toward the anlage of the medial amygdaloid nuclei (Figure 4.8B). Although few SP8+ cells were seen entering the cortex from the vCGE at 8 PCW, the number increased at 12 PCW (Figure 4.8B,G). SP8 was expressed in dividing cells (KI67+) in the SVZ in of LGE, but all SP8+ cells appeared to downregulate KI67 and therefore stop dividing before entering the cortex (Figure 4.12A).

In either the LGE or CGE, SP8 was predominately expressed in COUP-TFII+ cells and to a lesser extent in COUP-TFI+ cells. These double-labelled cells were mostly located in the SVZ of the LGE and CGE and in cells appearing to migrate tangentially into the cortex (Figure 4.9A-H,K-L). SP8 also co-localized with COUP-TFII in cells that appeared to be migrating ventrally from the vCGE into the posterior part of the mantle zone lateral and ventral to the GE (Figure 4.9C,D). However, similar to PAX6, SP8 was also expressed in the anterior part of this region, where these cells appeared to be migrating from the LGE (Figure 4.9C). In the cortical wall both COUP-TFI+/SP8+ and COUP-TFII+/SP8+ cells were mainly found in the SVZ and IZ; a few, if any, were found in the CP (Figure 4.9I,J). As SP8 was not expressed in the MGE or at the MGE/LGE boundary. SP8 and SOX6, a marker of post-mitotic MGE-derived cells (Batista-Brito *et al.*, 2009), were expressed in completely separate populations of cells either in the GE or the cortical SVZ and IZ (Figure 4.12B,C). cortical COUP-TFI+/SP8+ cells and COUP-TFII+/ SP8+ cells were most likely generated in the CGE (not the LGE) migrating either posteriorly or anterio-laterally into the cortex, confirmed by observing that a large

proportion of SP8+ cells in these two pathways co-expressed CalR (Figure 4.9M-O) a marker of CGE- derived interneurons (Kanatani *et al.*, 2008; Miyoshi *et al.*, 2010). However, we also found a population of SP8+ cells entering the cortex from the dLGE, similar to Ma *et al.* (2013) except that these cells did not co-express COUP-TFI, COUP-TFII, or CalR (Figure 4.9) but instead formed a population distinct from CGE derived cells. To confirm that SP8+ cells entering the cortex from the GE were GABAergic interneurons, we performed SP8 and GABA synthesizing enzyme glutamate decarboxylase 67Kd (GAD67) double labelling and observed proportion of SP+ cells entering the cortex from the LGE and vCGE co-localized GAD67; however, there was a number of cells entering the cortex expressing SP8 only (Figure 4.12D-F) which are most likely to be immature cells not yet expressing detectable levels of GAD67.

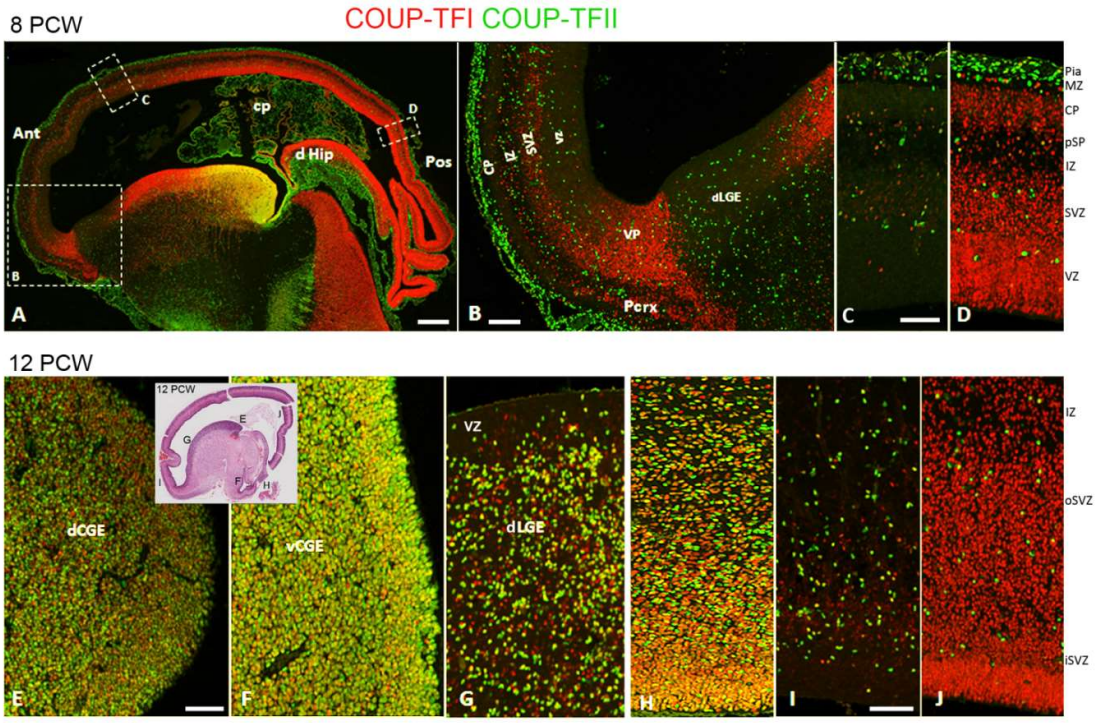

Figure 4.11: Double labelling for COUP-TFI (red) and COUP-TFII (green) in 8 and 12 PCW human fetal brain. (A) Sagittal section of 8 PCW fetal brain. The majority of cells in the caudal part of GE show co-localization of these two markers (yellow signal); while COUP-TFI was expressed in a decreasing posterior to anterior gradient; the anterior cortex was generally more populated with COUP-TFII+ cells than the posterior cortex however COUP-TFI (but not COUP-TFII) was markedly expressed in the anterior ventral pallium (B). Streams of COUP-TFI+ and COUP-TFII+ cells, and scattered COUP-TFI+/COUP-TFII+ cells appeared to migrate from the GE through the LGE toward the anterior cortex. (C, D) A

proportion of cells in the anterior and posterior cortex also showed co-localization for these two markers. (E-J). Differing proportions of COUP-TFI+/COUP-TFII+ cells were observed in the CGE compartments (E, F) dLGE (G), ventral cortex (H), anterior cortex (I) and the posterior cortex (J) of 12 PCW fetal brain. cp: choroid plexus, d Hip: dorsal hippocampus, dLGE: dorsal LGE, VZ: ventricular zone, SVZ: sub-ventricular zone, IZ: intermediate zone, CP: cortical plate, cp: choroid plexus, VP: ventral pallium, Pcrx: piriform cortex, dLGE: dorsal LGE, d and vCGE: dorsal and ventral CGE. A-D were taken from brain No. 5; E-J were taken from brain No. 20. Scale bars: 500 µm in A; 200 µm in B; 100 µm in C (and for D); 200 µm in E (and for F, G); 200 µm in I (and for H ,J).

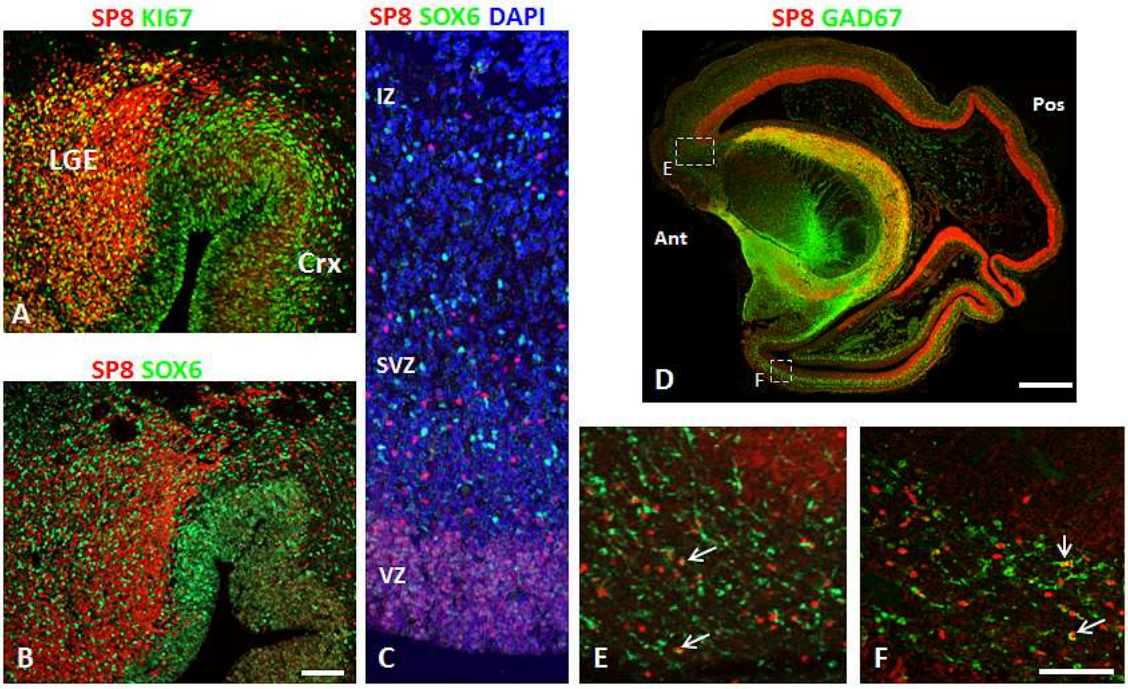

Figure 4.12: SP8 expression in cortical GABAergic interneurons. (A) SP8+ cells co-localized KI67 in LGE, all SP8+ cells downregulated KI67 before entering the cortex. (B,C) SP8 and SOX6 were expressed in separated population of cells in the LGE (B) and migrating cells in the VZ and IZ of the cortex (C). (D-F) proportion of SP8+ cells co-expressed GAD67 in the GE and cells entering the cortex from the LGE (E) and vCGE (F), Arrows indicate to examples of double labelled cells. boxed areas in D show where images (E and F) were taken. A-C were taken from brain No. 10; D-F were taken from brain No. 1. Scale bars: Scale bars: 200 µm in B (and for A); 2mm in D; 100 µm in F (and for C and E).

4.4.6 COUP-TFI expressed in both MGE- and CGE- derived cortical GABAergic interneurons.

The majority of COUP-TFI+ cells in the anterior cortex expressed GAD67 demonstrating that COUP-TFI was predominantly expressed in GABAergic interneurons in this region. As expected, a smaller proportion of COUP-TFI+ cells in the posterior cortex expressed GAD67 because there is a far higher density of cells co-expressing COUP-TFI with markers for glutamatergic neurons and their precursors (TBR1, PAX6 and TBR2; Figure 4.6; Figure 4.13A,B). In the cortex at 8 PCW GAD67+ cells were mostly found in either the IZ/SVZ or the MZ, the two major migration streams of GABAergic interneurons in the developing cortex (Lavdas *et al.*, 1999; Marín, 2013); however a considerable number of GAD67+ cells appeared to be migrating in the VZ and SVZ as well (Figure 4.13A-E). COUP-TFI/GAD67 co-localization was also observed in all these compartments with 45 ±2.4% of all GAD67+ cells in the proliferative zones (VZ/SVZ) co-expressing COUP-TFI. In particular, the majority of GAD67+ cells migrating in the VZ co-expressed COUP-TFI and some of these cells were shown to have a longitudinal rather than transverse morphology suggesting radial migration. A similar proportion was also seen in post-mitotic layers (47 ±2.7%) mostly located either at the CP/MZ border or just below in the pSP/ IZ; few, if any, were found in the CP (Figure 4.13A-C,F).

NKX2.1 is downregulated in migrating MGE-derived cells before they enter the cortex; the transcription factor SRY-box6 (SOX6) acts downstream of NKX2.1 and its expression is maintained in migrating interneurons in mouse (Batista-Brito *et al.*, 2009). At 8 PCW, SOX6 was expressed in the SVZ of the MGE, in cells probably migrating through the LGE, and in the cortex. SOX6+ cells were also broadly distributed throughout the cortical wall, the majority of which co-expressed GAD67 (Figure 4.13G) and a proportion of which also co-expressed COUP-TFI (Figure 4.13H) confirming that a population of COUP-TFI+ cortical interneurons are derived from the dMGE. This was apparent at 10 PCW, where COUP-TFI+ cells showed co-localization with SOX6 in the SVZ of the MGE and in a large number of cells migrating into the cortex from the LGE (Figure 4.13I,K). Moderate SOX6 immunoreactivity also appeared in both the cortical and dLGE VZ at this stage (Figure 4.13I) suggesting, as in rodents, a role in determining cortical progenitor identity (Azim *et al.*, 2009).

In addition, other interneurons expressing COUP-TFI were characterised by co-expression of either calretinin (CalR) or calbindin (CalB; Figure 4.13N,P). CalR, in particular, is characteristic of CGE- derived cortical GABAergic interneurons in rodents (Kanatani *et al.*, 2008; Miyoshi *et al.*, 2010). At 12 PCW, when the cortical wall was extensively populated with CalR+ cells, we found a sub-population of these cells co-localized COUP-TFI (Figure 4.13N). Overall, we estimated 39 ± 4.7% of all CalR+ cells in the various cortical regions co-expressed COUP-TFI. Although there were fewer CalB+ cells present at this stage, a proportion of these also co-expressed COUP-TFI (Figure 4.13P).

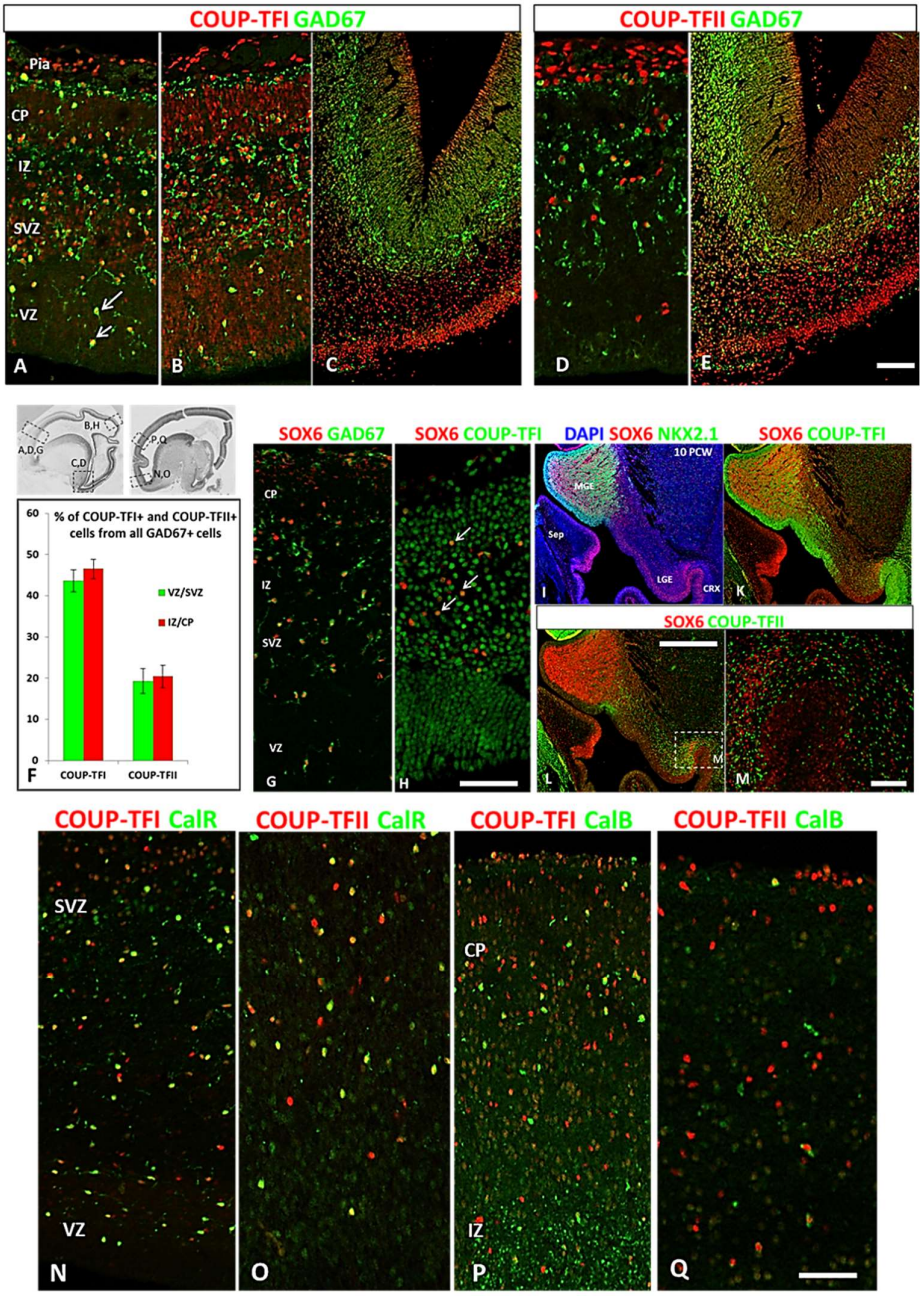

Figure 4.13: COUPT-FI and COUP-TFII expression in cortical GABAergic interneurons. (A–C) Double labelling for COUPT-FI and GAD67 in cortical wall at 8 PCW. COUP-TFI+/ GAD67+ cells broadly distributed in the cortical wall, mostly either just above the thin CP in the marginal zone or just below in the pre-subplate/IZ; (A, B) A considerable number also found in proliferative (VZ/SVZ) zone of anterior and posterior cortex with radial nuclear morphology (arrows). (C) A stream of COUP-TFI+/GAD67+ cells enter temporal cortex from vCGE, migrating tangentially mainly in the SVZ/IZ. (D, E) A smaller proportion of GAD67+ cells co-express COUP-TFII. (F) The percentage of COUP-TFI+ and COUPTFII+ cells from all GAD67+ cells in the proliferative (VZ/SVZ) and postmitotic zones (IZ/CP) of 8 PCW cortical wall. (G, H) Double labeling for SOX6 and GAD67 in the 8 PCW cortical wall. The majority of SOX6+ cells co-expressed GAD67 this stage (G), and a proportion of SOX6+ cells

also co-expressed COUP-TFI (H). (I) Double labeling for SOX6 and NKX2.1 in the GE of of 10 PCW fetal brain. SOX6 mainly expressed in NKX2.1+ cells in SVZ of MGE and in cells migrating through the LGE mantle zone; SOX6 also expressed in the cortical and dLGE VZ. (K–M) Double labeling for COUPT-FI or COUP-TFII with SOX6 in GE at 10 PCW; COUP-TFI highly expressed in SOX6+ cells in MGE and in cells entering cortex from LGE (L), no similar double labeling with COUP-TFII (L, M).(N, O) Double labeling for COUPT-FI and COUP-TFII with calretinin (CalR) in cortical wall of 12 PCW fetal brain. (P, Q) Double labeling for COUPT-FI and COUP-TFII with calbindin (CalB) in 12 PCW cortical wall. Scale bars: 100 μm in B, D (and for A); 200 μm in C, E; 100 μm in K (and for G, H, I); 100 μm in M (and for L) 500 μm in P (and for N, O); 100 μm in Q.

4.4.7 COUP-TFII expressed mainly by CGE-derived cortical GABAergic interneurons

The expression of COUP-TFII by a subpopulation of cortical GABAergic interneurons was also analysed by double immunofluorescence with GAD67, CalR, CalB, and SOX6 (Figure 4.13). Either in the proliferative zone or postmitotic layers, a far smaller proportion of GAD67+ cells expressed COUP-TFII than COUP-TFI; only 19 ± 3% of all GAD67+ cells expressed COUP-TFII in the proliferative zone (mainly in the SVZ), many COUP-TFII+ cells were seen in the VZ, but these cells did not co-express GAD67. In the postmitotic layers, 20 ± 2.8% of GAD67+ cells expressed COUP-TFII and were also mostly seen in the IZ/SP and above the CP in the marginal zone (Fig. 8D, E, F). Conversely, a higher proportion of COUP-TFII+ cells co-localized CalR (Figure 4.13O); 67± 6% of all CalR+ cells in the cortex of 12 PCW human brain co-expressed COUP-TFII, however a proportion of COUP-TFII+/ CalR+ cells are probably Cajal-Retzius cells (Meyer *et al.*, 2000; Meyer *et al.*, 2002; Zecevic *et al.*, 2011). Some COUP-TFII+ cells in the cortex were also immunoreactive for CalB (Figure 4.13Q). Although the MGE/LGE boundary could also be an origin for COUP-TFII+ cells (Chapter 3; Figure 4.2B) , similar to SP8, COUP-TFII was not co-expressed with SOX6 (Figure 4.13L,M) this demonstrates that COUP-TFII cells are not MGE derived, however, COUP-TFII+ cells in the cortex can co-express OLIG2 (Reinchisi *et al.*, 2012) and these cells could be derived from the MGE/LGE boundary.

4.5 Discussion

COUP-TFs and SP8 have been shown to be key regulators of telencephalic development in numerous experiments in rodents contributing to the protomap, controlling neurogenesis, determining phenotype and influencing rates and direction of cell migration (Waclaw *et al.*, 2006; Sahara *et al.*, 2007; Borello *et al.*, 2013; Alfano *et al.*, 2014b). It is important to determine the extent to which these roles have been retained or altered in human development particularly as mutations in COUP-TFI have been implicated in intellectual disability (Bosch *et al.*, 2014). The present study confirms that these transcription factors are likely to have equally important and largely similar roles in human, however it also revealed small differences that may be important in understanding the greater complexity of the human compared to the rodent brain and in neurodevelopmental disorders.

4.5.1 COUP-TFI, COUP-TFII and SP8 as regulators of cortical arealisation.

This study confirms that the observation that COUP-TFI and SP8 form counter-gradients of expression across the mouse pallium (O'Leary *et al.*, 2007; Rakic *et al.*, 2009; Sansom and Livesey, 2009; Borello *et al.*, 2013; Alfano *et al.*, 2014a) is also the case in the human cerebral cortex. A fundamental difference between mouse and primate, and in particular human, cerebral cortex is the substantially larger surface area with, more importantly, a more complicated pattern of functional arealisation and a considerably larger proportion devoted to higher functioning association cortex (Van Essen and Dierker, 2007; Krubitzer and Seelke, 2012; Buckner and Krienen, 2013). One mechanism that could contribute to the increased complexity is more variation in the combinatorial patterning of transcription factor expression to determine the human protomap. What the present chapter demonstrates is that whereas expression of SP8 and COUP-TFI overlap extensively in certain cortical regions, the expression of SP8 and COUP-TFII form distinct boundaries. In this way the cortical wall is divided up into regions that express SP8 only (the frontal pole excluding anterior ventral pallium) COUP-TFI/COUP-TFII (ventral temporal cortex, lateral and medial) and COUP-TFI/SP8 (central, posterior, and dorsal temporal cortex) (Figure 4.14; Table 4.1). This differs from the mouse protomap in which COUP-TFI and SP8 show little overlap (Borello *et al.*, 2013) and where COUP-TFII is confined to a very small portion of the posterior cortex (Qiu *et al.*, 1994) which is possibly the origin of the mouse secondary temporal cortex (Wree *et al.*,

1983). Combinatorial expression of COUP-TFI and SP8 could maintain a common genetic identity for some future primary sensory areas (visual, auditory and somatosensory) and a partially shared identity with SP8-expressing frontal motor cortex with which these sensory areas will interconnect, along with allied association cortex, via dorsal sensorimotor pathways (Baker, 2007; Hickok and Poeppel, 2007; Milner and Goodale, 2008). On the other hand, expansion of cortical COUP-TFII expressing territory in human fetal brain mirrors the increase in size and complexity, in human compared to mouse, of the association areas of the ventro-temporal cortex that includes the ventral stream of cognitive visual processing (Milner and Goodale, 2008; Kaas, 2013).

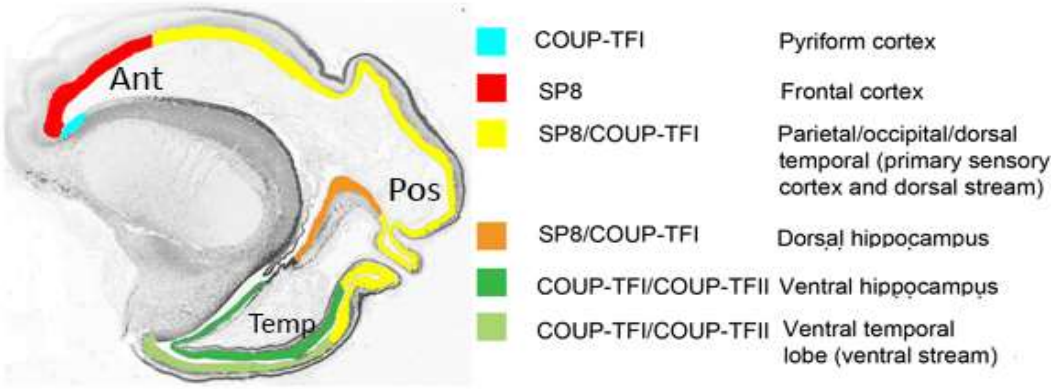

Figure 4.14: The roles of transcription factors COUP-TFI, COUP-TFII, and SP8 in cortical arealisation. Schematic sagittal section showing how the progenitor zones of the cortex are subdivided into compartments identified by unique or combined expressions of COUP-TFI, COUP-TFII, and SP8 that give rise to different functional areas of cortex in maturity. Ant: anterior; Pos: posterior; Temp: temporal.

An extension of this observation was that dorsal (posterior in adult human) and ventral (anterior in adult human) hippocampus are also differentiated by combinatorial expression of SP8/COUP-TFI and COUP-TFII/COUP-TFI respectively. The dorsal domain featured in our sections lies anterior and dorsal of the cortical hem but does not include that anterior-most portion that dissipates after 14 PCW as the corpus callosum develops (Kier *et al.*, 1995). Evidence from animal studies demonstrates that dorsal and ventral hippocampus have distinct roles; dorsal carries out primarily cognitive functions whereas ventral hippocampus function is primarily related to stress, emotion and affective states (Fanselow and Dong, 2010; Strange *et al.*, 2014) reflected by distinct patterns of gene expression in adult and post-natal rodents (Dong

et al., 2009; O'Reilly *et al.*, 2015) and by the distinct efferent and afferent connections each sub-compartment makes, also demonstrated in primates (Friedman *et al.*, 2002; Kondo *et al.*, 2009; Aggleton *et al.*, 2012). Neuroimaging studies show this holds true for human hippocampus also (Bunsey and Eichenbaum, 1996; Chua *et al.*, 2007; Strange *et al.*, 2014). It has recently been demonstrated in mouse that high to low expression of COUP-TFI along a septo-temporal gradient is important for the functional organisation of the hippocampus (Flore *et al.*, 2016). Here we provide evidence that the protomap for human hippocampal specialisation is laid down prior to formation of afferent and efferent connections, but that it is determined by complementary expression of SP8 and COUP-TFII rather than graded expression of COUP-TFI.

The ventral pallium, a cortical structure which forms the boundary between the cortex and the LGE and shown to contribute cells to the olfactory cortex, claustrum and amygdala in mouse (Puelles *et al.*, 2000; Medina *et al.*, 2004) has also been identified in human (Lindsay *et al.*, 2005). The present study shows it is characterized by COUP-TFI expression regardless of whether it was located posteriorly or anteriorly. Instead, anterior and posterior regions were defined by SP8 or COUP-TFII expression, respectively. This is in agreement with findings in mouse that have described expression of *Coup-TFI* at the anterior corticostriatal boundary (Lodato *et al.*, 2011) essentially part of the ventral pallium. In both human and mouse there is expression of COUP-TFI in post-mitotic cells of the nearby piriform olfactory cortex (Figure 4.9A) (Lodato *et al.*, 2011).

Interaction of COUP-TFI, COUP-TFII and SP8 with other transcription factor expression gradients demonstrated to exist in human fetal brain at this stage of development, for instance EMX2 and PAX6 (Bayatti *et al.*, 2008b; Ip *et al.*, 2010) CTIP2 (Ip *et al.*, 2011) and OLIG2 (Chapter 3) may produce a sufficiently complicated mosaic of organising maps that "tether" the subsequent rapid expansion of the human cerebral surface that occurs during development to produce the complex but largely stereotyped interconnection of primary, secondary and association cortex (Buckner and Krienen, 2013). For instance, it has been proposed that the early developing middle temporal (MT) and V1 areas of the visual cortex in non-human primates act as "molecular anchors" for the subsequent development of the dorsal and ventral visual streams respectively (Bourne and Rosa, 2006; Homman-Ludiye and Bourne, 2013).

These areas could be defined in early cortex by co-expression of SP8 with COUP-TFI (MT) or COUPTFII with COUP-TFI (V1).

Table 4.1: The expression level of COUP-TFI, COUP-TFII, and SP8 in the cortex and hippocampus.

	Ventral pallium	Anterior cortex	Central cortex	Posterior cortex	Temporal cortex	Dorsal hippocampus	Ventral hippocampus
COUP-TFI	++	-	+	++	++	++	++
COUP-TFII	-	-	-	-	++	-	++
SP8	-	++	++	++	-	+	-

(-) No expression (+) Moderate expression (++) Strong expression

4.5.2 Compartmentalisation of the ventral telencephalon

Taken together with our previous findings (chapter 3) the proliferative zones of the ventral telencephalon can be divided according to transcription factor expression (including COUP-TFI and COUP-TFII) and some predictions made as to how interneuron precursors derived from each compartment migrate into the telencephalon (Table 4.2). COUP-TFI immunoreactivity subdivided the MGE with NKX2.1 and OLIG2 expressed throughout but with COUP-TFI confined to the larger dorsal region. In rodents the dMGE is the birthplace of nearly all parvalbumin-positive (Pv+) and somatostatin-positive (Sst+) cortical interneurons, whereas the vMGE predominantly gives rise to globus pallidus neurons (Flandin *et al.*, 2010) although at later stages it may be the source of cortical chandelier cells (Taniguchi *et al.*, 2013). We observed co-expression of COUP-TFI with SOX6, a downstream regulator of NKX2.1 (Batista-Brito *et al.*, 2009) in the dMGE and in neuroblasts laterally migrating through the LGE and into the cortex, demonstrating co-expression of COUP-TFI and SOX6 in cortical interneurons in human for the first time. COUP-TFI may have a role in guiding migration (Boudot *et al.*, 2014) perhaps ensuring neurons migrate dorsally towards the cortex and not ventrally towards the basal ganglia.

Table 4.2: The subdivisions of GE and subcortical septum identified by the expression of certain transcription factors.

	vMGE	dMGE	MGE/LGE boundary	vLGE	dLGE	MCGE	LCGE	vCGE	MGE-LIKE Septum	LGE-LIKE Septum
NKX2.1	++	++	-	-	-	++	-	-	+	-
OLIG2	++	++	++	+	+	++	+	-	+	+
COUP-TFI	-	++	++	++	-	++	++	++	-	-
COUP-TFII	-	-	++	+ post-mitotic cells	+ post-mitotic cells	-	+ Post-mitotic cells	++	-	-
SP8	-	-	-	+ SVZ only	++ SVZ only	+	++ SVZ only	++ SVZ only	-	+ SVZ only
PAX6	-	-	-	+	+	-	++	++	-	+

(-) No expression (+) Moderate expression (++) strong expression

The confinement of COUP-TFI expression to its ventral region also divides the LGE into dorsal and ventral portions. Dorsal LGE is characterised by stronger PAX6 expression, SP8 expression in post-mitotic cells and few COUP-TFII+ cells. In the age range studied here COUP-TFII immunoreactivity in the dLGE appeared to belong only to anteriorly migrating cells arising from the vCGE. Instead, dLGE provided predominantly SP8+ only cells that migrated towards the RMS, amygdala and cortex and did not express CalR. The boundary zone of the MGE and LGE is identified by its own transcription factor expression profile, which is distinct from the adjacent dMGE and vLGE. Whereas COUP-TFI is continuously expressed across these three regions, COUP-TFII was exclusively expressed at the MGE/LGE boundary (Chapter 3; Figure 4.2A,B; Figure 4.4G,H) (Ma *et al.*, 2013); suggesting that this region could be an origin for COUP-TFII+ interneurons in addition to the vCGE (see below). In rodents, this boundary region is the source of COUP-TFII+/Sst+ cells that occupy cortical layer V (Cai *et al.*, 2013). However, no co-expression of COUP-TFII with SOX6, the developmental marker for Sst+ interneurons, was observed which suggests a difference between human and rodent models where a previous report indicated that one third of cortical COUP-TFII+ cells co-express SOX6 (Ma *et al.*, 2012).

Our previous analysis showed that the sub-cortical septum is divided into MGE-like (NKX2.1 expressing) and LGE-like (PAX6 expressing) domains and suggested that OLIG2+ cells from this compartment migrate medially into the cortex (See chapter 3). Here this was extended by confirming expression of SP8 in LGE-like septum (Ma *et al.*, 2013) and showing that septal SP8+ cells also migrate towards the RMS in addition to dLGE derived SP8+ cells (Ma *et al.*, 2013). However, COUP-TFI and COUP-TFII expression in both the MGE-like and LGE-like septum were confined to a very few, dispersed cells most likely to have migrated from other subcortical structures, making these septal compartments very similar to vMGE and dLGE, respectively.

The CGE is characterised by high expression of COUP-TFI and COUP-TFII, as well as SP8 and CalR. Dorsally, MGE-like CGE expresses NKX2.1, LGE-like CGE expresses PAX6 (Chapter 3) but neither compartment expresses COUP-TFII in dividing cells of the VZ, which is only seen in the vCGE (Chapter 3; Hansen et al., 2013; Ma et al., 2013). We have previously suggested that COUP-TFII+/CalR cells derived from the vCGE migrate both posteriorly into the temporal cortex and dorsally/laterally/anteriorly via the LGE towards more anterior cortex (Chapter 3). Here we show that many of these cells also express COUP-TFI and SP8 and that numerous cells in the LGE co-expressing SP8 and COUP-TFII are passing through rather than originating from the LGE as previously suggested (Ma *et al.*, 2013).

4.5.3 Migration pathways out of the ganglionic eminences

The idea that interneuron precursors principally enter the dorsal from the ventral telencephalon via two pathways; laterally from the MGE and caudally from the CGE (Faux *et al.*, 2012; Marín, 2013) has been challenged by recent observations. Medial migration of interneurons from the septum into the medial cortex has been described in shark (Quintana-Urzainqui *et al.*, 2015) and human (Chapter 3). Furthermore, studies in mouse (Touzot *et al.*, 2016) and human (Chapter 3) have suggested that CGE-derived interneurons reach their final location in the cortex via two distinct pathways. In addition to the caudal migratory stream (CMS) directing CGE-derived cells into the temporal cortex and hippocampus (Yozu *et al.*, 2005) these cells can also migrate anteriorly via the LGE into the anterior and lateral cortical regions. While COUP-TFII is important to establish the CMS (Kanatani *et al.*, 2008) COUP-TFI is proposed to control the lateral/anterior migratory stream of CGE-derived cells in mice (Touzot *et al.*,

2016). In addition, a third route that directs CGE-derived cells ventrally into the basal telencephalon, a region corresponding to the medial amygdala (Nery *et al.*, 2002; Touzot *et al.*, 2016) was confirmed to be the case in human in the present study.

Two reasons are proposed for the presence of multiple migration routes for CGE derived cells (Touzot *et al.*, 2016). Firstly, the CGE is the source of neurons for various telencephalic structures (cortex, hippocampus, and amygdala) and multiple routes are required for these cells to reach their specific targets. Possibly, the anterior pathway for CGE-derived interneurons might be more important in human to allow these interneurons to rapidly reach the expanded and more evolved frontal cortex where the majority (50%) of calretinin- expressing interneurons reside (Ma *et al.*, 2013; Hladnik *et al.*, 2014). Secondly, the temporal control of migration of later born CGE-derived interneurons allows proper laminar distribution in the cortex (Miyoshi *et al.*, 2010; Touzot *et al.*, 2016). We found that expression of COUP-TFI and COUP-TFII, and their downstream regulator SP8 are temporally distinct; however, the distribution of these three markers in each pathway might not be exactly the same as has been observed in mice by Touzot *et al.* (2016). The CMS seemed to be dominant at the early stages (8 PCW) where cells mainly expressed COUP-TFI and -TFII but not SP8. However, SP8 was highly expressed in COUP-TFII+ cells in particular in the CMS at older stages (12 PCW). In disagreement with observations in mice (Touzot *et al.*, 2016) SP8 was also highly enriched in COUP-TFII+ cells migrating ventrally from the vCGE into the amygdala even at the earliest age studied; furthermore COUP-TFI was much less expressed by cells following this pathway. This suggests that in human, as has been demonstrated in experiments with transgenic mice (Tang *et al.*, 2012) COUP-TFII is important in patterning the amygdala, whereas COUP-TFI does not play a role. However, it should be noted cells from dLGE migrating to anterior regions of the amygdala co-expressed PAX6 and SP8 but not COUP-TFII (Figure 4.2D,E; Figure 4.9C).

The anterior pathway for vCGE-derived cells via the LGE into the cortex became more prominent at older stages with COUP-TFII and SP8 more highly expressed than COUP-TFI in this migratory stream. In agreement with Ma *et al.* (2013) we observed that a proportion of SP8+ cells entering the cortex from the LGE appeared to originate locally in dLGE rather than migrating from the CGE. However, these particular cells were negative for COUP-TFI, COUP-TFII and CalR expression, thus our observations suggest that cells co-expressing any of these

three markers with SP8 are uniquely generated in the vCGE. In addition, a proportion of COUP-TFI+ and COUP-TFII+ cells entering the cortex from the LGE could also have originated from the MGE/ LGE boundary which can be also a birthplace for COUP-TFI+ and COUP-TFII+ cells.

It is proposed that diverse interneuronal subtypes generated in various domains in the ventral telencephalon use similar cellular mechanisms to translocate while tangentially migrating into the cortex but respond to different chemical cues based on distinct expression of specific surface receptors controlled by a specific transcriptional network (Marín, 2013). Regardless of their subtypes, it has been suggested that the appropriate responsiveness of GE cell sub-types to their particular guidance cues allowing them to migrate through the cortical wall and invade the CP is also unique to mammals and could contribute to the evolution of the neocortex (Tanaka *et al.*, 2011). In rodents migrating interneurons disperse in the cortex via two specific major migratory streams: a superficial stream located in the MZ and deep stream in the lower IZ, although smaller numbers of cells also migrate through the SP (Lavdas *et al.*, 1999; Marín and Rubenstein, 2001; Marín, 2013). The present study shows that the migration pattern of interneurons in the developing human cortex might be more dispersed with less distinguishable pathways than in rodents, at least in the early stage of development (8-12 PCW) where a considerable number of GAD67+ cells also appeared to be migrating through the VZ and SVZ. However, it has been reported that tangentially migrating interneurons, upon reaching the dorsal telencephalon, actively seek the cortical VZ before they migrate to their positions in the cortical plate, possibly to receive information related to their layer position (Nadarajah *et al.,* 2003). In the SVZ or the post-mitotic layers, GAD67+ migrating cells co-expressed COUP-TFI, COUP-TFII, or SOX6. However, interneurons in the VZ were solely MGE-derived cells co-expressing either SOX6 or COUP-TFI only (Figure 4.13) whereas SP8 expression was confined to cells migrating in the SVZ/IZ but not in the MZ, SP, or VZ (Figure 4.9) (Ma *et al.*, 2013). Therefore, the spatial origin and the expression of unique, or unique combinations of, transcription factors in migrating interneurons from the GE may control migration routes and thus their final laminar position in the developing cortex, which may be more complicated in human than in rodents, and could contribute to the evolution of the human neocortex as previously suggested (Tanaka *et al.*, 2011).

4.6 Conclusion

COUP-TFI, COUP-TFII and SP8 are likely to play important roles in human forebrain development. In conjunction with other transcription factors, their expression helps delineate the protomap of the human cortex with SP8 defining more anterior parts of the cortex, COUP-TFI expression more posterior parts of the cortex but COUP-TFII confined to ventro-temporal cortex, which is relatively enlarged in human in comparison to other species. COUP-TFI expression defines the VZ of nearly all the ganglionic eminence compartments contributing interneurons to the cortex whereas COUP-TFII is confined to the VZ of the MGE/LGE boundary and the vCGE, the latter being where distinct classes of interneurons more prominent in the primate brain are generated. Arealised expression of transcription factors in the cortical wall may, in turn, control expression of molecules that attract migrating cells expressing the same transcription factors, setting up the migratory pathways into the cortex for interneurons arriving anteriorly or posteriorly, medially or laterally from the ganglionic eminences. For instance, COUP-TFI expression in the anterior ventral pallium guides entry of COUP-TFI expressing interneurons from the MGE.

Note: Much of this chapter (data, figures and text) has been recently published in a research article (Alzu'bi *et al.*, 2017) under a creative commons licence and in a review article (Clowry *et al.*, 2017) which is reproduced here with permission (See appendices):

- Alzu'bi, A., Lindsay, S.J., Harkin, L.F., McIntyre, J., Lisgo, S.N. and Clowry, G.J. (2017) **'The Transcription Factors COUP-TFI and COUP-TFII have Distinct Roles in Arealisation and GABAergic Interneuron Specification in the Early Human Fetal Telencephalon'**, Cerebral Cortex, 27(10), pp. 4971-4987.

- Clowry, G.J., Alzu'bi, A., Harkin, L.F., Sarma, S., Kerwin, J. and Lindsay, S.J. (2017) **'Charting the protomap of the human telencephalon',** Seminars in cell & developmental biology, Online version.

Chapter 5: Potential Regional Variation and Generation of Subtype-specific Interneurons in the Dorsal Telencephalon of Early Fetal Human brain

5.1 Summary

The prefrontal associative area of the cerebral cortex provides the main biological substrate for higher cognitive abilities in human. In primates it forms a much larger proportion of the frontal lobe compared to all other species; this area contains 50% of all calretinin (CalR) expressing interneurons in the human cortex, and furthermore the proportion of interneurons that are calretinin positive is threefold higher in humans than in rodents. In cultures differentiated from isolated human cortical progenitors from anterior and posterior cortex of (9-12 PCW) human fetal brain, 19% of b-tubulin+ post-mitotic neurons expressed GABA in anterior cortex derived cultures, which was a significantly higher proportion than found in posterior cortex derived cultures (14%). Similarly, a higher proportion of CalR+ cells was observed in anterior derived cultures (37%) than posterior derived cultures (28%), more than half of CalR+ cells in either derived cultures also co-expressed GABA. Many cells expressed either of the COUP-TFs and 30% of these cells also co-expressed GABA, however no cells expressed the characteristic marker of MGE progenitor cells NKX2.1. Treatment of cortical cultures with exogenous sonic hedgehog (SHH) significantly increased cell proliferation but did not alter the regional identity for these cultures, which still lacked NKX2.1 expression and maintained similar proportions of GABA+, COUP-TFII+, and OLIG2+ cells. In RNAseq analysis for 18 genes expressed in GABAergic interneurons and their progenitors of fetal tissue samples taken at two developmental time points (9–10 PCW and 11–12 PCW), the expression of many genes, including *DLX1, DLX2, GSH2, ASCL1, ARX, OLIG2, CALB2* (calretinin) was significantly higher in samples derived from the anterior cortical region than posterior region. Collectively, these data suggest that, in addition to the GE, fractions of GABAergic interneurons could be generated intra-cortically, preferentially in the anterior cortical region. Most of these interneurons resemble CGE-derived interneurons, mostly express CalR and are generated from progenitors expressing either COUP-TFI or COUP-TFII.

5.2 Introduction

The remarkable expansion of human neocortex has been known as the main substrate providing the platform for the higher cognitive abilities compared to all the other species. It is well recognized that the prefrontal cortex (also known as frontal associative area) in humans is the key element for higher cognitive function and working memory (Teffer and Semendeferi, 2012). In primates higher order associative areas have expanded and these cover large part of the human cortical surface (Uylings and van Eden, 1991); in which, the frontal associative area represents 80% of all the associative areas including the parietal associative area (Teffer and Semendeferi, 2012; Hladnik *et al.*, 2014). The deep layer III pyramidal neurons in these associative areas form crucial elements for substantial number of connections with other cortical areas (Barbas *et al.*, 2005; Yeterian *et al.*, 2012) which have been reported as essential substrates involved in higher cognitive abilities (Selemon *et al.*, 2003; Wang *et al.*, 2006; Verduzco-Flores *et al.*, 2009). In addition to the cortical expansion and vast cortical-cortical connectivity, the intrinsic organization of cortical circuitries has been also shown to be evolutionary elements for higher functional properties of the neural networks in primates (Burkhalter, 2008; Forbes and Grafman, 2010). The efficiency of cortical circuitries is highly dependent on the function of inhibitory GABAergic interneurons, which act as intrinsic modulators essential to higher order processing (Whittington *et al.*, 2011; Buzsáki and Wang, 2012).

The majority of interneuronal subtypes are characterized by the expression of parvalbumin (Pv), somatostatin (Sst), or calretinin (CalR) (Wonders and Anderson, 2006; Fogarty *et al.*, 2007; Rudy *et al.*, 2011). In rodents, these interneuronal subtypes comprise 16% of total number of cortical neurons, represented by 7%, 5%, and 4% for Pv, Sst, and CalR expressing interneurons, respectively. The proportions of Pv (7%) and Sst (5%) expressing interneurons are also similar in primate; however, CalR interneurons become the dominant subtype increasing from 4% in rodents to 13% in primate. This notable increase in these interneuronal subtypes raises the total proportion of interneurons by 50% in primate compared to the rodents (Condé *et al.*, 1994; Gabbott *et al.*, 1997; Zaitsev *et al.*, 2005; Barinka and Druga, 2010; Hladnik *et al.*, 2014). Almost half of all these calretinin interneurons, in human, are found in the higher order associative areas (Hladnik *et al.*, 2014). Such a large increase in the proportion of CalR interneurons in the associative cortex could be accompanied by structural reorganization of neural networks in the human associative cortex, initiating different modes

of signal processing, which in turn, increase the cognitive functions of human brain (Burkhalter, 2008; Forbes and Grafman, 2010; Hladnik *et al.*, 2014).

Are interneuron generation mechanisms in human like those found in rodent models? In rodents cortical interneurons are generated in the ganglionic eminences and there is no evidence for generation in the cortex directly (See chapter 1, section 1.2). Are there additional sources for cortical CalR interneurons in human leading to the three-fold increase of these interneuronal subtypes? We have already demonstrated the presence of OLIG2+ and COUP-TFII+ progenitor cells in the cortex of 8-12 PCW fetal human brain (chapters 3 and 4); whether or not these could give rise to GABAergic interneurons remained an open question. Therefore we isolated and cultured progenitor cells from anterior and posterior cortex avoiding temporal cortex where it adjoins the ventral CGE. We then differentiated neurons from these progenitors to see if we produced cells that expressed GABA and markers of interneuron progenitors, like NKX2.1, OLIG2, and COUP-TFs.

This study investigated the spatial (anterior versus posterior cortex) mRNA expression levels for several transcription factors that promote the production, specification, and migration of cortical GABAergic interneurons (See chapter 1, section 1.4). At the top of the molecular hierarchy are the transcription factor ASCL1 and its downstream effectors DLX1/2. They are considered among the earliest transcription factors expressed within the GE, where they act together to coordinate the differentiation of interneuron precursors by regulating notch signalling. Ascl1 is expressed earlier and maintains the undifferentiated state of progenitors; DLX1/2 promotes the progenitor maturation and differentiation (Casarosa *et al.*, 1999; Marín *et al.*, 2000; Yun *et al.*, 2002). DLX1/2 induce the expression of DLX5/6, which are required for interneurons migration and differentiation, but not their generation (Liu *et al.*, 1997; Wang *et al.*, 2010). The ARX homeobox transcription factor also appears to be downstream of DLX genes, which is important for interneuron migration and integration into their laminar position in the cortex (Kitamura *et al.*, 2002; Vogt *et al.*, 2014). Upstream of ASCL1 and DLX1/2 genes are the transcription factors GSX1/2, they are among the earliest transcription factors that are enriched in the LGE and CGE progenitors, but weakly expressed in the MGE (Corbin *et al.*, 2003; Xu *et al.*, 2010). GSX2 maintains the undifferentiated state of progenitors, GSX1 promotes progenitor maturation and differentiation (Pei *et al.*, 2011). GSX1/2 are mainly required for the specification of CGE- derived interneurons; Gsx2 mutants have shown dramatic loss of bipolar CalR-expressing interneurons mainly derived from the CGE (Xu *et al.*,

2010). A sequential transcriptional cascade of Nkx2.1-Lhx6-Sox6 was identified for interneurogenesis in the MGE. NKX2.1 is the key regulator of MGE-derived interneuron specification (Sussel *et al.*, 1999; Xu *et al.*, 2004; Butt *et al.*, 2008; Du *et al.*, 2008); its expression is required for the specification of Sst+ and Pv+ interneurons in this region (Xu *et al.*, 2004; Butt *et al.*, 2008; Du *et al.*, 2008). NKX2.1 directly induce the expression of LHX6 in MGE cells, which is also required for specification of Pv+ and Sst+ interneurons and their migration into the cortex as well (Liodis *et al.*, 2007; Du *et al.*, 2008; Zhao *et al.*, 2008; Flandin *et al.*, 2011; Vogt *et al.*, 2014). SOX6 acts downstream of LHX6, it is mainly required for normal positioning and maturation, but not the specification, of MGE-derived interneurons (Azim *et al.*, 2009; Batista-Brito *et al.*, 2009).

Finally, this study explored the effect of exogenous SHH treatment on cortical cell cultures. In mice, Shh is a well-known ventralizing factor required for the patterning of the ventral telencephalon by promoting the generation of ventral cell types (Kohtz *et al.*, 1998; Rallu *et al.*, 2002). Shh induces the expression of NKX2.1 in the MGE, which is required for the specification of Pv and Sst expressing cortical interneurons (Kohtz *et al.*, 1998; Rallu *et al.*, 2002; Xu *et al.*, 2004). The ventral midline of the diencephalon has been known as the main source of Shh in the developing brain (Ericson *et al.*, 1995). The low level of Shh expression in the dorsal telencephalon is also required to regulate neural progenitor cell proliferation (Komada *et al.,* 2008; Dave *et al.,* 2011). Briefly, Shh binds and inactivates the transmembrane receptor Patched 1 (Ptch1) alleviating the inhibition of a second transmembrane receptor Smoothened (Smo), which in turn initiates a cascade of events lead to nuclear localization of glioma-associated (Gli) transcription factors, the terminal mediators of Shh signalling. In the absence of Shh, Suppressor of Fused (SuFu) binds to GLi transcription factors and anchoring them in the cytoplasm preventing the activation of GLi target genes (Figure 5.1) (Choudhry *et al.*, 2014; Rimkus *et al.*, 2016). In human, SHH and its downstream components (Ptch1, Smo, and Gli transcription factors) are expressed in the human developing cortex from 10th gestational week, their expression increases during development, including both progenitor cells and postmitotic neurons (glutamatergic and GABAergic neurons) (Radonjić *et al.*, 2016; Memi et al., 2018). *In vitro* treatment for RGC cultures, isolated from the dorsal telencephalon of human brain at mid-gestation, with exogenous SHH demonstrated that SHH can promote the MGE-like identity by inducing NKX2.1 expression in these cultures (Radonjić *et al.*, 2016).

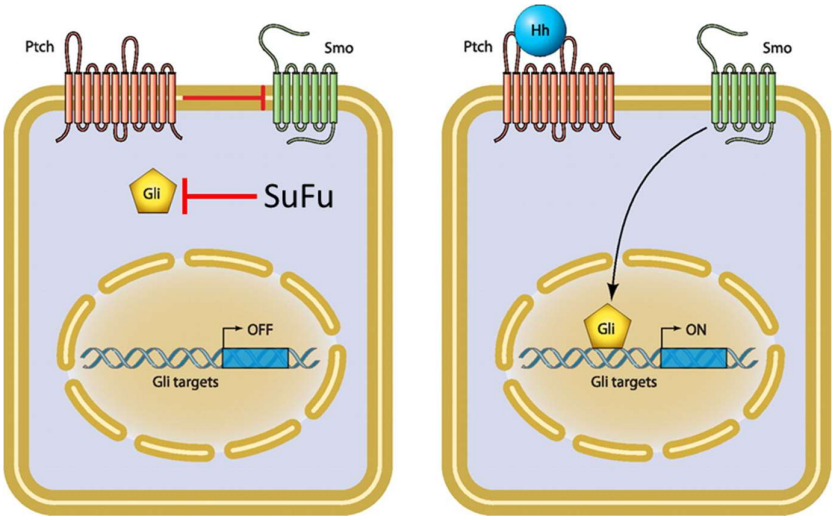

Figure 5.1: Schematic diagram of the Shh signalling pathways. In the absence of Shh ligand, Ptch inhibits Smo, SuFu anchoring Gli transcription factors in the cytoplasm preventing the activation of Gli target genes (left). Binding of Shh ligand to Ptch relieves the inhibition of Smo resulting in the nuclear localization of Gli transcription factors and activation of their target genes (right). Adapted from (Büller *et al.*, 2012).

5.3 Aim of Study

The present chapter firstly aimed to further investigate the potential dorsal origin of specific GABAergic interneuronal subtypes in dissociated cortical cell cultures from (9-12 PCW) human fetal telencephalon. Secondly, investigate the mRNA expression level of selected 18 genes expressed by GABAergic neurons and their progenitors at two developmental time points (9–10 PCW and 11–12 PCW). Finally, explore the effect of exogenous sonic hedgehog (Shh) treatment on cortical cell cultures.

5.4 Results

5.4.1 Expansion and differentiation of neural stem cells

The human fetal brains (n =7, 9-11 PCW; Table 2.1) were dissected, dissociated into single cell suspension, and plated in serum-free medium in the presence of a cytokines (rh EGF, rh bFGF) and heparin (See chapter 2, section 2.6). After 24 h of plating, round single cells were still suspended in the medium and translucent in appearance, progenitors continue to proliferate to form neurospheres in the presence of epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF) (Reynolds and Weiss, 1992; Siebzehnrubl *et al.*, 2011). In the next two days, the NSCs (progenitor cell) started to proliferate forming small clusters of cells (Figure 5.2A), the majority of these clusters became attached to the surface of the culture ware. However, these clusters grew and increased in size day 4 and day 5, detached from the surface and floated in the medium (Figure 5.2B). Over the next few days, the neurospheres increased in size and became rounded in shape; their centres were light in colour and translucent, with microspikes displayed from the cells on the outer surface (Figure 5.2C). The condition of the cultures was monitored every day; the medium was also replenished every two days. By day 13 and day 14, although neurospheres had various sizes, the majority of these neurospheres reached 100–200 μ in diameter, their centres were darkened in colour and became ready for passaging. If neurospheres grow too large, the darkened area will expand, and the centre of the neurosphers become blackened due to cell death, as these cells in the centre are not able to acquire the required oxygen and nutrients (Reynolds and Weiss, 1992; Siebzehnrubl *et al.*, 2011). Neurospheres were chemically and mechanically dissociated into single cell suspension and plated under the same conditions as the primary culture (See chapter 2, section 2.6.4).

Cells from dissociated neurospheres were spontaneously differentiated by withdrawal of the growth factors, adding small amount of serum, and plating the cells on poly-l-lysine (adhesive substrate) coated culture ware (Figure 5.2D). After 8 days, NSCs had become differentiated into the three primary cell types found in the developing human cortex (see next section).

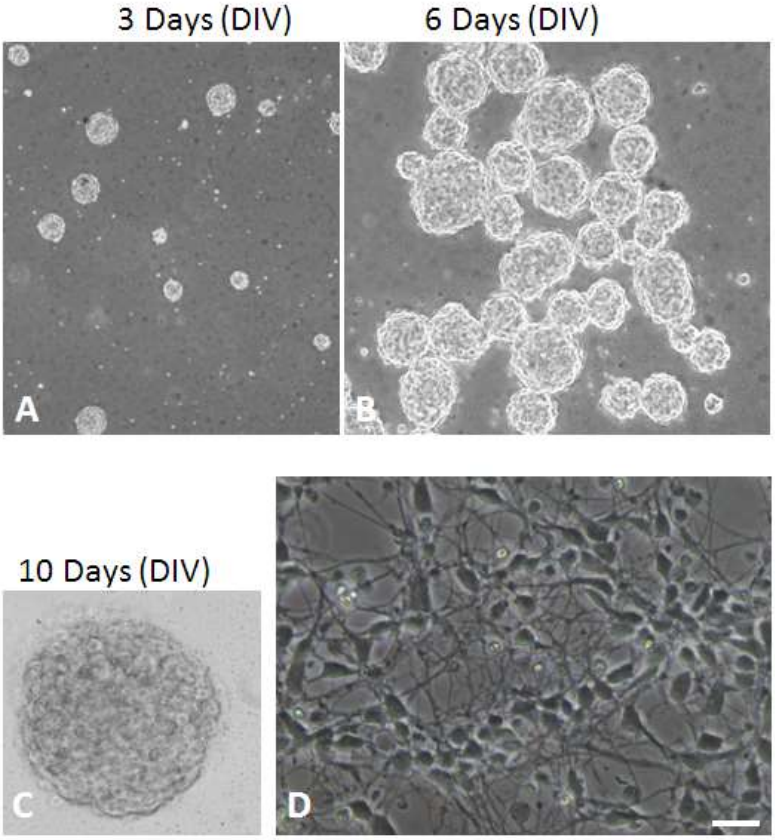

Figure 5.2: Phase contrast images of neurospheres and monolayer differentiating neurons. (A) 3 days *in vitro* (DIV), progenitor cells started to proliferate forming small clusters still attached to the surface of the culture ware. (B) By 6 DIV, neurospheres have grown in size and detached from surface. (C) Floating 10 DIV neurosphere, rounded in shape, light in colour and translucent, with microspikes displayed from the cells on the outer surface. (D) 8 days monolayer differentiating neurons. Scale bars: 50 μm.

5.4.2 Characterization of the cortical cell culture

The dissociated cortical cell cultures from anterior and posterior cortical regions (n=3) were first characterized by testing their immunoreactivity for general markers for various cell types after 8 days of plating in differentiating media, the time point when a high proportion of neural progenitors are present (Figure 5.3). The intermediate filament protein nestin, cell division marker KI67, and radial glia cell marker PAX6 were used to identify the human cortical neural stem/progenitor cells (Englund *et al.*, 2005; Bayatti *et al.*, 2008a), b-tubulin used for post-mitotic neurons (Katsetos *et al.*, 2003), TBR1 for cortically-derived glutamatergic neurons (Englund *et al.*, 2005; Bayatti *et al.*, 2008a), and the Glial fibrillary acidic protein (GFAP) to identify the glia cells like the astrocytes (Levitt and Rakic, 1980).

Generally, mixed populations of progenitor and post-mitotic cells were observed (Figure 5.3; Figure 5.4). 21 ± 1 % of the total number of cells identified by nuclear DAPI labelling expressed the cell division marker KI67, 25 ± 2 % expressed the radial glia marker PAX6, 35 ± 1% expressed b-tubulin, and only 3 ± 0.02 % expressed GFAP (Figure 5.4A). Almost 67 ± 3% of b-tubulin expressing neuroblasts/neurons were also TBR1 positive (Figure 5.3A) suggesting that a proportion of these postmitotic cells may be neuronal cell types other than glutamatergic neurons. When analysing anterior versus posterior cultures, statistically significant differences in the number of cells expressing these markers were found only for PAX6 and b-tubulin; more PAX6+ cells found in posterior (30 ± 2%) than anterior (21 ± 2%) derived cortical cultures (Figure 5.4C). Only a proportion of these PAX6+ cells were also dividing (Figure 5.3C) in either anterior or posterior cultures. Conversely, the number of b-tubulin + cells was generally higher in anterior (37 ± 2%) than posterior (30 ± 1%) derived cultures (Figure 5.4D). However, the number of dividing (KI67+) cells and astrocytes (GFAP+) was not significantly different in anterior and posterior derived cortical cultures (Figure 5.4B,E).

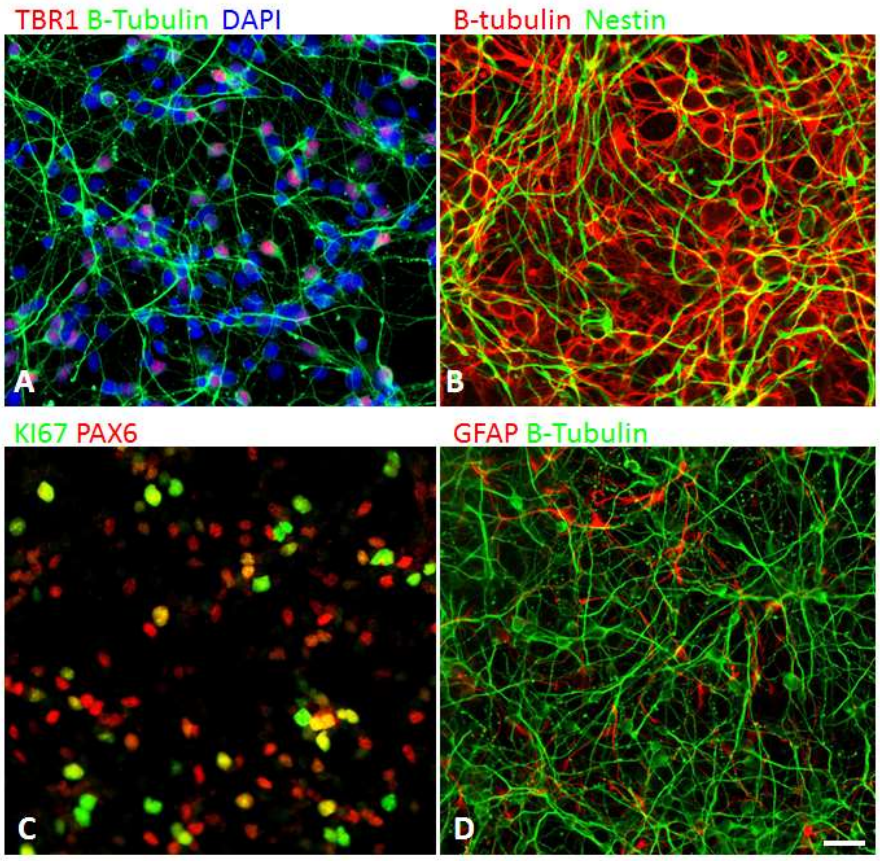

Figure 5.3: Characterization of the cortical cell cultures derived from human fetal cortex. Examples of double immunofluorescent staining for various markers of neuronal stem cells, post-mitotic neurons and glia cells like TBR1 and b-tubulin (A) nestin and b-tubulin (B) KI67 and PAX6 (C) GFAP and b-tubulin (D). Scale bar: 50 µm.

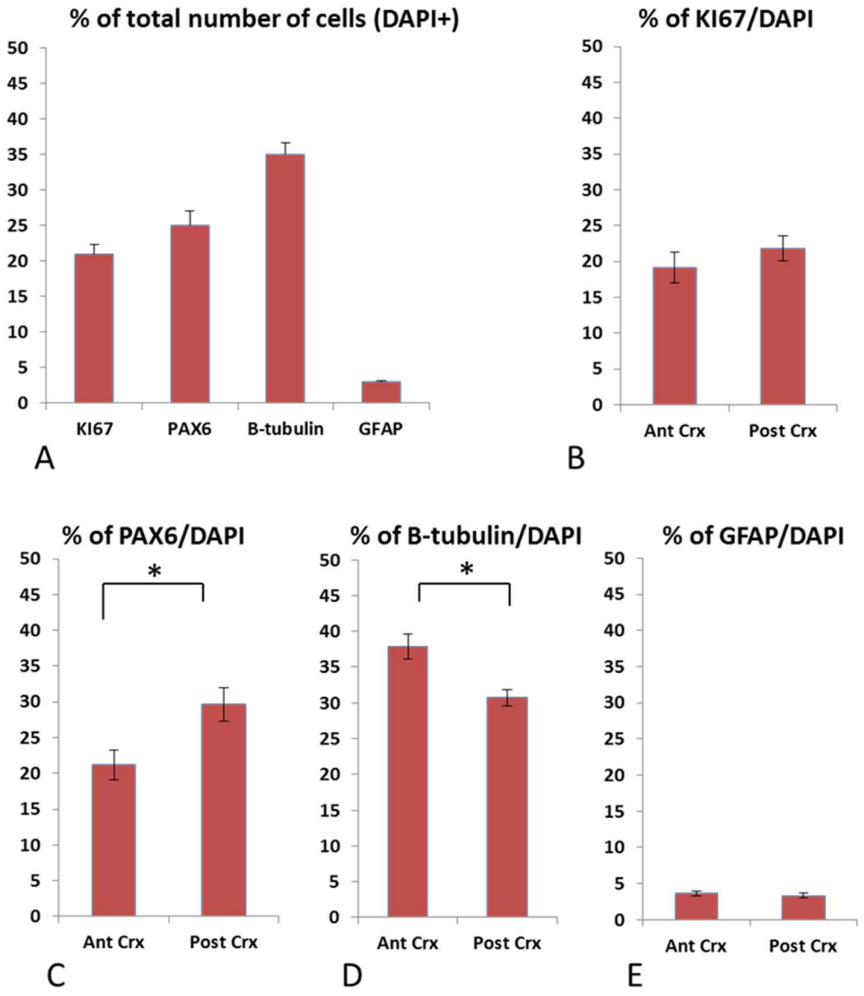

Figure 5.4: Cells quantification for general markers positive cells in cultures derived from anterior and posterior human fetal cortex (n=3). The overall percentage of KI67+, PAX6+, B-tubulin+, and GFAP+ cells from the total number of cells (DAPI+) in human fetal cortex cultures (A). No significant difference in the number KI67+ cells was found in cultures derived from the anterior and posterior cortex (B). Significantly higher PAX6+ cells were found in cultures derived from the posterior cortex (C). Conversely, higher B-tubulin+ cells were found in cultures derived from anterior cortex (D). No significant difference was found in the number GFAP+ cells in cultures derived from the anterior and posterior cortex (E). Asterisk denotes a statistically significant difference $P < 0.05$, 2-tailed t-test.

5.4.3 GABA+ and calretinin+ cells in cortical derived cell cultures

In further investigations for the diversity of neuronal progenitor cells in the cortex of early human fetal brain, we next sought to explore if the cortex contains populations of progenitor cells, similar to those found in the GE, that give rise to different pools of cortical GABAergic

interneurons. We tested cells in our cultures (from cortex and GE; n=3; Figure 5.5) for immunoreactivity to GABA, the neurotransmitter of inhibitory interneurons often proposed to derive almost entirely from progenitor cells of ganglionic eminence origin (Le Magueresse and Monyer, 2013) (Chapter 1, section 1.2). Cells were also tested for immunoreactivity to the calcium binding protein calretinin which is used as phenotypic marker for CGE-derived GABAergic interneurons (Kanatani *et al.*, 2008). Remarkably, immunoreactivity for these two markers was not confined to GE-derived cultures, but was also found in either anterior and/or posterior derived cortical cultures (Figure 5.5 A-C).

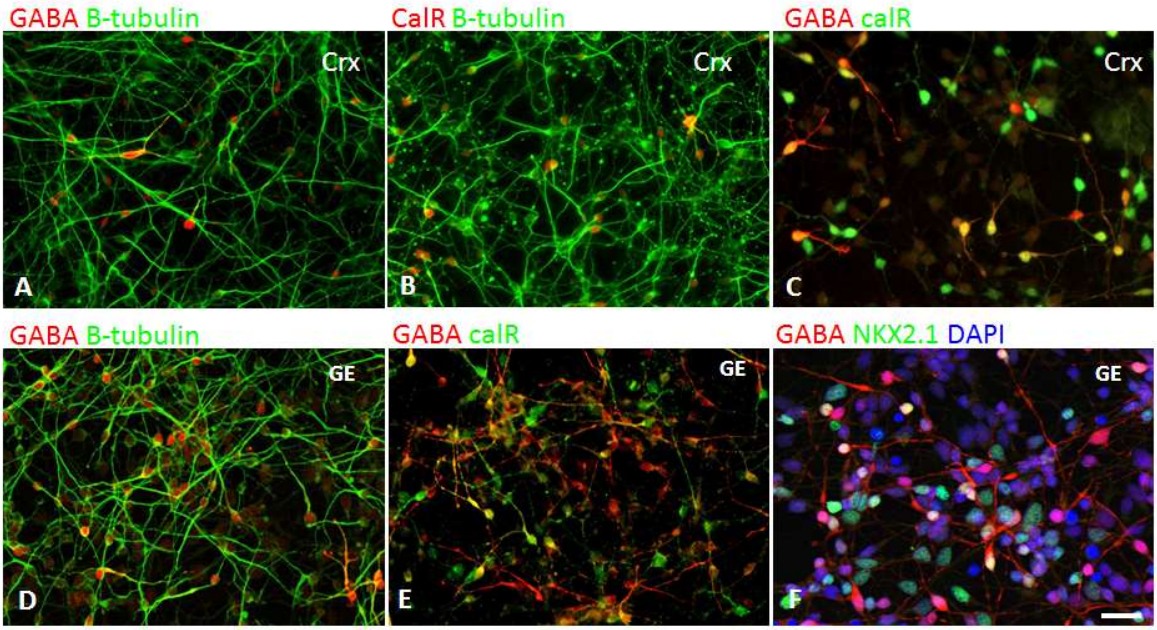

Figure 5.5: GABA+ and CalR+ neurons can be generated from cortically-derived progenitor cells. (A,B) Proportion of b-tubulin+ (green) co-expressed GABA and CalR (red) cortically-derived cultures. (C) Only proportion of ClaR+ cells were double labelled with GABA. (D,E) Higher proportion of GABA+ and CalR+ cells were observed in GE-derived cultures. (F) NKX2.1 expression was only found in GE-derived cultures (but not cortical cultures). Scale bar: 50µm.

As both GABA and calretinin are considered as post-mitotic markers, the expressions of these two markers were normalized to the expression of b-tubulin when investigating potential regional variations for GABAergic interneuron generation. Almost 19 ± 2% (177 b-tubulin+ cells and 34 GABA+ cells) of b-tubulin+ cells expressed GABA in cultures from the anterior cortex, which was higher than in cultures from the posterior cortex (14 ± 2%, 126 b-tubulin+ cells and 18 GABA+ cells; Figure 5.6A). As GABAergic interneurons are proposed to derive almost entirely from progenitor cells of the ganglionic eminences in primate (Hansen *et al.*, 2013b; Ma *et al.*, 2013; Arshad *et al.*, 2015) these observations may indicate either that cells have lost their regional identity during dissociation, expansion and culturing or that a proportion of interneurons are generated from cortical progenitor cells in developing fetal human brain as we proposed in chapter 1 and has been suggested previously (Zecevic *et al.*, 2011; Radonjić *et al.*, 2014a; Clowry, 2015). There was evidence, however, that the cultures retained regional identity; cultures of GE derived cells from the same brains showed a far higher proportion of b-tubulin+/GABA+ cells (53 ±2%, 132 b-tubulin+ cells and 70 GABA+ cells; Figure 5.5D,E; Figure 5.6A) and a proportion of cells expressed NKX2.1, characteristic of MGE derived progenitors (Figure 5.5F), whereas no NKX2.1 immunoreactivity was observed in cortical cultures (data not shown) as seen in forebrain sections immunostained for NKX2.1 before 12 PCW (See chapter 3, section 3.3.4) (Hansen *et al.*, 2013b; Pauly *et al.*, 2014).

In addition, significantly higher proportions of calretinin+ cells were also observed in culture from the anterior cortex than cultures from the posterior cortex; 37 ± 4% (157 b-tubulin+ cells and 59 CalR+ cells) and 28 ± 2% (172 b-tubulin+ cells and 48 CalR+ cells) of b-tubulin+ cells expressed calretinin in cultures from the anterior cortex and posterior cortex, respectively Figure 5.6B). However, only a proportion of CalR+ (49 ± 3%, 45 CalR+ cells and 22 GABA+ cells) cells co-expressed GABA (Figure 5.5C) confirming that calretinin expression in human fetal brain is not restricted to GABAergic interneurons but is also a marker for cortically derived pioneer neurons and Caja-Retzius cells (González-Gómez and Meyer, 2016). All these findings provide additional evidence that the cortex is capable of making its own interneurons. In addition, our findings suggest that the anterior cortex, origin of the highly evolved prefrontal cortex crucial to higher cognitive function, might be considered as favoured region for cortical interneurogenesis.

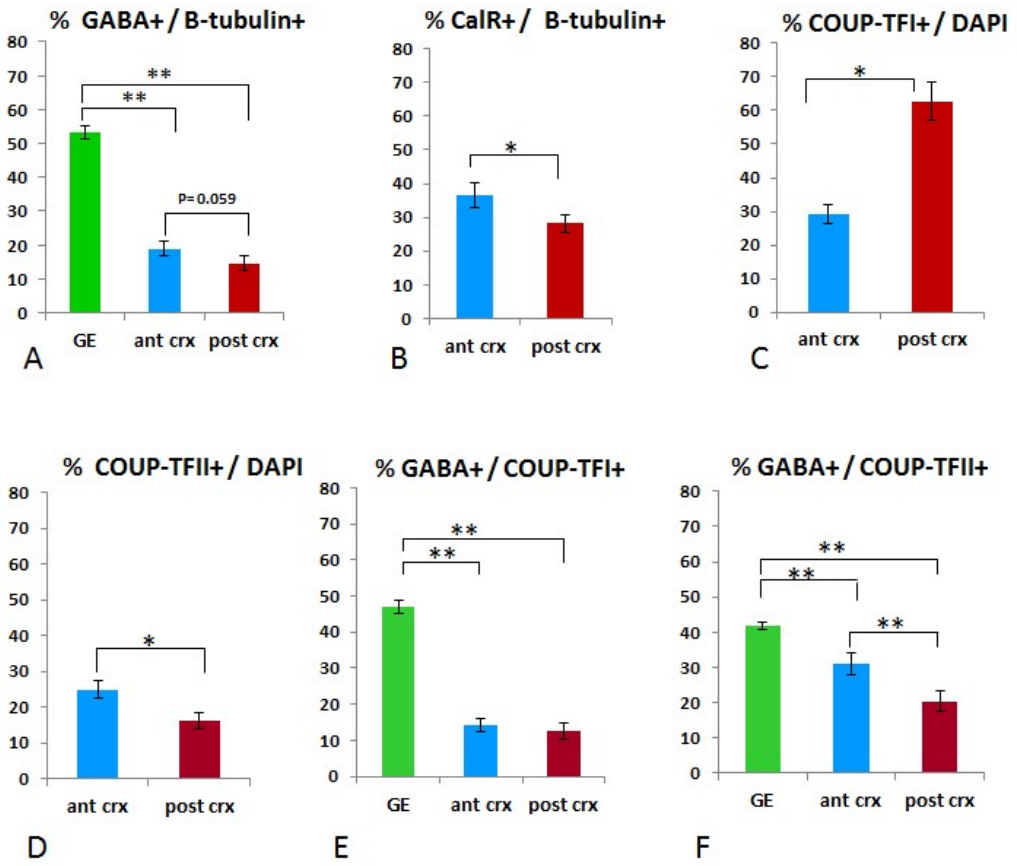

Figure 5.6: Cells quantification for GABAergic interneuron markers positive cells in cultures derived from anterior and posterior human fetal cortex (n=3). (A) The percentage of GABA+ cells of all B-tubulin+ cells in cultures derived from the GE, anterior cortex, and posterior cortex. (B) The percentage of CalR+ cells of all B-tubulin+ cells anterior and posterior derived cortical cultures. (C,D) The percentage of COUP-TFI+ and COUP-TFII+ cells of total number of cells (DAPI+) in anterior and posterior derived cortical cultures. (E,F) The percentage of GABA+ cells co-expressed COUP-TFI and COUPTFII in cultures derived from the GE, anterior cortex, and posterior cortex. Asterisk denotes a statistically significant difference $P < 0.05$, 2 asterisks $P < 0.0005$, 2-tailed t-test.

5.4.4 OLIG2+ cells but not NKX2.1+ cells in cortical derived cell cultures.

Analysis of immunoreactivity for NKX2.1 and OLIG2, MGE-derived GABAergic interneuron precursor transcription factors, on sections of 8-12 PCW forebrain has shown that NKX2.1 expressing progenitors were mainly confined to the MGE and the sub-pallial septum (MGE-like septum) and NKX2.1 appeared to be downregulated in cells migrating into the cortex. On the other hand, OLIG2 appeared continually expressed in migrating cells, but also seen

throughout the cortex in considerable numbers of actively dividing cells (See Figure 3.9). When testing cells in our cultures for these two markers, similar findings were also observed; NKX2.1 positive cells were only observed in culture from the GE but not from either the anterior or posterior derived cortical cultures (Figure 5.5F; data not shown). Conversely, cortical cultures were noticeably populated with OLIG2+ cells. Furthermore, 43 ± 2% of OLIG2+ cells were also dividing, actively expressing the cell division marker KI67 (Figure 5.7A). However, no statistically, significant difference was found in the numbers of OLIG2+ cells of all cells in the anterior (11 ± 1%) or posterior (8 ± 1%) derived cortical cultures, which was inconsistent with our previous findings in the immunostained sections where the anterior cortex was more heavily populated with OLIG2+ cells (see chapter 3, section 3.3.6).

Unfortunately, we were not able to investigate if OLIG2 expressing cells/ neuroblasts that we found in our cortical cultures are the source of GABA cells observed in the same cultures (see previous sections) because, firstly, double immunostaining for these two markers was not possible because the available commercial antibodies for these two markers were raised in the same species. Secondly, OLIG2 appeared to be expressed mainly in neuronal progenitor (dividing) cells, whereas GABA expression is found mainly postmitotic cells (b-tubulin expressing cells; Figure 5.5A). However, the absence of NKX2.1 expression in these cortical cultures indicates that these GABA cells may constitute interneuronal subtypes different from those generated in the MGE.

5.4.5 COUP-TFI and COUP-TFII expression in cortical derived cell cultures

We have previously demonstrated that COUP-TFs are expressed in populations of cortical GABAergic interneurons in human fetal brain; these two transcription factors were widely expressed in neuronal progenitor and postmitotic cells of ventral and dorsal telencephalon (Chapters 3 and 4). The expression, particularly of COUP-TFI, in the cortex was not confined to the inhibitory GABAergic interneurons but also observed in progenitor cells that give rise to populations of excitatory glutamatergic neurons (Figure 3.13H; Figure 4.6). Whether or not COUP-TFs expressing progenitors could give rise to GABAergic interneurons as well as glutamatergic pyramidal neurons remained an open question.

In additional analysis for cell type specification of *in vitro* cortical derived cultures, further evidence that our cultures retained regional identity was provided by the observation that both COUPTFI and COUPTFII were expressed in distinct gradients (Figure 5.6C,D; Figure 5.7B,C). COUP-TFI was expressed differentially between anteriorly and posteriorly derived cortical cultures as it is between anterior and posterior cortex; 29 ± 3 % of all cells in anterior derived cultures and 63 ± 6% of cells in posterior cortical derived cultures (Figure 5.6C). A proportion of COUP-TFI+ cells were double-labelled with either PAX6 (45 ± 3 %) or B-tubulin (20 ± 2 %), indicating that COUP-TFI was expressed in both progenitor and post-mitotic cortical cells (Figure 5.7B). A lower level of expression was found for COUPTFII than for COUP-TFI (Figure 5.6D; Figure 5.7C). All these observations are consistent with the immunohistochemical and RNAseq data from intact brains (chapter 4). Furthermore, and contrary to the findings for COUP-TFI expression, the proportion of DAPI labelled cells also immunoreactive for COUP-TFII+ was significantly higher in anterior cortex (25 ± 2%) than in posterior cortex cultures (16 ± 2%; Figure 5.6D).

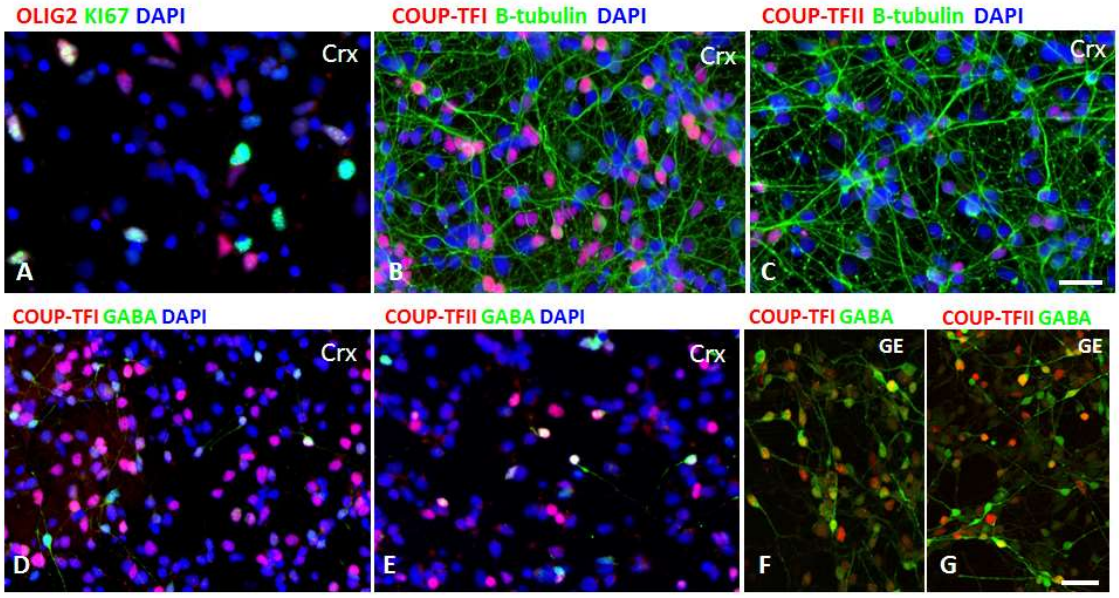

Figure 5.7: The expression of OLIG2, COUP-TFI, and COUP-TFII in cortically-derived cell cultures. (A) OLIG2 (B,C) Double labelling for COUP-TFI and COUP-TFII (red) with B-tubulin (green) in cortically-derived cell cultures.(D-G) Proportion of COUP-TFI+ and COUP-TFII+ cells co-expressed GABA in cortically-derived cell culture. (F,G) The majority of COUP-TFI+ and COUP-TFII+ cells co-expressed GABA in MGE-derived cell cultures. Scale bar: 50µm.

We hypothesised that COUP-TFI and COUP-TFII positive progenitors could provide the origin of GABAergic interneurons born in the cortex, as has been previously suggested, especially for COUP-TFII (Chapters 3 and 4) (Reinchisi *et al.*, 2012; Radonjić *et al.*, 2014a). In anterior cortex cultures 14 ± 2% of COUP-TFI+ and 31 ± 3% of COUP-TFII+ cells co-labelled for GABA and in posterior cortex cultures 13 ± 2% of COUP-TFI+ and 20 ± 2% of COUP-TFII+ cells were similarly double-labelled. However, a higher proportion of cells co-labelled for GABA and COUP-TFs in GE cultures (Figure 5.6E,F; Figure 5.7D-G). We conclude that there was no difference in the proportion of GABAergic neurons deriving from either COUP-TFI or COUP-TFII expressing precursors between anterior or posterior derived cultures, but, bearing in mind the higher proportion of COUP-TFI+ cells posteriorly and COUP-TFII+ cells anteriorly, there could be differences in the composition of populations of interneurons deriving from anterior and posterior cortex in terms of COUP-TFI and COUP-TFII expression. The conclusion still is that at the time of culturing there were progenitors present in the cortex that express COUP-TFI and/or COUP-TFII and are capable of generating GABAergic interneurons.

5.4.6 Investigating the gene expression level and gradient for GABAergic genes using RNAseq analysis

The largest RNAseq study to date of human cerebral cortex with 137 samples from different cortical regions with an age range of 7.5 to 17 PCW has been recently completed by our research group, from which we have produced normalised RPKM data for all genes for comparison of gene expression levels and can classify protein coding genes as very highly expressed (top 5% normalised RPKM >160) high expression (top 25%; 40-160) moderate expression (25-50%: 10-40) low expression (50-75%; 0.4-10) no expression above background (bottom quartile; <0.4) (Lindsay *et al.*, 2016; Clowry *et al.*, 2017; Harkin *et al.*, 2017).

In a subset of samples from 9-10 and 11-12 PCW (Table 2.3), we specifically analysed anterior versus posterior differences in the expression of 18 genes expressed by GABAergic neurons and their progenitors including several transcription factors, the two isoforms of GABA synthesising enzymes (*GAD1* and *GAD2*), and two GABA interneuron phenotypic markers (*CALB1* and *CALB2,* the genes for calbindin and calretinin respectively) (Table 5.1). As demonstrated earlier (see chapter 4, Section 4.4.1) analysis of the expression of several

transcription factor genes (*COUP-TFI*, *COUP-TFII*, and *SP8*) using the same set of RNAseq data confirmed that graded expression can occur in the human cortex at these developmental stages (Figure 4.1). In addition, RNA expression gradients for these genes were also consistent with the protein expression displayed by the immunohistochemical analysis indicating that these gradients were not an artefact of the experimental procedures (Figure 4.9A,C). In both studied stages, 9-10 and 11-12 PCW, the obtained data have shown that only three of our selected genes were classified as highly expressed genes (RPKM values 40-100) which are *ASCL1*, *ARX*, and *CALB2* (Figure 5.8). Conversely, three genes (*GSX1*, *LHX8*, and *CALB1*) have shown RPKM values at only the background level (RPKM <0.4). Whereas the remaining genes were classified as either moderately expressed (RPKM values 10-40) or expressed at low level (PRKM values 0.4-10).

Table 5.1: List of GABAergic genes used in RNAseq analysis with their description from rodent studies.

Gene Name	Description (from rodent studies)
DLX1	GABA interneuron progenitor*
DLX2	GABA interneuron progenitor*
DLX5	GABA interneuron *
DLX6	GABA interneuron *
ASCL1	GABA interneuron progenitor*
GSX1	GABA interneuron progenitor*
GSX2	GABA interneuron progenitor*
NKX2.1	MGE- GABA interneuron progenitor*
OLIG2	GABA interneuron/Oligodendrocyte progenitor marker*
LHX6	MGE-derived GABA interneuron*
LHX8	MGE-derived GABA interneuron*
SOX6	MGE-derived GABA interneuron*
PROX1	CGE-derived GABA interneuron *
ARX	GABA interneuron*
GAD1	GABA synthesizing enzyme (GAD67)
GAD2	GABA synthesizing enzyme (GAD65)
CALB1	GABA interneuron phenotypic marker calbindin
CALB2	CGE- derived GABA interneuron phenotypic marker calretinin

*Transcription factor.

At 9-10 PCW, a significant fold increase in anterior/posterior expression levels was detected in the overall average expression for the transcription factors *ASCL1* and its downstream effectors *DLX1/2* (Figure 5.8A; Figure 5.9), three transcription factors that are early expressed in the precursors of GABAergic interneurons, where they act together to coordinate their differentiation by regulating the notch signalling (Casarosa *et al.*, 1999; Marín *et al.*, 2000; Yun *et al.*, 2002). Although the overall expression level of *ASCL1* considerably decreased at 11-12 PCW, anterior/posterior fold increase was still significant (Figure 5.8B; Table 5.2). On the contrary, the expression levels *DLX1/2* increased at 11-12 PCW in both anterior and posterior cortex with a tendency for higher expression in anterior cortex (Figure 5.10; Table 5.2). These findings for these three transcription factors are also in agreement with previous quantitative PCR and microarray studies between 8-12 PCW, which identified an anterior to posterior gradient of expression for these three markers (Bayatti *et al.*, 2008a; Al-Jaberi *et al.*, 2015a). *DLX5/6* are another two genes of *DLX* family, located downstream of *DLX1/2*, that were also expressed in the developing human cortex. Far higher expression, 8 fold, was observed for *DLX5* compared to *DLX6* at 9-10 PCW (Figure 5.9); an increase of the cortical *DLX5/6* mRNA levels was recognised at 11-12 PCW, changing 7 and 5 fold for *DLX5* and *DLX6*, respectively (Figure 5.10). For both *DLX5/6*, a higher expression was always observed in the anterior cortex compared with posterior cortex in the studied stages of development, but was statistically significant only for DLX5 at 11-12 PCW (Figure 5.9; Figure 5.10; Table 5.2).

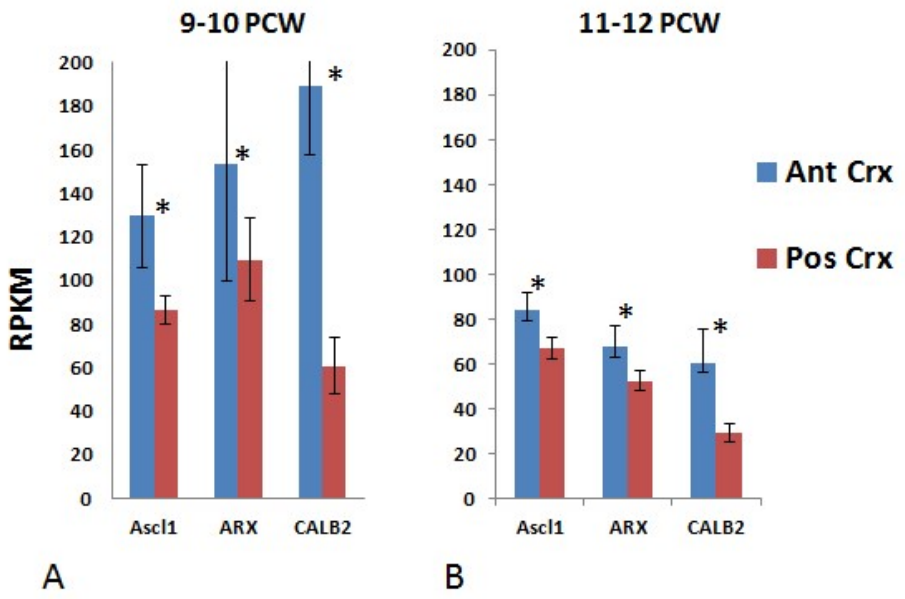

Figure 5.8: Gradients of *ASCL1*, *ARX*, and *CALB2* expression across the cortex of 9-10 and 11-12 PCW human brain by RNAseq. (A) Significantly higher expressions were observed for these three markers in anterior cortex than posterior cortex at 9-10 PCW. (B) The overall expression levels decreased at 11-12 PCW; however, anterior/posterior fold increase was still significant. (1-way ANOVA, Tukey's post hoc comparison, *P < 0.05).

Upstream to *ASCL1* and *DLX* genes are *GSX1* and *GSX2* which are enriched in the CGE and important for the generation of calretinin expressing interneurons in this domain (Corbin *et al.*, 2003; Wang *et al.*, 2009; Xu *et al.*, 2010). Very low expression was identified for these two transcription factors in the developing human cortex at 9-10 PCW, with RPKM values was close to background (<0.4; Figure 5.9); however, *GSX2* expression has greatly increased at 11-12 PCW, changing 20 fold from 9-10 to 11-12 PCW. Although it was not statistically significant, the trend for higher expression obtained in the anterior cortex was also identified (Figure 5.10; Table 5.2). Another transcription factor which is specifically important for migration and differentiation, but not production, of CGE-derived interneurons is Prox1 in rodents (Miyoshi *et al.*, 2015) which was also shown to have very low expression in fetal human cortex at both 9-10 and 11-12 PCW (Figure 5.9; Figure 5.10).

The transcription factors that are specifically expressed by MGE derived GABAergic interneurons and their progenitors (like NKX2.1, OLIG2, LHX6, LHX8, and SOX6) also showed a low (*NKX2.1* and *LHX8*) and moderate (*OLIG2*, *LHX6*, *SOX6*) level of expression in our RNAseq data. The mRNA level for *NKX2.1* and *OLIG2* followed the trend seen for their

protein expression in either dissociated cell culture (see section 5.4.4) and/or in forebrain sections immunostained for NKX2.1 and OLIG2 (Chapter 3). Very low expression was observed for *NKX2.1* at 9-10 PCW, with slightly higher expression found at 11-12 PCW (Figure 5.9; Figure 5.10, Table 5.2); this is inconsistent with our previous findings where NKX2.1 protein expression was only found in very small number of cells in sections of 12 PCW human cortex (see chapter 3, section 3.3.4); similarly, no NKX2.1+ cells were found in dissociated cell culture from the cortex of 9-12 PCW human brain (see above, section 5.4.4). Unlike *NKX2.1*, *OLIG2* mRNA has shown a considerably higher level of expression at 9-10 PCW, with significantly higher expression seen in the anterior cortex than posterior cortex. The overall expression doubled at 11-12PCW; although it was not statistically significant, higher expression was observed in the anterior cortex (Figure 5.9; Figure 5.10; Table 5.2). These findings are also in agreement with our previous findings, where significant numbers of OLIG2+ cells, mostly dividing cells, were populating the cortex of 8-12 PCW brains; either in immunostained sections or dissociated cell cultures, OLIG2+ cells were more common in the anterior cortex (Figure 3.9A,B; Figure 5.7A). LHX6, LHX8, SOX6 and ARX are located downstream of NKX2.1, but their expression is maintained in migrating MGE-derived interneurons where they are required for their specification, migration and integration in the cortex (Zhao *et al.*, 2003; Liodis *et al.*, 2007; Du *et al.*, 2008; Batista-Brito *et al.*, 2009; Vogt *et al.*, 2014). Moderate expression was observed for *LHX6* in both developmental stages, with no significant difference in the expression level in the anterior and posterior cortex. However, The expression of other member of *LHX* family *LHX8* was only at the background level indicating no expression for LHX8 in the human fetal cortex at these stages of development (<0.4; Figure 5.9; Figure 5.10). The effectors downstream of LHX6 are SOX6 and ARX (Zhao *et al.*, 2003; Liodis *et al.*, 2007; Batista-Brito *et al.*, 2009; Vogt *et al.*, 2014) Moderate expression was also observed for *SOX6* and it was the only transcription factor that showed significantly higher posterior expression at 9-10 PCW, however there was no significant difference in the expression level observed in the anterior and posterior cortex at 11-12 PCW (Figure 5.9; Figure 5.10). *ARX* was generally more highly expressed (RPKM > 100) than *LHX6* and *SOX6*, with significantly higher expression obtained from the anterior cortex than the posterior cortex (Figure 5.8; Table 5.2) suggesting that ARX may be expressed in neuronal subtypes other than MGE-derived interneurons for example CGE-derived interneurons or even in the glutamergic neurons. Similar to *ASCL1* significantly higher expression obtained from the anterior cortex than the posterior cortex at 9-10 PCW, with considerable decrease in the

expression level observed at 11-12 PCW; however, anterior/posterior fold increase was still significant (Figure 5.8A,B; Table 5.2).

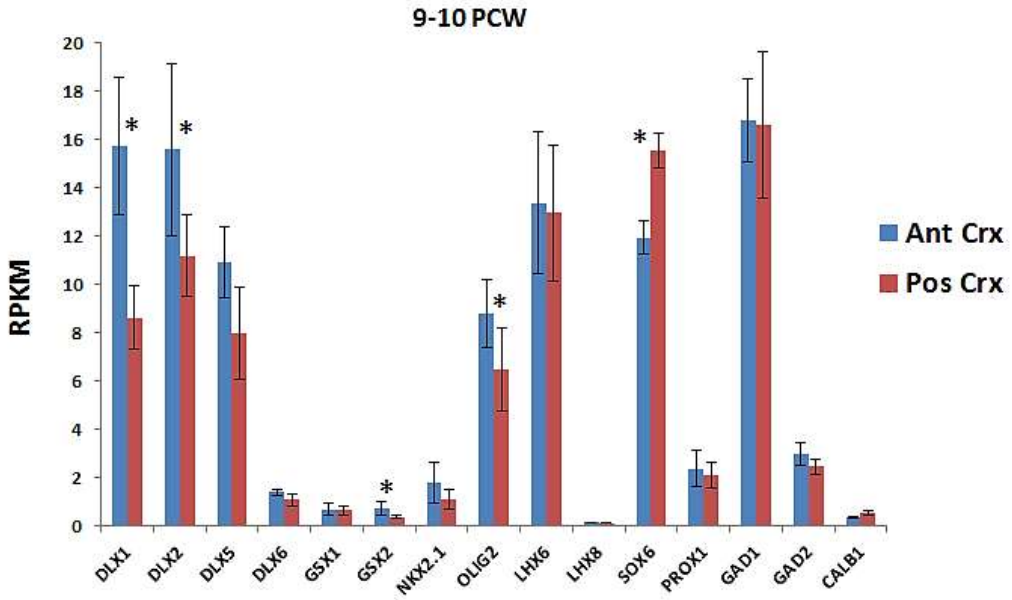

Figure 5.9: Gradients of GABAergic gene expression across the cortex of 9-10 PCW human brain by RNAseq. *DLX1, DLX2, GSX2* and *OLIG2* showed significant higher expressions in anterior cortex than posterior cortex. Only *SOX6* showed significant higher expression in posterior cortex at this stage (1-way ANOVA, Tukey's post hoc comparison, *P < 0.05).

Finally, we have also analysed the mRNA expression for the two glutamate decarboxylase isoforms of GABA synthesising enzymes (*GAD1* and *GAD2*) and two GABA interneuron phenotypic markers (*CALB1 and CALB2*). *GAD1* was 5 fold more highly expressed than *GAD2* at 9-10 and 11-12 PCW, with no significant difference in the expression in anterior and posterior cortex (Figure 5.9; Figure 5.10). Far higher expression was observed for *CALB2* than *GAD* genes (up to more than 10 fold) suggesting, as mentioned above, that *CALB2* is expressed in cells other than inhibitory interneurons like cortically derived pioneer neurons and Caja-Retzius cells (Meyer *et al.*, 2000; González-Gómez and Meyer, 2016). In addition, anterior cortex showed significantly higher expression of *CALB2* than the posterior cortex at 9-10 PCW (Figure 5.8A). Although there was a dramatic decrease of *CALB2* expression in the anterior cortex at 11-12PCW, it was still significantly higher than in the posterior cortex (Figure 5.8B). In contrast, the expression level of other calcium binding protein *CALB1* was only at the background level in both anterior and posterior cortical regions at these stages of development (<0.4, Figure 5.9; Figure 5.10).

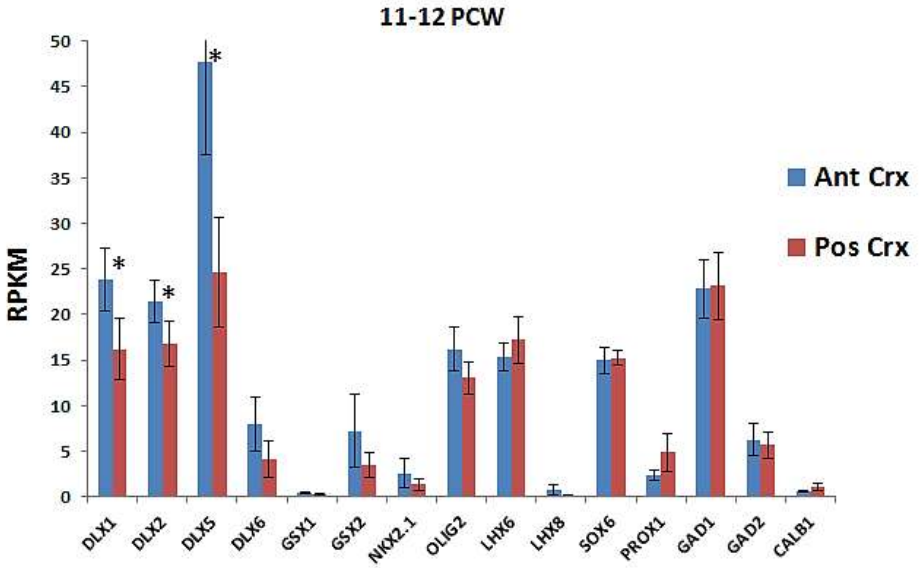

Figure 5.10: Gradients of GABAergic gene expression across the cortex of 11-12 PCW human brain by RNAseq. *DLX1, DLX2*, and *DLX5* showed significant higher expressions in anterior cortex than posterior cortex. (1-way ANOVA, Tukey's post hoc comparison, *P < 0.05).

Table 5.2: The fold difference of GABAergic genes expression in anterior (A) and posterior (P) cortex.

Gene Name	Fold Difference A>P	
	9-10 PCW	11-12 PCW
DLX1	1.8	1.5
DLX2	1.4	1.3
DLX5	1.4	1.9
DLX6	1.3	1.9
ASCL1	1.5	1.3
GSX1	1	1.3
GSX2	2	2
NKX2.1	1.6	1.9
OLIG2	1.4	1.2
LHX6	1	0.9
LHX8	1	4.2
SOX6	0.8	1
PROX1	1.1	0.5
ARX	1.4	1.3
GAD1	1.2	1
GAD2	1	1.1
CALB1	0.7	0.5
CALB2	3.1	2

5.4.7 The effect of exogenous Shh treatment on cortical cell cultures

The final aim of this project was to explore the effect of exogenous SHH treatment on cortical cell cultures. We firstly evaluated our RNA seq data for the expression levels of *SHH* mRNA and other downstream components of Shh signalling pathway (*PTCH1*, *SMO*, *SUFU*, *GLI1*, *GLI2*, and GLI3). For these genes we used the full age range of samples available from 7.5 to 17 PCW (Figure 5.11). Distinct expression levels were observed for these various components; low level of expression was detected for *SHH* (RPKM values 0.4-10) at any time point between 8-12 PCW (Figure 5.11A). Nevertheless, a remarkable higher expression level was observed for the Shh receptor *PTCH1* (RPKM values 10-40) and *SMO* (Figure 5.11B,C; RPKM values up to 70). Similarly, the negative regulator of Shh signalling pathway *SUFU* showed moderate level of expression (Figure 5.11D; RPKM values 20-25). The terminal effectors of the pathway *GLI1*, *GLI2*, and *GLI3* also showed distinct expression levels. Similar to *SHH*, very low expression was observed for *GLI1* (Figure 5.11E; RPKM values 0.4-4); whereas moderate expression observed for *GLI2* (Figure 5.11F; RPKM values 20-40). The highest expression level GLI family genes was for *GLI3* (RPKM values 60-120) which also showed noticeable decrease with age (Figure 5.11G). Consistent with previous findings at mid-gestation (Radonjić *et al.*, 2016) these results demonstrate that various components of Shh signalling pathway are expressed in the dorsal telencephalon of human fetal brain at earlier stages of development (the end of first trimester). However, the very low level of Shh mRNA in the cortex observed in this study compared to higher expression observed at mid-gestational period (Radonjić *et al.*, 2016) could suggest that the incidence of Shh expression in the human fetal cortex increases with age.

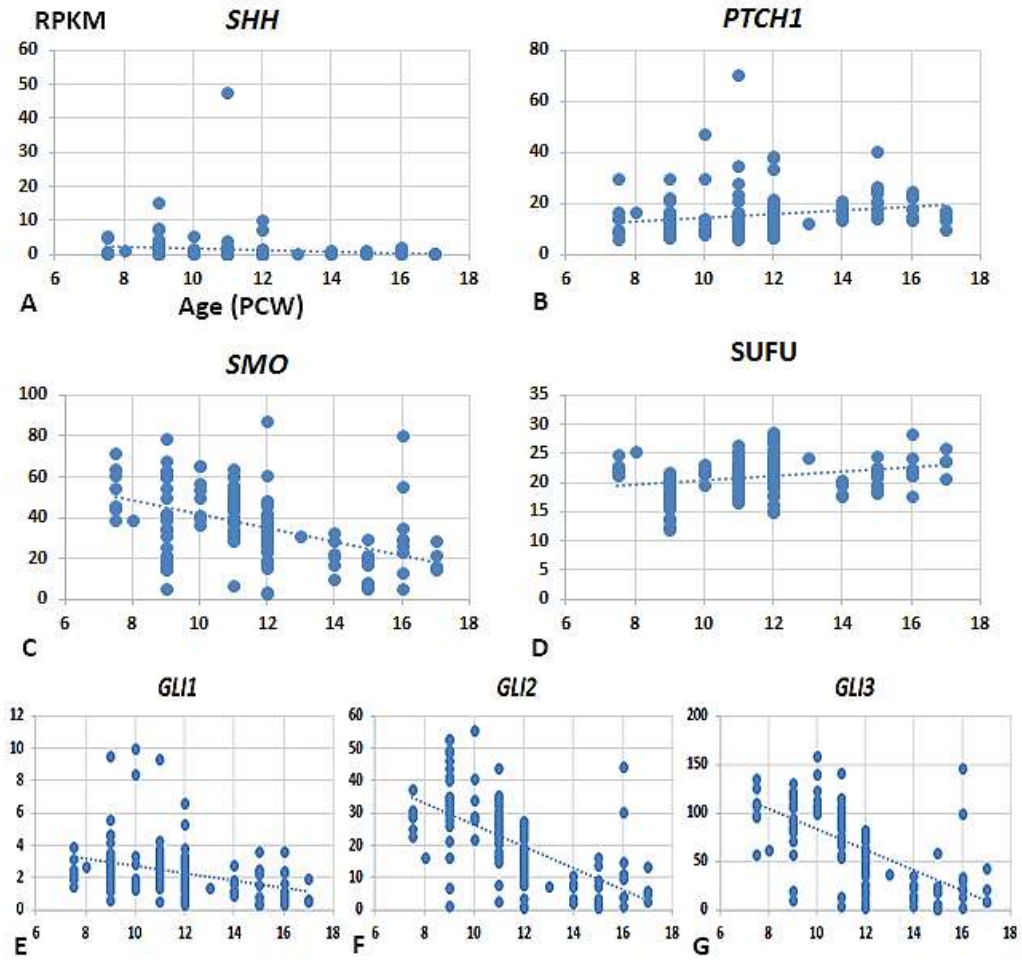

Figure 5.11: The expression levels of *PTCH1*, *SMO, SUFU, GLI1, GLI2*, and *GLI3* mRNA in human fetal cortex (7.5-17 PCW) by RNAseq. Very low level of expression was detected for *SHH* (A). Moderate to high expression for *PTCH1* (B) SMO (C) and *SUFU* (D). *GLI1* was expressed at low level expression (E) moderate expression was observed for *GLI2* (F) whereas the highest expression level observed for *GLI3* (G). Note: the scales for the vertical axes used in the figures are different. For these genes we used the full age range of samples available (7.5 to 17 PCW).

As SHH receptors appear to be expressed in the human fetal cortex, we next sought to investigate the effect of exogenous SHH treatment on cortical cell cultures. Cells isolated from the cortex of 9 and 11 PCW were differentiated for 12 days, cultures were treated with recombinant human SHH (200 ng/mL) every other day (Chapter 2, section 2.6.5). After 12 days, we observed that cell density was apparently higher in treated cultures compared to control cultures (Figure 5.12A,B) indicating that Shh promote cell proliferation of neural precursors; which was also confirmed when our cultures were tested for immunoreactivity for KI67 (cell division marker) and TBR1 (post-mitotic glutamatergic neuronal marker; Figure

5.12C). Significantly higher numbers of KI67+ cells (of total number of cells) were found in treated (25 ± 1%) than control (17 ± 1%) cultures (Figure 5.12D). Conversely, the number of TBR1+ cells was significantly higher in controls (54 ± 3%) than treated (41 ± 1%) cultures (Figure 5.12E). These findings are consistent with many *in vivo* and *in vitro* studies in both human and rodent models which reported that SHH maintains the proliferative state of neural progenitor cells (Rowitch *et al.*, 1999; Gulacsi and Lillien, 2003; Komada *et al.*, 2008; Shikata *et al.*, 2011; Radonjić *et al.*, 2016). Investigating the identity of dividing cells in treated cultures; 74 ± 4% and 16 ± 2% of KI67+ cells in treated cultures co-expressed PAX6 and OLIG2, respectively (Figure 5.12F,G), which weren't significantly different from control cultures where 69 ± 5% and 16 ± 1% of KI67+ cells co-expressed PAX6 and OLIG2, respectively. When testing cultures for GABAergic markers, Shh treatment failed to induce any NKX2.1 expression in these cultures; furthermore, the treated cultures maintained similar proportions of GABA+, CalR+, OLIG2+, and COUP-TFII+ cells compared to control cultures (See Figure 5.6). Collectively, all these findings suggest that although the human cortex has the necessary components for Shh signalling pathway at the early stage of development, exogenous Shh isn't sufficient to alter the differentiation pathway of dorsal neural progenitor cells switching their fate from glutamatergic neurons into GABAergic interneurons.

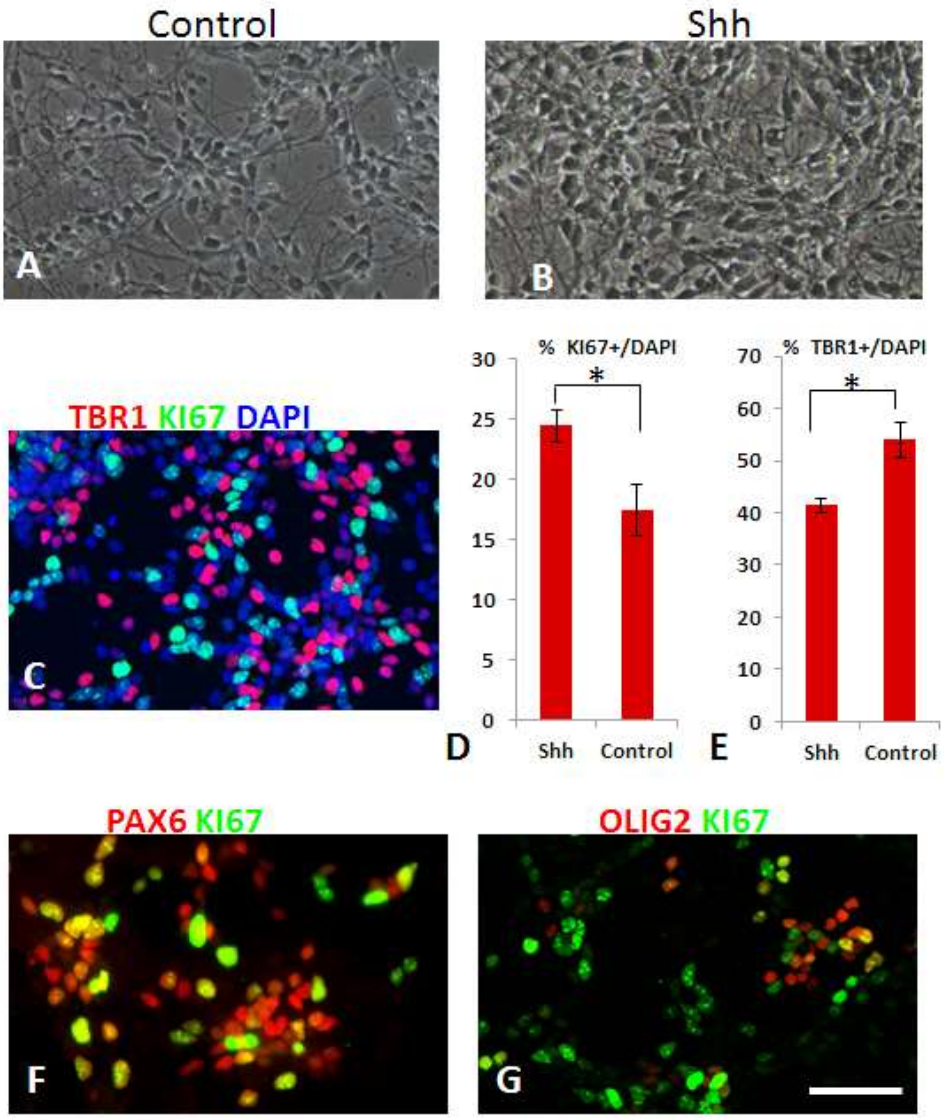

Figure 5.12: The effect of exogenous Shh treatment on cortical cell cultures. (A,B) Phase contrast images of 12 days monolayer differentiating neurons in control (A) and Shh treated (B) cortical cultures showing higher cells density in treated cultures compared to control cultures. (C-E) Immunofluorescent analysis for and quantification TBR1 and KI67 of in control and SHH cultures after 12 days of treatment illustrating that exogenous SHH promote cells proliferation. (F,G) Most of dividing (KI67+) cells in treated cultures expressed PAX6 (F) proportion expressed OLIG2 as well. Scale bar: 50μm in G (and A-C, and F).

5.5 Discussion

Several studies have reported that during the early stage of development, cortical GABAergic interneurons in primate, like rodents, are mainly generated in the GE; whereas in the second trimester (15–24 PCW in human and E64–E75 in macaque Monkey) the proliferative zone of the dorsal telencephalon could also contribute to a proportion of cortical GABAergic interneurons (Letinic *et al.*, 2002; Petanjek *et al.*, 2009a; Zecevic *et al.*, 2011; Radonjić *et al.*, 2014a). In addition, only one study suggested a potential regional variation of interneuron generation in the dorsal pallium (Al-Jaberi *et al.*, 2015a). In the present study, we confirmed that the dorsal pallium of human fetal brain can also give rise to a proportion of cortical interneurons at earlier stages (9-12 PCW), mainly CGE-like interneuronal subtypes. We have also shown that these interneurons are preferentially generated from anterior cortical regions.

5.5.1 Is dorsal interneurogenesis limited to subtypes associated with the CGE?

To a large extent the organisation and function of cortical GABAergic interneurons is shared between species, however primates have a higher proportion of cortical interneurons (25–34% of cortical neurons) than rodents (15–25%) particularly in frontal cortex (Hladnik *et al.*, 2014). CalR+ GABAergic interneurons are more common in adult primates than in rodents (Condé *et al.*, 1994; Gabbott *et al.*, 1997; Barinka and Druga, 2010). The ratio of parvalbumin positive interneurons to projection neurons is similar in mouse and human but the ratio of CalR positive interneurons to projection neurons has increased exponentially accounting for the overall increased proportion of interneurons in the human cortex (Hladnik *et al.*, 2014). It is established that the human fetal brain has evolved an expanded outer SVZ in the ganglionic eminences (Hansen *et al.*, 2013b) and the CGE has increased in complexity extending ventrally as the temporal lobe has increased in size (Chapter 3 and 4) (Hansen *et al.*, 2013b; Ma *et al.*, 2013). Together, these changes might underlie the relative increase in CGE-derived CalR+ interneurons. However, a more contentious proposal is that CalR+ interneurons, in particular, may be generated intra-cortically in primates especially at later developmental stages (Petanjek *et al.*, 2009a; Zecevic *et al.*, 2011; Reinchisi *et al.*, 2012; Hladnik *et al.*, 2014; Radonjić *et al.*, 2014b) which is supported by findings in cases of human holoprosencephaly with severe hypoplasia in ventral telencephalon, where interneurons expressing nitric oxide synthase 1

(NOS1), NPY, and Sst were absent or largely reduced, while CalR interneurons were present in the cortex of these cases (Fertuzinhos *et al.*, 2009) indicating that this subtype is not merely produced in the ventral telencephalon, but locally in the dorsal telencephalon as well.

The present study shows that at 8-11 PCW a proportion of neurons derived from the cultured cortical progenitors are GABAergic and can co-express CalR, COUP-TFI or COUP-TFII. We showed also that our cortical cultures retain positional information in terms of gene expression from the tissue from which they were cultured. This therefore provides another piece of evidence in favour of cortical interneurogenesis in human. There are three possible origins of GABAergic interneuron progenitor cells in culture; either 1) they are of cortical origin and/or 2) they are ventral progenitors that have migrated into the cortex retaining their proliferative capacity (Radonjić *et al.*, 2014a) or 3) as has been shown in previous rodent studies, cortical progenitors in *vitro* can "abnormally" generate GABAergic interneurons (Götz and Bolz, 1994; He *et al.*, 2001) possibly in response to exogenous factors (Trinh *et al.*, 2006). If we consider possibility 1) the lack of NKX2.1 expression in our cultures rules out cortex being able to produce interneurons associated with the MGE, such as basket cells and others, at this stage of development. However, for possibility 2) COUP-TFI+ progenitors, that have down-regulated NKX2.1, could have reached the cortex from dMGE and undergone further division. For 3) it is possible that FGF2 added to proliferating progenitors may have induced a ventral phenotype via induction of SHH signalling (Gabay *et al.*, 2003). However, expression of FGF2 and its receptors is robust in developing human cortex *in vivo* (Ip *et al.*, 2011; Lindsay *et al.*, 2016) and cortical SHH expression possibly increases with age (Miller *et al.*, 2014; Radonjić *et al.*, 2016) although we found no strong evidence to support the third possibility in our RNAseq analysis. Nevertheless, the conditions we employed in culture may not be so far removed from the *in vivo* condition of the developing human brain. We propose that COUP-TFI and particularly COUP-TFII positive progenitors for GABAergic cells could have reached the cortex from the CGE, or be generated in the cortex, given the high levels of CalR expressed by GABAergic cells from cortical cultures. It would seem that if cortical interneurogenesis exists it is primarily to contribute to CGE-like interneuron populations.

5.5.2 Anterior cortex is preferred region for dorsal interneurogenesis

The presence of the higher order associative areas suggests that different developmental mechanisms are used in different species and these led to such cortical expansion in primate (Teffer and Semendeferi, 2012). Two associative areas, frontal and parietal, were identified as unique to, or at least more highly developed in, the primate cortex; the frontal associative area is the largest and covers the frontal part of the frontal lobe (almost covering 80% of the entire frontal lobe) and one third of the total cortical surface (Teffer and Semendeferi, 2012; Hladnik *et al.*, 2014). Calretinin expressing interneurons are the major interneuronal subtype in this area, representing almost 50% of all GABAergic interneurons. Remarkably, one half of all calretinin expressing interneurons in human are in this region (Condé *et al.*, 1994; Gabbott *et al.*, 1997; Zaitsev *et al.*, 2005; Barinka and Druga, 2010; Hladnik *et al.*, 2014). Similarly, in a study on the developing fetal brain of human and monkey, the frontal and parietal regions were more highly populated with calretinin interneurons compared to other cortical areas (Ma *et al.*, 2013).

Why are there significantly higher proportions of CalR interneurons in the frontal lobe when it is apparently anatomically distant from the posteriorly positioned CGE, the major source of calretinin expressing interneurons (Hladnik *et al.*, 2014)? We have previously demonstrated that these CGE- derived interneurons may reach the frontal lobe more rapidly than expected through the anterior pathway via LGE (See chapter 3); Recently this migration route has been also described in rodents (Touzot *et al.*, 2016). As mentioned in the previous section, we propose that a proportion of CalR GABAergic interneurons are generated intra-cortically, more likely from the newly evolved outer sub-ventricular zone in primates (Rakic, 2009; Geschwind and Rakic, 2013; Sousa *et al.*, 2017). But is dorsal interneurogenesis only confined to, or more prominent in, the frontal region explaining the three-fold increase of CalR interneurons in this region? In this study, we found a higher proportion of GABA+, CalR+ and COUP-TFII+ progenitors were present in anterior compared to posterior cortex derived cultures (Figure 5.6). In addition, the mRNA levels for several genes expressed in interneurons progenitors and involved in the genetic regulatory pathway of interneuron specification (like *DLX*, *GSH*, *ASCL1* genes) were generally higher in samples derived from the anterior cortical region than posterior region. These findings also reflect previous qPCR studies suggesting elevated expression of various "GABAergic" genes anteriorly including CalR, OLIG2, GAD and DLX genes (Ip *et al.*, 2011; Al-Jaberi *et al.*, 2015a). Collectively all these data indicate an anterior

preference for cortical interneurogenesis, at least over posterior if not temporal cortex; however, the regional variation of dorsal interneurogenesis needs further investigation.

Finally, we propose various potential sources for CalR-expressing GABAergic interneurons destined for the expanded frontal associative area in human. The CGE, or more precisely vCGE, is still the main source for these interneuronal subtypes, but probably at higher proportions (Hansen *et al.*, 2013b) and more characteristic anterior migration, via LGE, into the frontal area than in rodents (Chapter 3). Additionally, proportions of CalR interneurons are generated intra-cortically and preferentially in the anterior cortical region, corresponding to the anatomical position of the frontal associative area. Such distinct mechanisms for CalR interneuron generation could provide an explanation for the exponential increase of these interneurons in the higher order associative areas in human (Hladnik *et al.*, 2014) which could provoke substantial change in the intrinsic organization of cortical circuitries resulting in higher cognitive abilities (Burkhalter, 2008; Forbes and Grafman, 2010).

5.5.3 Exogenous Shh doesn't alter the fate dorsal neural progenitor cells

In human, Shh is expressed in the dorsal telencephalon at the mid-gestational period (Miller *et al.*, 2014; Radonjić *et al.*, 2016). Radonjić *et al.* (2016) have reported the presence of *SHH* mRNA and protein in radial glia cells and postmitotic neurons; the same study has also reported the expression of all other necessary components of Shh signalling pathway including PTCH1, SMO, SUFU, GLI1, GLI2, and GLI3 at the mid-gestational period. In our study, we also confirm moderate to high expression for PTCH1, SMO, SUFU, GLI2, and GLI3 at earlier stage of development around the end of first trimester. However, although these four elements were expressed at relatively high levels, the very low levels of SHH expression in the cortex at this stage of development suggest that SHH signalling is inactive unless SHH is diffusing into the cortex from external signalling centres in appreciable amounts. However, GLI1 expression, which is used as an indicator of cells actively responding to high levels of Shh signalling (Bai *et al.*, 2002) also exhibited very low levels of expression (RPKM values 0.4-4). The higher expression levels for Shh and GLI1 reported at mid-gestation (Radonjić *et al.*, 2016) suggest that the incidence of Shh expression in the human fetal cortex increases with age, but our RNAseq data indicates this happens after 17 PCW at the earliest.

The prospective age-related role of Shh signalling in the specification of neural progenitor cells in human cortex can be estimated firstly, as Shh is important to induce NKX2.1 expression in ventral progenitor cells (Kohtz *et al.*, 1998; Rallu *et al.*, 2002) by the incidence of NKX2.1+ cells in the human cortex, which are mainly observed around mid-gestational period (Petanjek *et al.*, 2009b; Radonjić *et al.*, 2014a). Secondly, exogenous Shh treatment in our cortical cultures (from 9-12 PCW) could not induce NKX2.1 expression and did not reduce the number of CalR+ and COUP-TFII+ cells in these cultures either; whereas applying the same treatment on cortical cultures at mid-gestation increased the proportion of NKX2.1+ cells and downregulated CalR expression (Radonjić *et al.*, 2016). However, although exogenous Shh treatment in our study has showed no influence on the plasticity of dorsal neural progenitor cells, our results showed that this treatment affected the cell cycle kinetics by increasing proliferation and decreasing the number of differentiated cells (Figure 5.12), which is also consistent with many *in vivo* and *in vitro* studies which reported that Shh promotes the proliferative state of neural progenitor cells (Rowitch *et al.*, 1999; Gulacsi and Lillien, 2003; Komada *et al.*, 2008; Shikata *et al.*, 2011; Radonjić *et al.*, 2016). The selective influence of exogenous Shh treatment on cell cycle but not the plasticity of dorsal neural progenitor cells in our studied stage could suggest that these cortical cells lack crucial factors important for promoting the MGE-like identity in these cells as identified by NKX2.1 expression.

5.5.4 Conclusion

These studies provide additional evidence that the human cortex is capable of making its own interneurons; however, intra-cortically generated interneurons seem to be confined to specific subtypes similar to those generated in the CGE (Calretinin interneurons in particular). COUP-TFs expressing GABAergic neurons were generated from cortical progenitors in culture suggesting that these transcription factors may direct the expansion of cortical interneuron populations in developing primate brain. In addition, our findings suggest that the anterior cortex, origin of the highly evolved prefrontal cortex crucial to higher cognitive function, might be considered as a favoured region for cortical interneurogenesis. Exogenous Shh treatment promotes the progenitor state of cortical cells but did not influence the phenotypic plasticity of these cells.

Note: Data from this chapter has been recently published in an original article (Alzu'bi *et al.*, 2017, see appendices):

- Alzu'bi, A., Lindsay, S.J., Harkin, L.F., McIntyre, J., Lisgo, S.N. and Clowry, G.J. (2017) **'The Transcription Factors COUP-TFI and COUP-TFII have Distinct Roles in Arealisation and GABAergic Interneuron Specification in the Early Human Fetal Telencephalon'**, Cerebral Cortex, 27(10), pp. 4971-4987.

Chapter 6: General Discussion and Future Work

The availability of genetic mutants in murine studies has allowed us to make significant advances in our understanding of mammalian brain development. Although the mouse is considered a reliable experimental model to study the basic steps of neurogenesis, cell type specification, and cell migration in the developing brain, its ability to model the unique composition and higher cognitive function of the human brain, which has larger and folded cortex, and a higher proportion of inhibitory interneurons among other differences, is limited. Therefore, despite limitations in the experimental approaches that can be employed, working directly on the developing human brain has become a necessity in order to unveil the main molecular and cellular divergences between human and other species, which could provide further understanding for several neurodevelopmental conditions such as autism, schizophrenia and epilepsy. This thesis investigated the generation of cortical GABAergic interneurons in human early fetal forebrain between 8 -12 PCW, a stage of development that has not been thoroughly studied before. This study characterized the developmental expression patterns for several interneuron precursor transcription factors in the ventral and dorsal telencephalon. A more detailed description of the subdivisions of the GE compartments and septum into several neurogenic domains has been provided. The migration routes of interneurons from the ventral telencephalon to dorsal telencephalon, identified by the expression of several transcription factors, have been described. Finally, this study has investigated the possibility of interneuron generation in the proliferative zones of dorsal human telencephalon; in addition, the potential regional variation for dorsal interneurogenesis has been also inspected.

6.1 There are complex subdivisions of the human ventral telencephalon

Using extensive immunohistochemical analysis, this thesis described the organization of the MGE, LGE, CGE and septum in human ventral telencephalon (Figure 6.1; see chapters 3 and 4). NKX2.1 and OLIG2 were expressed throughout the MGE; whereas COUP-TFI immunoreactivity subdivides the MGE into vMGE and dMGE, with COUP-TFI confined to the larger dorsal region (Figure 6.1A). In rodents, spatial segregation for interneurons generation has been described within the MGE, the dMGE is the birthplace of nearly all Pv+ and SST+ cortical interneurons, whereas the vMGE predominantly gives rise to globus pallidus

neurons (Flandin *et al.*, 2010) although at later stages it may be the source of cortical chandelier cells (Taniguchi *et al.*, 2013). COUP-TFI was expressed in SOX6+ cells, a downstream regulator of NKX2.1 (Batista-Brito *et al.*, 2009) in the SVZ of dMGE and the cortex perhaps ensuring Pv+ and SST+ interneurons migrate dorsally towards the cortex and not ventrally towards the basal ganglia, as COUP-TFI is known to have a role in guiding migration (Boudot *et al.*, 2014). Neither vMGE nor dMGE expressed COUP-TFII, SP8, and PAX6.

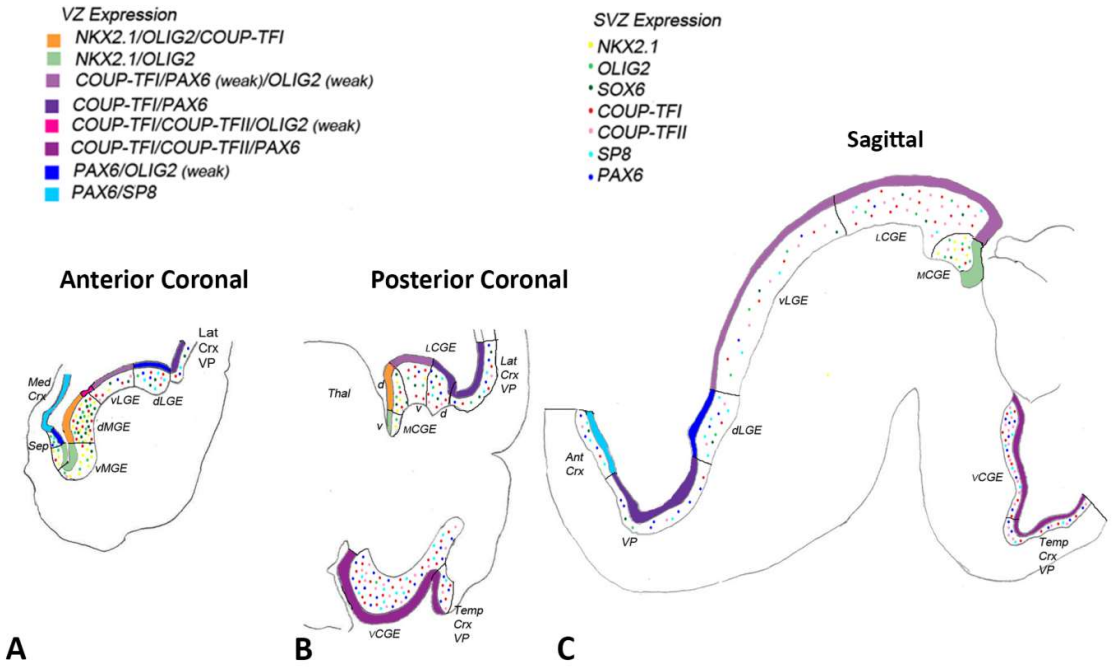

Figure 6.1: The complex subdivisions of human ventral telencephalon. Schematic coronal (A,B) and sagittal (C) sections showing the subdivisions of ventral telencephalon identified by the expression of certain transcription factors in the proliferative zones (solid colour) and post-mitotic cells (dots).

The confinement of COUP-TFI expression in the VZ to its ventral region also divides the LGE into vLGE and dLGE (Figure 6.1A). OLIG2 was moderately expressed in the VZ and SVZ of both vLGE and dLGE. However, PAX6 was expressed in a decreasing gradient from high in dLGE to low in vLGE, which also applied to SP8 expression but only in the SVZ. COUP-TFII was expressed in post-mitotic cells in the dLGE. The expression patterns of both SP8 and

COUP-TFII delineated a clear pallial/sub-pallial boundary; although SP8 and COUP-TFII were expressed either side of this boundary, there was markedly higher expression of both in the dLGE. COUP-TFII immunoreactivity in the dLGE appeared to belong only to anteriorly migrating cells arising from the vCGE (See chapter 3, section 3.3.7). Instead, the dLGE provided predominantly SP8+ only cells that migrated towards the RMS, amygdala and cortex and did not express CalR. The proliferative zone of MGE/LGE boundary was molecularly distinct from the adjacent dMGE and vLGE, characterized by strong expression of COUP-TFII suggesting that this region could be a site of origin for COUP-TFII+ interneurons in addition to the vCGE. This boundary region was positive for OLIG2 and COUP-TFI expression but negative for NKX2.1. In rodents, this is the source of COUP-TFII+/Sst+ cells that occupy cortical layer V (Cai et al., 2013); however, no co-expression of COUP-TFII with SOX6, the developmental marker for Sst+ interneurons (Batista-Brito *et al.*, 2009) was observed in the cortical wall in human fetal brain.

This study also provided a better demonstration of CGE compartments than previous attempts along with the distinguishing features of their progenitor cells. We have shown that the CGE can be divided into at least three compartments, two located dorsally, the MCGE and LCGE which are considered as caudal extensions of the MGE and LGE respectively, sharing the expression patterns of OLIG2, NKX2.1 and PAX6 see in more the more anterior ganglionic eminences (Figure 6.1B). However, in addition to these two compartments, the extension of the CGE along the lateral ventricle into the greatly enlarged temporal lobe has produced a third compartment called vCGE distinguishable by its characteristic co-localisation of intense COUP-TFII, SP8 and PAX6 expression in the proliferative layers (Figure 6.1B,C). Dividing COUP-TFII+ cells were confirmed as being confined to this ventral region of the CGE (this study, Hansen et al., 2013). While COUP-TFII appeared to be continually expressed throughout the vCGE and ventro-temporal cortex, although pallial/sub-pallial boundary was still clearly delineated by the expression of TBR1 and PAX6; TBR1 was exclusively expressed in the post-mitotic zone of the cortex; whereas PAX6 was expressed in a gradient, high in all cortical proliferative zones to progressively lower across the proliferative zones from vCGE.

Finally, transcriptional patterning showed that the septum shares common molecular features of the ganglionic eminences along the dorsoventral axis. The sub-cortical septum was divided into the ventrally located MGE-like septum characterized by the immunoreactivity for NKX2.1 and OLIG2 but not PAX6 expression; and more dorsally LGE-like septum was characterized by the expression of PAX6, SP8, and OLIG2 but not NKX2.1. The most dorsal part of septum

had a cortical rather than sub-cortical identity, manifested by higher PAX6 expression in the VZ and SVZ and expression of TBR1 by post-mitotic cells.

6.2 Additional migration pathways for interneurons from the ventral to dorsal telencephalon

The migration routes of interneurons identified by the gradients of expression of several transcription factors have been revealed in this thesis. In addition to the well-known lateral (MGE derived) and posterior (CGE derived) pathways of migration for interneuron precursors from ventral to dorsal telencephalon (Wonders and Anderson, 2006) this thesis has described two new migration routes of interneurons from the ventral to dorsal telencephalon in human that have not been described before.

Unlike in rodents, where septal derived interneurons are reported not to enter the cortex at all (Rubin *et al.*, 2010) and interneurons populating medial wall derived structures such as the hippocampus are derived from the MGE and CGE via lateral migration (Pleasure *et al.*, 2000; Wonders and Anderson, 2006; Morozov *et al.*, 2009; Faux *et al.*, 2012) this study presented evidence that OLIG2+ and NKX2.1+ progenitors reside in the septum and OLIG2+ cells, at least, can reach the cortex via medial migration pathway (See chapter 3, section 3.3.5). Although this route of migration has not been reported, or has been overlooked, in rodent models, the medial migratory pathway for Nkx2.1+ precursors from the MGE to the medial pallium has recently been reported in the shark (Quintana-Urzainqui *et al.*, 2015) making this pathway not evolutionarily novel to the human brain.

This study described three migration routes for CGE (or more precisely vCGE) cells to reach their specific targets; we have also shown that these routes are identified by the expression of specific transcription factors (COUP-TFI, COUP-TFII, and SP8) and controlled in a temporal manner. In addition to the well-known CMS that guides CGE cells into the posterior cortex (Yozu *et al.*, 2005; Kanatani *et al.*, 2008) this study and a recent study in mouse (Touzot *et al.*, 2016) have shown that CGE cells can also migrate anteriorly via LGE to reach the anterior cortex. We have also shown that migrating interneurons may more rapidly invade the anterior than the posterior cortex, even from apparently caudal structures such as the vCGE. While the CMS is controlled by COUP-TFII (Kanatani *et al.*, 2008) COUP-TFI is required to guide the cells in the anterior migratory stream (Touzot *et al.*, 2016). Some vCGE-derived cells also appeared to be migrating ventrally to the caudal regions of basal telencephalon, a region

corresponding to the medial amygdala (Nery *et al.*, 2002; Tang *et al.*, 2012; Touzot *et al.*, 2016). At the earliest studied stage (8 PCW) the caudal and ventral migratory streams of vCGE cells appeared to be dominant; however, cells migrating in anterior routes considerably increased with age. The expression of COUP-TFI, COUP-TFII, and SP8 in each pathway (see chapter 4, section 4.5.3 for more details) was not exactly the same as has been observed in mice (Touzot *et al.*, 2016). However, the distinct expression of these transcription factors in these three routes in human could differentially control their responsiveness to their particular guidance cues which is unique to mammals, and could contribute to the evolution of the neocortex (Tanaka *et al.*, 2011).

In addition, this study proposes that regionalised expression of transcription factors in both cortex and the GE controls the migration pathways from ventral to dorsal telencephalon. COUP-TFI is expressed in the ventral pallium along the lateral border of the dorsal telencephalon, even in more anterior regions of the cortex, and in interneuron precursors migrating laterally from either MGE or CGE into the cortex (see chapter 4). COUP-TFI is proposed to control the lateral/anterior migratory stream of CGE-derived cells in mice (Touzot *et al.*, 2016) in addition to the lateral migration from the MGE to cortex (Faux *et al.*, 2012; Marín, 2013). In mouse COUP-TFII is important in establishing a caudal migratory stream (CMS) directing CGE-derived cells into temporal cortex and hippocampus (Yozu *et al.*, 2005; Kanatani *et al.*, 2008). In human, COUP-TFII is expressed in temporal and ventral anterior cortex, as well as interneuron precursors generated in the vCGE (Chapters 3 and 4; Reinchisi et al., 2012; Hansen et al., 2013; Ma et al., 2013). Again it appears that COUP-TFII is expressed in gateway regions of the dorsal telencephalon for the entrance of COUP-TFII expressing interneurons into the cortex. Similarly, at early developmental stages (8PCW) SP8+ cells migrate from dLGE to SP8+ anterior cortex, but SP8+/COUP-TFII co-expressing cells were not seen within the CMS migrating towards SP8- temporal cortex (Chapter 4) however this distinction broke down at later developmental stages where SP8+ cells where present in the CMS in abundance. In addition, we have observed increased expression of OLIG2 in the anterior-medial cortex at 7-8 PCW (Chapter3; Ip et al., 2010; Al-Jaberi et al., 2015) in conjunction with a migratory stream of MGE and sub-cortical septum derived OLIG+ cells entering the cortex via the medial wall. Thus arealised expression of transcription factors in the cortical wall may, in turn, control expression of cell adhesion molecules and chemokine secretion locally that attracts migrating cells expressing the same transcription factors, setting

up the migratory pathways into the cortex for interneurons arriving anteriorly or posteriorly, medially or laterally, The much larger human cortex may require additional migratory pathways compared to smaller mammalian brains, although some pathways may not be missing but relatively small and overlooked in rodent.

6.3 Evidence for a cortical origin of cortical GABAergic interneurons in human fetal brain

Over the past decade, a considerable amount of literature has been published on the developmental origins of cortical interneurons in human brain. However, to date there has not been universal agreement to what degree the developmental rules of cortical interneuron generation, in human, are similar to those found in our experimental models. The first serious discussion of potential divergence of interneuron origins in human brain has been raised by Letinic *et al.* (2002), using retroviral labelling of DLX2+ and ASCL1+ progenitors in slice cultures of human fetal brain, this study suggested that perhaps up to 65% of cortical interneurons in human fetal brain are locally generated in the proliferative zone of the dorsal telencephalon. Whilst more recent studies have arrived at similar conclusions in both human and macaque monkey, but perhaps with a smaller dorsal contribution to cortical interneurons (Petanjek *et al.*, 2009a; Zecevic *et al.*, 2011; Reinchisi *et al.*, 2012; Radonjić *et al.*, 2014b; Al-Jaberi *et al.*, 2015a) two other influential studies reported that the origins of cortical interneurons in human and monkey are similar to those found in rodents where cortical interneurons are almost solely generated in the proliferative zone of ventral telencephalon and tangentially migrate into the dorsal telencephalon (Hansen *et al.*, 2013b; Ma *et al.*, 2013). In addition, these two studies also rejected the possibility that interneurons precursors generated in ventral telencephalon could retain their proliferative capacity after migrating into the dorsal telencephalon.

This thesis revisited the generation of cortical GABAergic interneurons in human early fetal brains between 8-12 PCW. Our findings indicate that the GE is the main source of cortical GABAergic interneurons; however, this study supports previous findings and provides further evidences that a proportion of interneuron precursor cells may, if not intra-cortically derived, at least undergo division in the cortex. This study also suggests that at the early stage of development, intracortical interneuogenesis could be more prominent in specific types of interneuron precursor cells most likely similar to those generated the CGE. In addition, we

have also shown that the anterior cortex may be the favoured region for intracortical interneurogenesis. In immunostained forebrain sections (See chapter 3) we demonstrated the presence of OLIG2+ and COUP-TFII+ progenitor cells (KI67+) in the cortex of 8-12 PCW fetal human brain; the expression of NKX2.1, the characteristic marker of MGE cells, was limited to a few number of cells found only in 12 PCW cortex. Similarly, in cultures differentiated from human cortical progenitors from anterior and posterior cortex (see chapter 5) GABA+ and CalR+ cells were present in these cultures in considerable numbers but most of these cells were expressing either of the COUP-TFs, the CGE-derived interneuron markers (Kanatani *et al.*, 2008; Miyoshi *et al.*, 2010). Consistent with the findings in the immunostained forebrain sections, these cultures entirely lacked NKX2.1 expression. The incidence of GABA+, CalR+, and COUP-TFII+ cells was always higher in cultures derived from anterior cortex compared with cultures derived from posterior cortical regions. The anterior regional preference for the expression of GABAergic interneuron markers was also observed by RNAseq analysis for several "GABAergic" genes like *DLX1, DLX2, GSH2, ASCL1, ARX, OLIG2, CALB2*. All these findings lead us to propose that a proportion of cortical interneurons could be generated intra-cortically, preferentially in the anterior cortical region; most of these interneurons resemble CGE-derived interneurons (CalR+) which could provide a sensible explanation for the higher proportion of CalR-expressing interneurons reported in the highly evolved human prefrontal cortex (Uylings and van Eden, 1991; Teffer and Semendeferi, 2012; Hladnik *et al.*, 2014).

6.4 Future work

The present work described the presence of additional migration pathways for interneurons from the ventral to dorsal telencephalon; however, the experimental approaches for the identification of these migration routes was mainly based on the expression gradients of transcription factors like COUP-TFI, COUPTFII, and SP8. So, it will be important to further study these pathways using more specific techniques; for example, by using the micromanipulator to inject the lipophilic tracer (DiI) into the proliferative zone of individual subcortical structures (like CGE, MGE, septum) in whole hemisphere slice cultures, and tracking the migrating labelled cells over a period of time. Similarly, migrating cells in slice

cultures could also be transfected with green fluorescent protein GFP- expressing virus and tracked.

Although this study provided evidence for the presence of COUP-TF+ and OLIG2+ interneuron progenitors in immunostained forebrain sections and cortical cell cultures, it is still not determined if these cells are generated intra-cortically or if they are ventral telencephalon derived progenitors that have retained their proliferative capacity after migrating into the cortex. Again, organotypic slice culture experiments might be able to shed some light. Migrating cells from the ventral telencephalon could be tracked to see if they undergo intra-cortical cell division. Dyes or viruses could be targeted to the cortical ventricular zone to see if any of the progeny of these cell co-expressed GABAergic markers such as GAD67.

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Appendices

Appendix A: Publications arising from this study.

Brain Struct Funct
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ORIGINAL ARTICLE

Distinct cortical and sub-cortical neurogenic domains for GABAergic interneuron precursor transcription factors NKX2.1, OLIG2 and COUP-TFII in early fetal human telencephalon

Ayman Alzu'bi[1,2] · Susan Lindsay[2] · Janet Kerwin[2] · Shi Jie Looi[1,2] · Fareha Khalil[1,2] · Gavin J. Clowry[1]

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Abstract The extent of similarities and differences between cortical GABAergic interneuron generation in rodent and primate telencephalon remains contentious. We examined expression of three interneuron precursor transcription factors, alongside other markers, using immunohistochemistry on 8–12 post-conceptional weeks (PCW) human telencephalon sections. NKX2.1, OLIG2, and COUP-TFII expression occupied distinct (although overlapping) neurogenic domains which extended into the cortex and revealed three CGE compartments: lateral, medial, and ventral. NKX2.1 expression was very largely confined to the MGE, medial CGE, and ventral septum confirming that, at this developmental stage, interneuron generation from NKX2.1+ precursors closely resembles the process observed in rodents. OLIG2 immunoreactivity was observed in GABAergic cells of the proliferative zones of the MGE and septum, but not necessarily co-expressed with NKX2.1, and OLIG2 expression was also extensively seen in the LGE, CGE, and cortex. At 8 PCW, OLIG2+ cells were only present in the medial and anterior cortical wall suggesting a migratory pathway for interneuron precursors via the septum into the medial cortex. By 12 PCW, OLIG2+ cells were present throughout the cortex and many were actively dividing but without co-expressing cortical progenitor markers. Dividing COUP-TFII+ progenitor cells were localized to ventral CGE as previously described but were also numerous in adjacent ventral cortex; in both the cases, COUP-TFII was co-expressed with PAX6 in proliferative zones and TBR1 or calretinin in post-mitotic cortical neurons. Thus COUP-TFII+ progenitors gave rise to pyramidal cells, but also interneurons which not only migrated posteriorly into the cortex from ventral CGE but also anteriorly via the LGE.

Keywords Ganglionic eminences · Inhibitory interneurons · Neurodevelopment · Neuronal fate specification · Pallium · Subpallium

Abbreviations

COUP-TFII	Chicken ovalbumin upstream promotor-transcription factor 2
DLX2	Distal-less homeobox 2
NKX2.1	NK2 homeobox 1
OLIG2	Oligodendrocyte lineage transcription factor 2
PAX6	Paired box 6
TBR1 and TBR2	T-box brain 1 and 2
CGE	Caudal ganglionic eminence
LGE	Lateral ganglionic eminence
MGE	Medial ganglionic eminence

Electronic supplementary material The online version of this article (doi:10.1007/s00429-016-1343-5) contains supplementary material, which is available to authorized users.

✉ Susan Lindsay
susan.lindsay@ncl.ac.uk

✉ Gavin J. Clowry
gavin.clowry@ncl.ac.uk

[1] Institute of Neuroscience, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK

[2] Institute of Genetic Medicine, Newcastle University, International Centre for Life, Parkway Drive, Newcastle upon Tyne NE1 3BZ, UK

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ORIGINAL ARTICLE

The Transcription Factors COUP-TFI and COUP-TFII have Distinct Roles in Arealisation and GABAergic Interneuron Specification in the Early Human Fetal Telencephalon

Ayman Alzu'bi[1,2], Susan J. Lindsay[2], Lauren F. Harkin[1,2,3], Jack McIntyre[1,2], Steven N. Lisgo[2] and Gavin J. Clowry[1]

[1]Institute of Neuroscience, Newcastle University, Newcastle upon Tyne NE2 4HH, UK, [2]Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK and [3]Present address: School of Healthcare Science, Manchester Metropolitan University, UK

Address correspondence to Gavin J. Clowry, Institute of Neuroscience, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK. Email: gavin.clowry@ncl.ac.uk; Susan J Lindsay, Institute of Genetic Medicine, Newcastle University, International Centre for Life, Parkway Drive, Newcastle upon Tyne NE1 3BZ, UK. Email: susan.lindsay@ncl.ac.uk

Abstract

In human telencephalon at 8–12 postconceptional weeks, ribonucleic acid quantitative sequencing and immunohistochemistry revealed cortical chicken ovalbumin upstream promotor-transcription factor 1 (COUP-TFI) expression in a high ventro-posterior to low anterior gradient except for raised immunoreactivity in the anterior ventral pallium. Unlike in mouse, COUP-TFI and SP8 were extensively co-expressed in dorsal sensory neocortex and dorsal hippocampus whereas COUPTFI/COUPTFII co-expression defined ventral temporal cortex and ventral hippocampus. In the ganglionic eminences (GEs) COUP-TFI immunoreactivity demarcated the proliferative zones of caudal GE (CGE), dorsal medial GE (MGE), MGE/lateral GE (LGE) boundary, and ventral LGE whereas COUP-TFII was limited to ventral CGE and the MGE/LGE boundary. Co-labeling with gamma amino butyric acidergic interneuron markers revealed that COUP-TFI was expressed in subpopulations of either MGE-derived (SOX6+) or CGE-derived (calretinin+/SP8+) interneurons. COUP-TFII was mainly confined to CGE-derived interneurons. Twice as many GAD67+ cortical cells co-labeled for COUP-TFI than for COUP-TFII. A fifth of COUP-TFI cells also co-expressed COUP-TFII, and cells expressing either transcription factor followed posterior or anterio-lateral pathways into the cortex, therefore, a segregation of migration pathways according to COUP-TF expression as proposed in mouse was not observed. In cultures differentiated from isolated human cortical progenitors, many cells expressed either COUP-TF and 30% also co-expressed GABA, however no cells expressed NKX2.1. This suggests interneurons could be generated intracortically from progenitors expressing either COUP-TF.

Key words: cerebral cortex development, ganglionic eminences, hippocampus development, interneuron migration, SP8, ventral pallium

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Review

Charting the protomap of the human telencephalon

Gavin J. Clowry[a,*], Ayman Alzu'bi[a,b], Lauren F. Harkin[c], Subrot Sarma[d], Janet Kerwin[b], Susan J. Lindsay[b]

[a] Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, United Kingdom
[b] Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom
[c] School of Healthcare Science, Manchester Metropolitan University, Manchester, United Kingdom
[d] Department of Chemistry, Indian Institute of Technology, Mumbai, India

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ABSTRACT

The cerebral cortex is divided stereotypically into a number of functionally distinct areas. According to the protomap hypothesis formulated by Rakic neural progenitors in the ventricular zone form a mosaic of proliferative units that provide a primordial species-specific cortical map. Positional information of newborn neurons is maintained during their migration to the overlying cortical plate. Much evidence has been found to support this hypothesis from studies of primary cortical areas in mouse models in particular. Differential expansion of cortical areas and the introduction of new functional modules during evolution might be the result of changes in the progenitor cells. The human cerebral cortex shows a wide divergence from the mouse containing a much higher proportion of association cortex and a more complicated regionalised repertoire of neuron sub-types. To what extent does the protomap hypothesis hold true for the primate brain? This review summarises a growing number of studies exploring arealised gene expression in the early developing human telencephalon. The evidence so far is that the human and mouse brain do share fundamental mechanisms of areal specification, however there are subtle differences which could lead us to a better understanding of cortical evolution and the origins of neurodevelopmental diseases.

Contents

1. Introduction

Each individual, excepting those suffering a gross environmental or genetic insult, possesses a telencephalon built in development to the same plan. Different functional modules always appear in the same place, connections between areas are universal and the brain tissue architecture at the cellular level in each module is the same [1] however subtle differences in these parameters may under-

* Corresponding author at: Institute of Neuroscience, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, United Kingdom.
E-mail address: gavin.clowry@ncl.ac.uk (G.J. Clowry).

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Appendix B: Other publications

Journal of Anatomy

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Distinct expression patterns for type II topoisomerases IIA and IIB in the early foetal human telencephalon

Lauren F. Harkin,[1,2] Dianne Gerrelli,[3] Diana C. Gold Diaz,[3] Chloe Santos,[3] Ayman Alzu'bi,[1,2] Caroline A. Austin[4] and Gavin J. Clowry[1]

[1]*Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, UK*
[2]*Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK*
[3]*HDBR Resource, UCL Institute of Child Health, London, UK*
[4]*Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK*

Abstract

TOP2A and TOP2B are type II topoisomerase enzymes that have important but distinct roles in DNA replication and RNA transcription. Recently, TOP2B has been implicated in the transcription of long genes in particular that play crucial roles in neural development and are susceptible to mutations contributing to neurodevelopmental conditions such as autism and schizophrenia. This study maps their expression in the early foetal human telencephalon between 9 and 12 post-conceptional weeks. TOP2A immunoreactivity was restricted to cell nuclei of the proliferative layers of the cortex and ganglionic eminences (GE), including the ventricular zone and subventricular zone (SVZ) closely matching expression of the proliferation marker KI67. Comparison with sections immunolabelled for NKX2.1, a medial GE (MGE) marker, and PAX6, a cortical progenitor cell and lateral GE (LGE) marker, revealed that TOP2A-expressing cells were more abundant in MGE than the LGE. In the cortex, TOP2B is expressed in cell nuclei in both proliferative (SVZ) and post-mitotic compartments (intermediate zone and cortical plate) as revealed by comparison with immunostaining for PAX6 and the post-mitotic neuron marker TBR1. However, co-expression with KI67 was rare. In the GE, TOP2B was also expressed by proliferative and post-mitotic compartments. *In situ* hybridisation studies confirmed these patterns of expression, except that *TOP2A* mRNA is restricted to cells in the G2/M phase of division. Thus, during early development, TOP2A is likely to have a role in cell proliferation, whereas TOP2B is expressed in post-mitotic cells and may be important in controlling expression of long genes even at this early stage.

Key words: autism susceptibility genes; cortical development; DNA replication; ganglionic eminences; RNA transcription.

Introduction

The helical structure and supercoiling of DNA is essential for nuclear packaging; however, processes such as DNA replication and transcription require complete separation and partial separation of the strands, respectively. During transcription, the partial separation of the DNA allows RNA polymerase and transcription factors to access specific gene regions, creating tension in the DNA. Topoisomerase enzymes govern the topological state of DNA in both prokaryotic and eukaryotic cells, allowing unwinding of the DNA and relieving the torsional strain created by supercoiling (Nitiss, 2009a). Cells have type I and II topoisomerase enzymes. Type I topoisomerases (TOP1 and TOP3) are able to break a single strand of DNA, allowing the intact strand to pass through it before re-joining the broken strand, whilst type II topoisomerases (TOP2) carry out ATP-mediated strand breakage of one or both strands. Human topoisomerases comprise distinct alpha and beta isoforms (Austin & Marsh, 1998). Topoisomerase poisons are effective anti-cancer drugs as they prevent cell replication and induce apoptosis (Nitiss, 2009b).

In rodents, expression of Top2a and Top2b in the brain is higher during early embryogenesis compared with the later stages (Capranico et al. 1992). However, there is a surge of Top2b expression in the brain of newborn mice that is not observed for Top2a or the marker of cell proliferation thymidylate synthase. Top2a expression is most apparent

Correspondence
Dr Gavin Clowry, Institute of Neuroscience, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK. E: gavin.clowry@ncl.ac.uk
Prof. Caroline Austin, Institute of Cell and Molecular Biosciences, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK. E: caroline.austin@ncl.ac.uk

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ORIGINAL ARTICLE

Neurexins 1–3 Each Have a Distinct Pattern of Expression in the Early Developing Human Cerebral Cortex

Lauren F. Harkin[1,2,3], Susan J. Lindsay[2], Yaobo Xu[2,4], Ayman Alzu'bi[1,2], Alexandra Ferrara[1,2], Emily A. Gullon[1,2], Owen G. James[1,2,5] and Gavin J. Clowry[1]

[1]Institute of Neuroscience, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK, [2]Institute of Genetic Medicine, Newcastle University, International Centre for Life, Parkway Drive, Newcastle upon Tyne NE1 3BZ, UK, [3]Present address: School of Healthcare Science, Manchester Metropolitan University, Manchester, M1 5GD, UK, [4]Present address: Wellcome Trust, Sanger Institute, Cambridge, CB10 1SA, UK and [5]Present address: MRC Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, EH16 4UU, UK

Address correspondence to Gavin J. Clowry, Institute of Neuroscience, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK. Email: gavin.clowry@ncl.ac.uk or Susan J. Lindsay, Institute of Genetic Medicine, Newcastle University, International Centre for Life, Parkway Drive, Newcastle upon Tyne NE1 3BZ, UK. Email: susan.lindsay@ncl.ac.uk

Abstract

Neurexins (NRXNs) are presynaptic terminal proteins and candidate neurodevelopmental disorder susceptibility genes; mutations presumably upset synaptic stabilization and function. However, analysis of human cortical tissue samples by RNAseq and quantitative real-time PCR at 8–12 postconceptional weeks, prior to extensive synapse formation, showed expression of all three *NRXN*s as well as several potential binding partners. However, the levels of expression were not identical; *NRXN1* increased with age and *NRXN2* levels were consistently higher than for *NRXN3*. Immunohistochemistry for each NRXN also revealed different expression patterns at this stage of development. NRXN1 and NRXN3 immunoreactivity was generally strongest in the cortical plate and increased in the ventricular zone with age, but was weak in the synaptogenic presubplate (pSP) and marginal zone. On the other hand, NRXN2 colocalized with synaptophysin in neurites of the pSP, but especially with GAP43 and CASK in growing axons of the intermediate zone. Alternative splicing modifies the role of NRXNs and we found evidence by RNAseq for exon skipping at splice site 4 and concomitant expression of KHDBRS proteins which control this splicing. NRXN2 may play a part in early cortical synaptogenesis, but NRXNs could have diverse roles in development including axon guidance, and intercellular communication between proliferating cells and/or migrating neurons.

Key words: cortical development, neurexins, neurodevelopmental disorders, subplate