The expression of autism susceptibility genes in the earliest stages of human cerebral cortex development

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Abstract

Autism susceptibility gene (ASG) mutations are suspected to perturb developmental pathways essential for the correct formation and organisation of the brain. Many ASG products, including neurexins (NRXNs), neuroligins (NLGNs) and SHANKs, have established functions in mature synapses. However, a previous microarray study suggested that ASGs are expressed in the human cortex from as early as 8 PCW, when synapses are sparse.

RNA sequencing of human cortical samples revealed that a number of ASGs that code for cell adhesion molecules, enzymes that synthesise neurotransmitters, neurotransmitter receptors, molecules involved in neurotransmitter transport, synaptic transmission, cell-cell signalling and neuronal differentiation are differentially expressed both spatially and temporally between 9 and 12 PCWs. *NRXNs*, *NLGNs* and *SHANKs*, were studied in further detail using RNA sequencing, qPCR and IHC. Many of the *NRXN* and *NLGN* genes showed an increase in expression between 8 and 12 PCW and were significantly increased in either the anterior or temporal cortex. The *SHANK* genes did not increase in expression with age and there was high variation between the RNA sequencing data and the qPCR data. Immunohistochemistry revealed that NRXN proteins have distinct distribution patterns within the cortex at these ages. NRXNs 1 and 3 were predominantly found in the cortical plate (CP) whilst NRXN2 immunopositivity was strongly co-localised with the presynaptic vesicle protein, SYP and the marker of outgrowing axons, GAP43, largely found in the pre-subplate, marginal zone and intermediate zone. Regulators of *NRXN* splicing, were expressed primarily in the CP whereas TOP2B, a regulator of *NRXN* transcription, was found throughout the cortex.

The distribution of NRXN proteins suggests that, in addition to their established function in synaptogenesis, they may have diverse roles in development including axon guidance and intercellular communication between proliferating cells and/or migrating neurons.

Dedication

For my mum and dad,

Jean Harkin & Patrick Harkin

For always believing in me.

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I am sincerely grateful to Professor Susan Lindsay and Dr Gavin Clowry for their supervision, support and encouragement. I am extremely thankful for the opportunity to join their lab and will forever appreciate their time, expertise and understanding.

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I would also like to thank Professor Caroline Austin for her support and advice. Also, for providing the TOP2A and TOP2B antibodies and providing the chance for collaboration.

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Finally, I thank my family and my partner as without their support I would not be in this position.

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Abbreviations

5hmc	5-hydroxy-methyl-cytosine
5mc	5-methyl-cytosine
A1	Primary auditory region
ADD	Attention deficit disorder
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANK2	Amkyrin 2
ANOVA	Analysis of variance
ANR	Anterior neural ridge
APC	adenomatous polyposis coli
ASD	Autism spectrum disorder
ASG	Autism susceptibility gene
BDNF	Bone derived neurotropic factor
BMP	Bone morphogenic protein
bp	base pairs
CA1	Region I of hippocampus proper
CASK	calcium/calmodulin-dependent serine protein kinase
CBLN1	Cerebellin 1
CDH4	R Cadherin
CDH6	Cadherin 6
CNS	Central nervous system
CNTNAP2	Contactin associated protein like 2
CNV	Copy number variant
COUPTF1/2	Chicken ovalbumin upstream promoter (COUP) transcription factor 1/2
CP	Cortical plate
CPG	Cytosine-phosphate-guanine
CRD	Cysteine rich domain
CS	Carnegie stage
Ct	Cycle threshold
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DKK	Dickopf
DLX	Distal-less homeobox
DNA	Deoxyribonucleic acid
DVL	Dishevelled

E	Embryonic
EGF	Epidermal growth factor
ELF1	E74 like factor 1
EMX1/ 2	Empty spiracles homeobox1/2
EN2	Engrailed homeobox 2
EPSP	Excitatory post synaptic potential
ER81	E-twenty six (Ets) variant 1
FBS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FMR1	Fragile X mental retardation 1
FMRP	Fragile X mental retardation protein
FOXO1	Forkhead box protein O1
FP	Forward primer
FRAP	Fluorescence recovery after photobleaching
FZD	Frizzled
GABA	Gamma aminobutyric acid
GABRQ	gamma amino butyric acid A receptor theta
GAD1	Glutamic acid decarboxylase
GAP43	Growth-associated protein 43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GE	Ganglionic eminence
GFAP	Glial fibrillary acidic protein
GO	Gene ontology
GO	Gene ontology
GRID	Glutamate receptor delta
GRIP2	Glutamate Receptor Interacting Protein 2
GRM	glutamate receptor metabotropic
GSH2	Genetic screened homeobox 2
GSK	Glycogen synthase kinase
H&E	Haematoxylin and eosin
HBSS	Hank's balanced salt solution
HDBR	Human Developmental Biology Resource
hESC	human Embryonic stem cell
HGFR	human growth factor receptor
hIPSC	human Induced pluripotent stem cell

HOX	Homeobox domain
HRP	Horseradish peroxidase
IC	Internal capsule
ICC	Immunocytochemistry
IGFBP3	Insulin like growth factor binding protein 3
IGM	Institute of Genetic Medicine
IHC	Immunohistochemistry
IPSP	Inhibitory post synaptic potential
IQ	Intelligent quotient
ISVZ	Inner sub-ventricular zone
ITG	Integrin
IZ	Intermediate zone
KCC2	K+ Cl- cotransporter 2
KHDRBS	KH domain containing, RNA binding, signal transduction associated
KLF4	Kruppel-like factor 4
KO	Knock-out
LGE	Lateral ganglionic eminence
LHX2	LIM homeobox 2
LIF	Leukaemia Inhibitory Factor
linc	long non-coding
LNS	laminin/neurexin/sex hormone-binding globulin-domain
LRRTM	Leucine rich repeat transmembrane
LTP	Long term potentiation
m	messenger
M1	Primary motor cortex
MBD	Methyl CpG binding domain
MECP2	Methyl-CpG Binding Protein 2
mEPSC	miniature Excitatory post synaptic current
MET	hepatocyte growth factor receptor (HGFR)
MGE	Medial ganglionic eminence
mi	micro
mIPSC	mouse Induced pluripotent stem cell
ml	Millilitre
MMR	Measles, mumps, rubella
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid

MZ	Marginal zone
n	Number
n	number
NCBI	Nation centre for biotechnology information
ng	Nano gram
NGN2	Neurogenin2
NKX2.1	Nk2 homeobox 1
NLGN	Neuroligin
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
no.	number
NPTX1	Neural pentraxin
NRG	Neuregulin
NRXN	Neurexin
NURR1	Nuclear receptor-related protein 1
NXPH	Neurexophilin
OCT	octamer-binding transcription factor
OXT	Oxytocin
OXTR	Oxytocin receptor
P	Postnatal day
P/M	Primary motor region
P13/AKT	Phosphatidylinositol-3/
PAX6	Paired-box 6
PCA	Principal component analysis
PCDH	Protocadherin
PCW	Post-conceptional weeks
PCW	Post conceptional weeks
PFA	Paraformaldehyde
PP2A	Protein phosphatase 2
PSD95	Post synaptic density
PTEN	Phosphatase and tensin homolog
PTPRZ	Protein Tyrosine Phosphatase, Receptor Type Z1
PTX3	Pentraxin related protein
r	ribosomal
RA	Retinoic acid
RELN	Reelin

RNA	ribonucleic acid
RNA Seq	Ribo nucleic acid sequencing
ROBO	Roundabout
RP	Reverse primer
RPH3A	*rabphillin 3A*
RPKM	Reads per kilo base per million
RTO	Rostral telencephalic organiser
RTT	Rett syndrome
S1	Primary somatosensory area
SAM68	Src-Associated substrate in Mitosis of 68 kDa
SAT1B/2B	spermidine/spermine N1-acetyltransferase 1B/ 2B
SC-CA1	Schaffer-collateral-CA1 pyramidal
SDHA	Succinate dehydrogenase complex subunit A
SEMA	Semaphorin
SFRP2	Secreted frizzled-related protein 2
SH3	SRC Homology 3
SHANK	SH3 and multiple ankyrin repeat domains
SHH	Sonic hedgehog
si	small interfering
siRNA	RNA interference
SLC32A1	Solute carrier family 32 member 1
sn	small nuclear
sno	small nucleolar
SNP	Single nucleotide polymorphism
SOX2	Sex determining region Y (SRY) box 2
SP	Subplate
SP8	Specificity protein 8
SVZ	Subventricular zone
SYN2	Synapsin 2
SYTL5	Synaptogamin like 5
TBR1/2	T-box brain 1/2
TBS	Tris based buffer
TGFα	Transforming growth factor alpha
TOP	Topoisomerase
TRD	transcription repression domain
TRKB	Tropomyosin receptor kinase B

V1	Primary visual area
VZ	Ventricular zone
WGCNA	Weighted correlation network analysis
Wnt	Wingless
µg	Microgram
µl	Microliter
µm	Micrometre
µM	Micro molar

Statement of Contributions

- **Chapter 3**

Dissection of those samples sent for RNA sequencing was carried out by the HDBR. AROS Applied Biotechnology (Aarhus, Denmark) carried out the RNA extraction, library preparation and sequencing. The bioinformatician, Dr Yaobo Xu, Newcastle University, carried out the quality control of the sequencing reads, mapping of reads to the human genome, the creation of RPKM values and the resultant gene list, PCA and hierarchical cluster analysis, and finally the differential expression analysis to create gene lists used in the analysis. All of the work regarding the RNA sequencing was carried out under the advice of myself, Professor Susan Lindsay and Dr Gavin Clowry and the supervision of Professor Mauro Santibanez Koref. Venn diagrams, gene ontology analysis and general analysis of the data set was carried out by myself.

- **Chapter 4**

Dissection of the embryonic and foetal brain, further processing pre RNA collection and the majority of primer design, quantitative PCR (qPCR) and analysis was carried out by myself. Emily Gullon, MSc, contributed to the primer design and qPCR for the *Neuroligin* genes.

- **Chapters 5 and 6**

The majority of immunostaining and the analysis was carried out by myself under the supervision of Dr Gavin Clowry and Professor Susan Lindsay. Ayman Alzubi and, to a lesser extent, Alex Fererra, under the supervision and guidance of myself, Professor Susan Lindsay and Dr Gavin Clowry, double labelled many of the human cortical sections using fluorescent immunohistochemistry. The processing, majority of tissue sectioning, creation of slides for use in IHC and quality control was performed by the HDBR.

Chapter 1 Introduction

1.1 Human Brain development

Human prenatal development takes, on average, 280 days or 40 weeks. Conception marks the beginning of the embryonic period of development which lasts for approximately 9 weeks, after which the fetal period of development begins (O'Rahilly and Muller, 2010). By the end of the embryonic period, the forebrain, midbrain, hindbrain and spinal cord are established along the anterior/ posterior axis of the neural tube. The fetal period sees further subdivisions of these primary regions. During early neural development, molecular organisers and surrounding tissues secrete morphogens that diffuse outwards creating signalling gradients. Varying concentrations and combinations of these molecules, in addition to varying concentrations of agonists, enable the primary patterning of the human central nervous system (CNS).

The neural tube arises from the neuroectoderm, which is a group of cells located along the midline of the upper embryonic layer at the end of gastrulation (figure 1.1A). A mediolateral axis is determined by bone morphogenic protein (BMP) signalling, with higher BMP concentrations laterally. The lateral ridges of this region fold inwards and join to create the hollow neural tube structure (Copp *et al*., 2003) with the laterally positioned cells constructing the dorsally positioned roofplate of the neural tube whilst the medial cells of the neuroectoderm make up the ventrally positioned floorplate. There is a high concentration of Wingless (Wnts) proteins in the roofplate and a high concentration of Sonic Hedgehog (Shh) proteins in the floorplate, which diffuse outwards creating signalling gradients (figure 1.1B). In addition to these dorsoventral signalling gradients, anteroposterior gradients exist. There is a higher concentration of Wnts at the posterior and Shh at the anterior ends of the neural tube (Lumsden and Krumlauf, 1996).

The cells at the anterior end of the neural tube give rise to the midbrain and forebrain whilst the more caudally located cells give rise to the hindbrain and spinal cord. The complex patterning of the most anterior part of the forebrain, the telencephalon, during the late embryonic/ early fetal period is discussed further below.

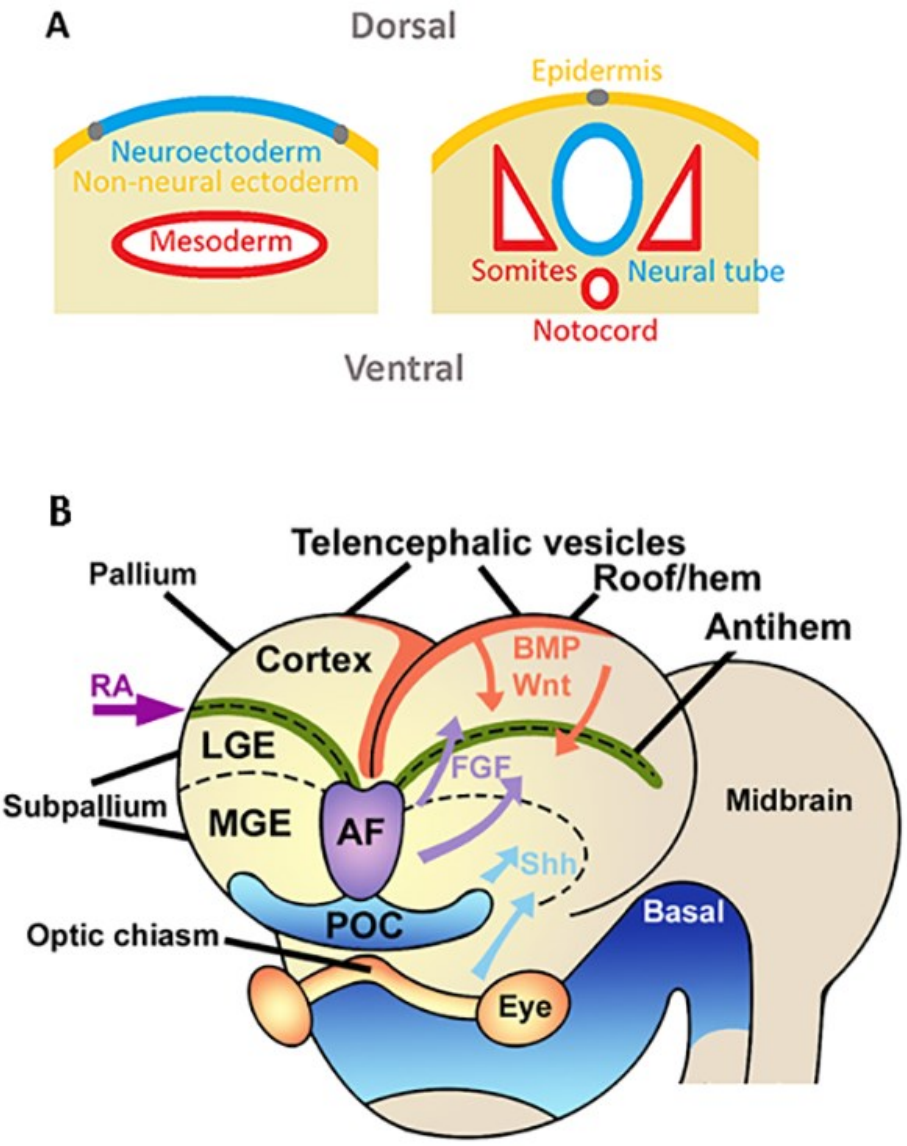


Figure 1.1 Development of the human central nervous system (CNS) adapted from Montiel & Aboitez (2015). (A) The human CNS develops from the neuroectoderm that folds inwards to form the neural tube. **(B)** This structure is subjected to multiple morphogen gradients enabling the distinction of the telencephalon, diencephalon and mesencephalon. Wnt signalling originates from the roof plate creating a dorsal ventral expression gradient. Shh signalling originates from the floor plate creating a ventral –dorsal gradient. FGF signalling is essential in the development of the midbrain-hindbrain boundary. The developmental progression of the telencephalon sees the separation of the pallium and sub-pallium.

1.1.1 Development of the telencephalon

The forebrain becomes further subdivided into the telencephalon and diencephalon. From the telencephalon develops the cerebral cortex and the ganglionic eminences whilst the diencephalon structures include the thalamus, sub thalamic nuclei, hypothalamus and epithalamus. The dorsal and ventral regions of the early telencephalon give rise to the pallium and sub pallium respectively, which develop into the cerebral cortex, caudate, putamen, globus pallidus and nucleus acumbens that we see in the postnatal brain. The pallium and sub pallium contain different sets of transcription factors with *Empty spiracles homeobox 1 (EMX1), 2 (EMX2)*, *Paired-box 6* (*PAX6)* and *T-box brain* (*TBR1)* expressed dorsally and *Distal-less homeobox* (*DLX*) and *Nk2 homeobox* (*NKX*) genes ventrally which are involved in further patterning of these separate regions (Figure 1.2A).

In part, the division of the pallium and sub pallium occurs due to the opposing effects of the transcription factors PAX6 and Glutathione synthetase homeobox2 (GSH2) (Toresson and Campbell, 2001, Yun *et al*., 2001). *Pax6* mutations in mice result in the loss of the dorso/ ventral telencephalic boundary and ectopic DLX expression in cells of the cortex (Stoykova *et al*., 1997). Using a β galactosidase reporter, cells of the dorsal telencephalon were found misplaced in the sub pallial regions of *PAX6*$^{sey/sey}$ mutants at E18.5 (Kroll and O'Leary, 2005). *Gsh1/2* double mutants show ectopic expression of PAX6 and NGN2 and a decreased expression of DLX at E12.5 but the expression of NKX2.1 is not affected (Toresson and Campbell, 2001). Mutations in these genes also result in the abnormal expression of downstream markers of regional identity such as *E-twenty six (Ets) variant 1* (*Er81*) and *Specificity protein 8* (*Sp8*) (Carney *et al*., 2009). Another factor in the separation of these two regions is the expression of cell adhesion molecules R cadherin (CDH4) and cadherin 6 (CDH6). Cells of the sub pallium express CDH4 whilst cells of the pallium express CDH6 and when mixed, these cells adhere to those expressing the same cadherin as themselves (Inoue *et al*., 1997, Gotz *et al*., 1996, Matsunami and Takeichi, 1995). Fluorescently tagged pallial and sub pallial cells collected from PAX6$^{sey/sey}$ mutant mice, after being mixed, show a significant reduction in their adherence to cells of the same region. This is most likely due to a decrease in *Cdh4* expression in cells of the pallium (Stoykova *et al*., 1997).

In addition to GSH1 and 2, the transcription factors NKX2.1 and DLX1 and 2 can also be found in the sub-pallial regions (figure 1.2A). Mice with *Nkx2.1* mutations, that render the protein non-functional, have missing pallial structures and the striatum, which is derived

from the LGE, is expanded suggesting that this protein is essential for the development of the MGE and its derivatives (Sussel *et al*., 1999). Immunohistochemistry and in situ hybridisation in human brain sections shows that in the late embryonic/ early fetal period of development, *NKX2.1* is confined to the medial ganglionic eminence (MGE) as it is in the mouse at E12 (Onorati *et al*., 2014, Pauly *et al*., 2013, Sussel *et al*., 1999). In humans and rodents, DLX2 positive cells are found throughout the sub pallium. Co expression with either PAX6 or NKX2.1 identifies cells of the LGE and MGE respectively (Pauly *et al*., 2013). *Dlx1/2* double mutant mice show a failure of cells to migrate out of the proliferative regions of the sub pallium into the mantle zone (Anderson *et al*., 1997).

1.1.2 The complex patterning of the developing cortex

Originally, the protocortex theory proposed that that the cortex develops as a homogenous group of cells that receive patterning information from the incoming thalamic efferents during the fetal stage of development. Although these efferents are clearly important in cortical regionalisation, the protomap hypothesis, recognises that differences in gene and protein expression arise before these efferents innervate the cortex (Rakic and Lombroso, 1998; reviewed in Sansom and Livesey, 2009a). Cortical gene and protein expression patterns that develop before thalamic input are discussed below.

Identified organisers that are thought to regulate early patterning include the cortical hem, the rostral telencephalic organiser (RTO) and the antihem. The cortical hem is positioned in the dorsomedial telencephalon (1.2A) and is a source of BMPs and WNTs (Grove *et al*., 1998, Furuta *et al*., 1997). Its formation is regulated by LHX2, which also acts to prevent its expansion into the cortex (Bulchand *et al*., 2001). The absence of the cortical hem in mice decreases the size of the cortical hemispheres and perturbs patterning along the rostro-caudal axis (Caronia-Brown *et al*., 2014). Rostral areas were expanded at the expense of caudal areas.

The RTO is positioned rostrally and is a source of FGF signalling (Cholfin and Rubenstein, 2008, Fukuchi-Shimogori and Grove, 2001). One of the main signalling molecules secreted from the RTO is FGF8, which diffuses towards the caudal telencephalon creating a gradient of expression (Assimacopoulos *et al*., 2012, Garel *et al*., 2003, Fukuchi-Shimogori and Grove, 2001). WNTs and BMP proteins have opposing effects to those of FGF8. This information

suggests that the cortical hem and the RTO release morphogens that create signalling gradients that act antagonistically in order to produce the anterior/ posterior axis of the cerebral cortex.

The cells of the antihem, located near the boundary of the LGE and the ventro-lateral neocortex, express *Transforming growth factor alpha* (*Tgfα*)*, Neuregulin* (*NRG*) *1* and *3* and *Fgf7* (Assimacopoulos *et al*., 2012, Assimacopoulos *et al*., 2003). *Secreted frizzled-related protein 2 (SFRP2)*, a known Wnt antagonist, is also secreted from the antihem (Kawano and Kypta, 2003, Ladher *et al*., 2000). It interacts with Wnts via either the C terminal or CRD domain (Uren *et al*., 2000, Lin *et al*., 1997). SFRP proteins share similarities with the Frizzled (FRZ) proteins which act as receptors for Wnts. The interaction between SFRP2 and Wnts may render the Wnts non-functional by preventing them from binding Frizzled proteins (Ladher *et al*., 2000).

These primary signalling molecules released from cells within organiser regions can activate the production of secondary signalling molecules, via the activation of transcription factors that can themselves act as morphogens or can act in a concentration dependent manner. In addition to their role in the formation of the pallial-sub pallial boundary, transcription factors play an important role in the arealisation of the cerebral cortex. In both mice and humans, *EMX2* expression is higher in the posterior cortex compared to the anterior cortex (Bayatti *et al*., 2008b, Mallamaci *et al*., 1998, Gulisano *et al*., 1996) and *PAX6* expression is higher in the anterior cortex (Bayatti *et al*., 2008b, Stoykova and Gruss, 1994) (figure 1.2B). The *PAX6* expression gradient disappears by 9PCW in humans (Bayatti *et al*., 2008b). Homozygous *PAX6* $^{sey/sey}$ and homozygous *EMX2*$^{-/-}$ mutant mice die soon after birth (Bishop *et al*., 2000, Mallamaci *et al*., 2000) but analysis of the primary visual (V1), motor (P/M), auditory (A1) and somatosensory (S1) regions revealed gross abnormalities in cortical patterning. The rostro-lateral regions, F/M and S1, were reduced in *PAX6* $^{sey/sey}$ mice whereas caudo-medial regions, A1 and V1, were reduced in the *EMX2*$^{-/-}$ mice (figure 1.2C) (Bishop *et al*., 2002, Bishop *et al*., 2000, Mallamaci *et al*., 2000).

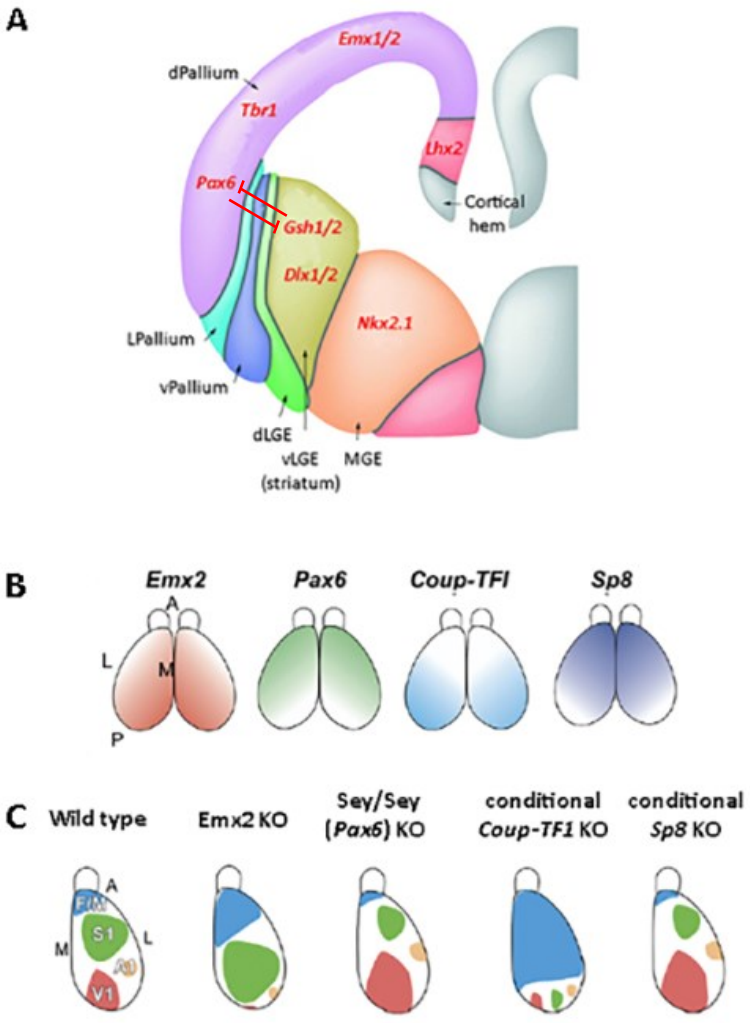


Figure 1.2 Gene expression patterns in the developing brain (adapted from Schuurmans and Guillemot, 2002 & O'Leary & Sahara, 2008). (A) The telencephalon is divided into the pallium and sub pallium due to the opposing expression of *Pax6* and *Gsh1/2*. Transcription factors Tbr1 and Emx1.2 are specific to the dorsal (d) pallium, Dlx1 and 2 are found in the lateral ganglionic eminence (LGE) and NKX2.1 is specific to the medial (M) GE. **(B)** The transcription factors *Emx2* and *Coup-TF1* are expressed in a posterior-anterior gradient across the mouse cortex, whilst PAX6 and SP8 show higher expression in the anterior cortical regions. Anterior (A), posterior (P), medial (M) and lateral (L) **(C)** Wildtype mouse cortex contains the primary auditory (A1), visual (V1), somatosensory (S1) and motor (F/M) regions. The F/M region of EMX2 and COUP-TF1 Knockout (KO) mice is increased in size reducing the size of the other cortical regions. PAX6 and SP8 KO mice have expanded V1 regions, pushing A1, S1 and F/M anteriorly and reducing the size of these areas.

Chicken ovalbumin upstream promoter (COUP) transcription factor 1 (Coup-tf1) is expressed in a posterior-anterior and lateral -medial gradient (figure 1.2B) and functions to repress frontal/ motor cortical regions. Conditional *Coup-tf1* knockout mice, in which the gene is only deleted in the cortex, results in F/M cortical areas being greatly expanded at the expense of the V1, S1 and A1 regions (figure 1.2C; Armentano *et al*., 2007). *Sp8* is expressed in an anterior- posterior and medial- lateral gradient (figure 1.2B). Sp8 can also activate the transcription of Fgf8. An *Sp8* conditional knockout mouse, like the *PAX6* knockout, shows an increase in the primary visual area (figure 1.2C; Zembrzycki *et al*., 2007).

1.1.3 Neurogenesis and neuronal differentiation in the cerebral cortex

The generation of cortical neurons requires a sufficient number of neural progenitor cells. Only when there is an adequate progenitor population can these cells switch their mode of division from symmetrical (producing two progenitor cells) to asymmetrical (producing one progenitor cell and one differentiating neuronal cell). This switch occurs in humans around E42 (5 weeks) which is when the first neurons begin to appear in the developing brain. Neurons continue to be produced through the fetal period of development (Rakic, 1995) which is best characterised by the 'production, migration and differentiation of neurons'. Some neurogenesis even continues after birth (Stiles and Jernigan, 2010b).

The newly formed neuronal cell leaves its progenitors in the proliferative zone and begins to travel into the neocortex (figure 1.3). As more and more neuronal cells are produced, the neocortex expands and eventually we can see the formation of a six-layered structure consisting of a mixture of old and newly formed neurons. The formation of this structure is described as 'inside out' due to the fact that the most recently formed neurons migrate past older neurons to form the more peripheral layers (Tiffin and Goffinet, 2003).

The preplate is formed by the first neurons that leave the proliferative layer. A further population of migrating neurons then splits the preplate into the marginal zone (MZ) and the subplate (SP). These layers play a vital role in the development of the neocortex. Cajal-Retzius cells subside in the MZ and release a protein called Reelin. Reelin is present at the periphery of the newest layer of neuronal cells and signals to cells when to halt migration and take up their positions in the neocortex (Valiente and Marín, 2010; Huang, 2009; Bielle *et al*., 2005). Once migrating cells pass the previously formed layer, they are signalled to

stop and therefore form the most superficial layer. The subplate is required at a later stage of cortical development when it plays a role in creating the primary sensory inputs including the thalamocortical and corticothalamic pathways (Kostović and Jovanov-Milošević, 2006).

The six layers of the neocortex are produced after the establishment of the SP and MZ and contain different types of neurons. It is thought that neural progenitors become more restricted in the type of neurons that they can produce as development progresses. Whereas progenitor cells at the beginning of neurogenesis are capable of becoming any layer of the neocortex, cells later in development are restricted to becoming the more superficial layers (McConnell and Kaznowski, 1991).

Immature neurons have a lack of or poorly developed axons and dendrites. The axons and dendrites are branching protrusions of the cell body that enable signalling between cells. Dendrites receive electrical signals while axons carry the signals and relay them to other neurons by means of synapses.

Growth cones at the end of each axon allow for extension (Dent and Gertler, 2003). Molecules in the vicinity of the growth cone will guide the axon towards target areas and synapses with target neurons will form. Myelin sheathes, which are white in colour, insulate the axons to allow efficient transduction of the signal. The grey matter of the brain signifies where the cell bodies of neurons are concentrated while the white areas contain the myelinated axons. Grey areas of the brain include but are not limited to the neocortex, the cerebellum, the basal ganglia and the thalamus.

There are two methods of neuronal migration used when cells travel away from the ventricular zone. Somal translocation involves the cell adjusting its shape to reach the outer (pial) surface of the brain to aid migration over very short distances (Nadarajah and Parnavelas, 2002). As the brain and therefore, neocortex grows the distance over which the neurons have to travel increases. Especially since newer cells migrate through already positioned cells, somal translocation is not feasible. Radial glial guides are now used to assist the migration of newly differentiated neurons into the neocortex (Nadarajah and Parnavelas, 2002). Radial glial cells can extend their basal process to reach the pial surface whilst keeping their cell body in the VZ (figure 1.3).

Sometimes, cells migrate even longer distances for example those that originate from the basal ganglia and finish in the cortex. This mode of migration is referred to as tangential

migration. The ganglionic eminence consists of three regions – medial, lateral and caudal. These regions are the origin of a population of inhibitory cortical interneurons (Anderson *et al*., 2001; Corbin *et al*., 2001). The migrating neurons encounter a number of signalling and guidance molecules along their way that will direct them to their final positions in the cortex (Valiente and Marín, 2010, Huang, 2009, reviewed in Marin and Rubenstein, 2001).

1.1.4 Synapse formation and stabilisation within the cortex

After neurons reach their final position in the neocortex (the outermost part of the cerebral cortex), they differentiate further and extend their dendrites and axons to reach areas of the brain in which they will eventually develop connections or synapses (figure 1.4B). The human neocortex is thought to contain over 160×10^{12} synapses (Tang *et al*., 2001).

Synapses act to relay electrical signals from one neuron to the next. There are two types of synapses, characterized by the distance between the pre and postsynaptic membranes. Electrical synapses have a gap of < 4nm and the electrical signal can pass from one cell to another due to the presence of membrane channels connecting the two cells (Hormuzdi *et al.,* 2004). Chemical synapses, however, have a larger gap of 20-40nm. An electrical signal/ action potential travels along the neuron and down its dendrites until it reaches the presynaptic membrane. An influx of calcium ions causes synaptic vesicles containing neurotransmitters such as glutamate to fuse with the pre-synaptic membrane releasing the neurotransmitter into the synaptic cleft. The neurotransmitters then bind to receptors on the postsynaptic membrane resulting in a change in membrane potential and influx or efflux of ions. In the case of excitatory synapses, the result is the generation of another action potential in the receiving neuron (Benarroch, 2013).

An excitatory postsynaptic potential (EPSP) is one that increases the likelihood that the neuron will fire an action potential. Neurotransmitters associated with EPSPs such as acetylcholine, glutamate and serotonin, once bound to the receptors on the postsynaptic terminal, cause depolarisation of the target neuron. If summation of EPSPs reaches threshold then an action potential is generated, transmitting the signal from the original neuron. An inhibitory post synaptic potential (IPSP) reduces the chance that the target neuron will fire an action potential by raising (hyperpolarising) the membrane potential and thereby increasing the threshold for an action potential to occur. Gamma aminobutyric acid

(GABA) is the major inhibitory neurotransmitter in the brain. When acting at ionotropic GABA-A receptors, it causes a rapid influx of across the postsynaptic membrane, raising the membrane potential. Other inhibitory receptors include g protein coupled receptors, e.g. GABA-B receptors that hyperpolarise cells by modulating potassium channel opening, or dopamine receptors that attenuate EPSPs generated at excitatory synapses. Synapses have first been shown to form between 7 and 8 post conceptional weeks (PCW) (figure 1.4A; de Graaf-Peters and Hadders-Algra, 2006). The first synapses to form in the cerebral cortex are present in the marginal zone followed by those of the sub-plate (SP) (Zecevic, 1998). Synaptogenesis increases with the appearance of the cortical plate including a large increase between 10-12 PCWs (Zecevic, 1998). The emergence of synapses and increase in synapse production seems to be synchronized across the brain although studies on postnatal brains suggest that some brain regions cease synaptogenesis before others (Huttenlocher and Dabholkar, 1997). Following the rapid increase in synaptogenesis and the generation of new synaptic connections, there is a successive period of pruning that takes place to eliminate unnecessary synapses (figure 1.4B; Huttenlocher *et al*., 1982, Rakic *et al*., 1986).

Neurogenesis in the cortex is thought to cease postnatally except for the few neurons that are produced for the olfactory bulb and the hippocampus throughout life. Recent studies however, have shown that elevated transcription of synaptic genes continues up to 5 years of age in the prefrontal cortex and may be a defining factor in human evolution (Liu *et al*., 2012b). Some glial progenitors continue to be produced postnatally (figure 1.4A) and injury causes their differentiation into mature glial cells such as oligodendrocytes and astrocytes. The differentiation of glial cells plays a large role in establishing mature neural circuits. The ongoing differentiation of these cells is likely to assist in the maturation of the human brain and its plasticity (Stiles and Jernigan, 2010b). MRI imaging of children's brains shows the changes in myelination along with the significant changes in structure over the first few years of life (Barkovich, 2000, Barkovich, 2005).

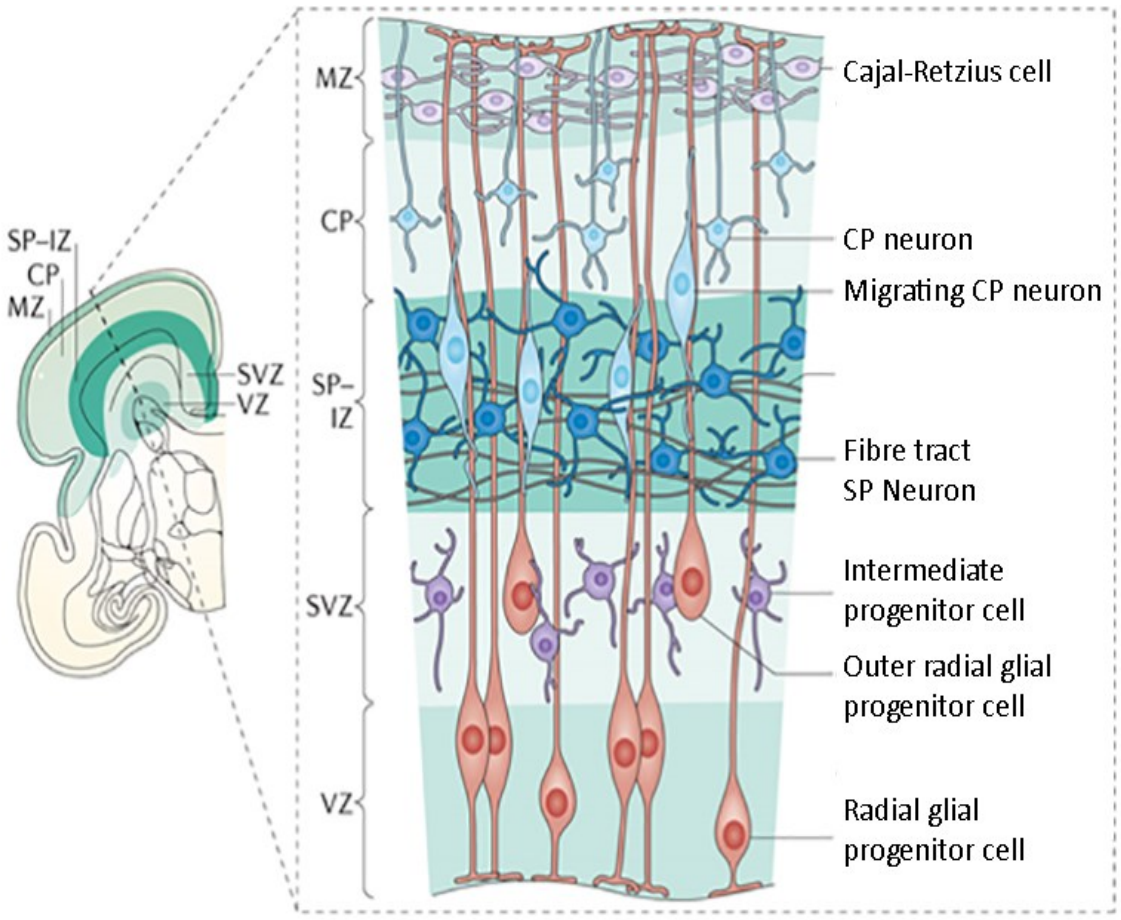


Figure 1.3 Generation of the neocortex (adapted from Hoerder-Suabedissen & Molnár., 2015). The neocortex begins as a collection of proliferating cells referred to as the ventricular zone (VZ) and then also the sub ventricular zone (SVZ). Some of these cells switch their mode of proliferation from symmetric to asymmetric, which produces both proliferating cells and cells that begin differentiation. Radial glial cells extend their processes to produce a platform along which differentiating cells can migrate outwards. Differentiating cells migrate, via radial glial cells, to form the preplate which is eventually split into the sub plate (SP) and marginal zone (MZ) by incoming migratory cells. These cells will form the cortical plate. The cortex is formed in an 'inside out' manner enabling progenitor cells to migrate past newly formed cortical layers to establish new layers. The migratory region between the proliferative regions and the sub plate is known as the intermediate zone (IZ).

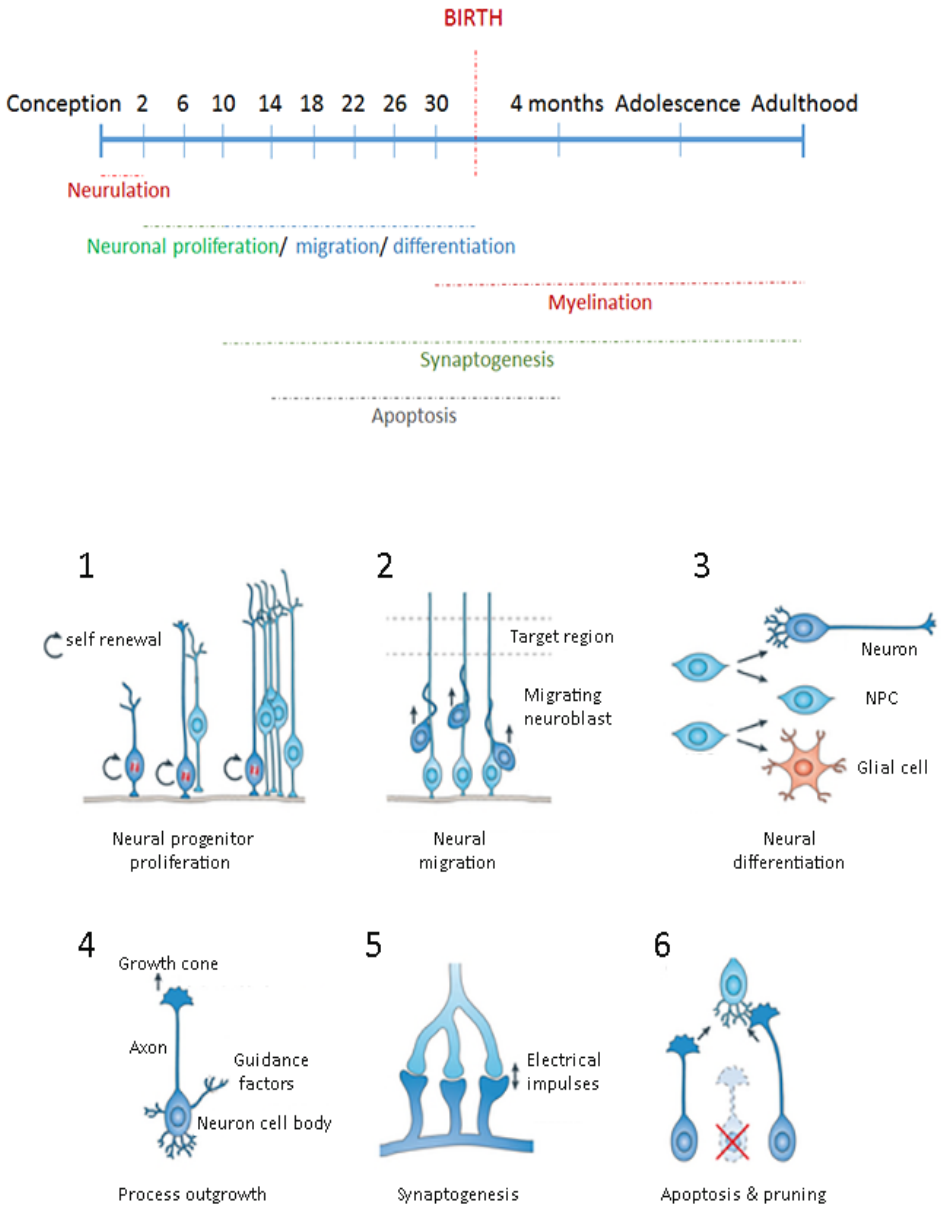


Figure 1.4 The different stages of neural development (adapted from Ronan *et al*., 2013). **(A)** Early development consists of neurulation, neuronal proliferation, migration and differentiation. This period spans from 2PCW until birth. Synaptogenesis begins as early as 8 PCW although this process will be minimal at early stages of brain development and in adulthood. Myelination begins much later than synaptogenesis and persists into adulthood. Apoptosis begins at about 12 PCW and continues until approximately 6 months of age. **(B)** Neural development begins when cells switch from symmetric to asymmetric division. They begin to differentiate and migrate to target positions within the brain. Neural progenitor cells (NPCs) can differentiate into glial cells or neurons. Neurons will develop axons and dendrites and eventually form synaptic connections with other neurons or muscle cells. Any abnormal connections will be pruned by apoptosis.

1.2 AUTISM

1.2.1 Autism is a heterogeneous developmental disorder

Autism is a developmental disorder and is characterised by impairments in social interaction and communication and restrictive, repetitive behaviours (American Psychiatric Association, 2013). Autism is now included in the diagnosis of autism spectrum disorder (ASD), which also includes Asperger's syndrome, Rett syndrome, childhood disintegrative disorder and other pervasive developmental disorders that don't fall into any of the above diagnoses (Committee on Children with Disabilities, 2001). Autism can be reliably diagnosed at around two years of age, however, some symptoms of autism may present before this age and may be useful when trying to analyse the autistic brain at earlier stages of brain development.

The prevalence of autism differs between countries, likely due to variable methodologies (Zaroff & Uhm, 2012). The majority of autism prevalence research is carried out in the UK and US where, on average, the prevalence estimate is around 1% (Baron-Cohen *et al.,* 2009; Rice, 2009; Kogan *et al.*, 2008; Baird *et al.,* 2006). Estimates of ASD are higher than those for autism and a review of more recent studies has estimated that the prevalence of ASDs including other PDDs is increasing due to amended diagnostic criteria and improved awareness (Blumberg *et al*., 2013; Fombonne, 2009). In addition, there is a higher incidence of ASD in males compared to females with current estimates at around 4:1 (Fombonne, 2009).

There is an increasing body of evidence showing that the neuroanatomy and neurophysiology of the autistic brain differs from that of typically developing children and adults. MRI scans of children's brains between 1.5 and 5 years of age revealed that both the cerebral white and grey matter was enlarged in children with autism at 2.5 years, with the most significant increases occurring in the frontal, temporal and cingulate cortex (Courchesne *et al.,* 2011; Schumann *et al.,* 2010). This increase in size is predicted to be due to hyperplasia rather than hypertrophy (Carper *et al.,* 2002). Hazlett *et al.,* (2011) compared the cortical thickness of children with autism to those without at age 2 and 6 years and found no significant difference (Hazlett *et al.,* 2011). However, between 3 and 39 years, Zielinski *et al.,* (2014) presented evidence of cortical thinning in the individuals with autism (Zielinski *et al.,* 2014). In addition to increased size and cell number, there has also been observed an increase in cortical folding in the brain of autistic people (Hardan *et al.,* 2004).

Anatomical changes have been reported in autistic brains as shown by sulcus mapping (Levitt *et al.,* 2003). Functional MRI studies also show significant differences in the activation of multiple cortical areas between autistic and typically developing children when processing speech (Redcay & Courchesne, 2008), in response to emotional faces (Kim *et al.,* 2015) and in social reward learning (Choi *et al.,* 2015).

Impairments in language have been observed in children with autism and these impairments were originally used as one of the diagnostic criteria for the condition (Ritvo & Freeman, 1977). They vary in severity from some articulatory errors to not developing language at all, and a large number of children with ASD are reported to have delayed language acquisition (Boucher, 2012). Using functional magnetic resonance imaging, Harris *et al.* (2006) and Knaus *et al.* (2008) have shown a reduced activation of Broca's area, which controls the motor functions required for speech, but an increased activation in Wernicke's area, which controls language comprehension, after semantic processing. Another feature in many people with ASD is intellectual disability (Chakrabarti, 2001; Ghaziuddin, 2000). While the current diagnosis criteria for intellectual disability are being examined, it has been usual in the past to use an intelligence quotient test for diagnosis. Almost 40% of children with ASD are also diagnosed with intellectual disability (CDC, 2014). Diagnosis with both ASD and ID can lead to specific adaptive and challenging behaviours not seen in the individual conditions and the co-occurrence of the two results in a poor prognosis (Matson & Shoemaker, 2009). In contrast to this, Patients with Asperger's syndrome and high functioning autism present with a higher than average IQ and no language impairments (Schopler, 1996).

Many other medical disorders are commonly reported in people with autism. The incidence of epilepsy in people diagnosed with ASD rages from 6-27% (reviewed in Jeste & Tuchman, 2015). There is a higher rate of epilepsy in those people with autism who have more severe intellectual disability (Amiet *et al.,* 2013) and also the risk of developing epilepsy increases with age (Bolton *et al.,* 2011; Hara *et al.,* 2007). The molecular and cellular links between the development of these two conditions revolve around the synaptic theory of autism (see section 1.3). A change in the proportions of inhibitory and excitatory synapses and changes in synaptic plasticity and connectivity are risk factors for both autism and epilepsy (Brooks=Kayal, 2010). Autism has also been associated with a higher than usual occurrence of schizophrenia, bipolar disorder, attention deficit disorder (ADD), anxiety and depression (Mazefsky *et al.,* 2008; Stewart *et al*., 2006, Gillott *et al*., 2001, Kim *et al*., 2000). Again, a

change in the brains circuitry is hypothesised to be responsible for these conditions and there is genetic overlap between the conditions (see section 1.2.2). It is important to recognise the presence of comorbid psychiatric conditions in people with ASD as specific treatment of each condition is more effective than a single treatment for both. Distinguishing between these conditions can be problematic but psychiatric co-morbidity estimates reach as high as 72% (Leyfer *et al.,* 2006).

Immune system dysregulation is a symptom in a number of people with ASD and there have been familial links of ASD with multiple immunological conditions including polyendocrine autoimmune disorder, maternal asthma, celiac disease, type I diabetes and rheumatoid arthritis (Keil *et al.,* 2010; Atladóttire *et al.,* 2009; Mostafa & Kitchener, 2009; Croen *et al.,* 2005; Money *et al.,* 1971). Whilst paternal immunological conditions are likely to influence prenatal development as a result of heritable factors, maternally, immunological conditions can play an environmental role, influencing conditions within the womb. Maternal antibodies have been shown to have an effect on brain growth and social behaviour in the offspring of rhesus macaques (Bauman *et al.,* 2013). Maternal infection in humans is also linked with an increased incidence of ASD and schizophrenia (Atladóttir *et al.,* 2010; Brown & Derkits, 2010; Libbey *et al.,* 2005). For an in depth review of immune system dysregulation and autism see Bjorklund *et al.* (2016) and Careager & Ashwood, (2012).

There are reported gastrointestinal (GI) symptoms in up to 70% of people with ASD (Valicenti-McDermott *et al.,* 2006). There is also evidence that the gut microbiota of autistic individuals is different from controls in terms of the proportion of each bacterial species (De Angelis *et al.,* 2013). Whether the GI symptoms are causative or secondary to ASD remains unclear. Research in mice has suggested that prenatally manipulating the gut microbiota can result in autistic like symptoms of increased repetitive behaviours and impaired social communication and interaction (Hsiao *et al.* 2013). A 'leaky gut' may enable toxins to leak from the gut and elicit an autoimmune response (reviewed in Samsam *et al.,* 2014). However, despite the high incidence of GI symptoms reported in ASD, some studies suggest that they may be neurobehavioral. A study by Ibrahim *et al.* (2009) found no significant increase in the incidence of GI symptoms, diarrhoea, gastroesophageal reflux/vomiting, or abdominal bloating/discomfort/irritability.

Autism spectrum disorder, as shown by the variety of co-occurring symptoms and conditions, is best addressed as a spectrum of disorders with different individuals having

different needs and therefore, potentially, being suited to different modes of treatment. In addition to the symptoms reported at the time of diagnosis or later on, there are a number of symptoms reported in autistic children before the age of diagnosis. These early symptoms preceding the onset of the condition are known as the autistic prodrome. Identification of early behavioural abnormalities in autistic children has been possible by using home video footage recorded by family members. By analysing these home videos, researchers hope to pinpoint any behavioural abnormalities that could relate to the later autistic diagnosis. However, the home videos differ in both content and length and family members do not tend to film 'abnormal' behaviour. Nevertheless, some interesting insights have been gained from such studies. When compared to the videos from children who did not develop autism, the autistic children tended to have impairments in sensorimotor development, less goal directed actions, reduced social interaction, gaze avoidance, an absence of emotional expression, a reduced response to their name and visual stimuli and show less pointing (reviewed in Yirmiya & Charman, 2010). The screening of high risk siblings has also provided insight into the prodrome, revealing delays in language, gestural communication, repetitive behaviours, abnormal paly behaviour and diminished gaze (reviewed in Yirmiya & Charman, 2010). An ASD prodrome would be a useful tool to predict the onset of ASD and to try to treat and possibly prevent the condition(s), or at least the more severe symptoms, before they occur.

1.2.2 The history of autism genetics

Family and twin studies have been used for decades to examine the link between genetics and autism. It is reported that approximately 10-15% of the siblings of autistic individuals also suffer from autism which is significant in comparison to its prevalence in the general population (Ozonoff *et al.,* 2011). Comparing the concordance rates of autism between monozygotic twins and dizygotic twins has provided more evidence that the condition has a high genetic component. Between 1977 and 1995, separate studies presented a concordance percentage of between 36-96 for monozygotic twins compared to 0-24% for dizygotic twins (Bailey *et al*., 1995; Steffenburg *et al.,* 1989; Ritvo *et al*., 1985; Folstein & Rutter, 1977). More recently, Tick *et al.,* (2016) presented a concordance rate of 98% for monozygotic twins and 53% for dizygotic twins. Other studies have identified a higher than

average incidence of cognitive abnormalities in the siblings of autistic individuals (Gizzonio *et al.,* 2014; Piven *et al.,* 1997; Baird & August, 1985; Minton *et al.,* 1982; August *et al.,* 1981).

Research into the genes responsible for the ASD phenotype started with cytogenetic testing that aimed to identify their chromosomal loci. Some studies identified as many as 48% of people with autism as having chromosomal abnormalities (Hagerman *et al.,* 1986) whilst more recent studies put estimates at between 3-7.4% (reviewed in Bergbaum *et al.,* 2016). Chromosomal abnormalities resulting in autism include fragile 16q23, 15q11-13 and 17p11.2 (reviewed in Lauritsen, 2001). Changes at these loci are also associated with the syndromes Fragile X, Praeder-Willi and Angelman. Mutations within specific genes located in these chromosomal regions are often sufficient to cause the syndrome, for example, mutations in the genes *FMR1* and *UBE3A* lead to Fragile X and Angelman syndrome respectively. The association of autism with these separate developmental diagnoses has led to the term syndromic ASD in which the diagnosis of autism is often secondary to the previously mentioned syndromes. Other examples of syndromes with a high incidence of autism include Rett syndrome and Tuberous Sclerosis caused by mutations in the MECP2 and TSC genes. These syndromes are discussed in more detail in section 1.2.4.

Alternatively to syndromic autism, there are many cases of non-syndromic or idiopathic autism in which autism is the primary diagnosis. In these cases, it is often harder to pinpoint genetic causes. It became clear that autism was not the result of mutations in a single gene due to its pattern of inheritance, its low penetrance and its presence in multiple monogenetic syndromes. Linkage and association studies have helped to further identify candidate susceptibility genes. These techniques identify regions of the DNA sequence that likely contain genes that when mutated confer a risk to the condition. The risk loci identified from these studies is summarised in Yang & Gill, (2007).

Attention has shifted to identifying specific susceptibility genes and their specific functions. Recent research has examined if autism is caused by common or rare variants or, what is most likely, by both (Gupta & State, 2007). Gaugler *et al.* (2014) has shown that the heritability of autism is predominantly due to common variants. After the completion of the human genome project and the decrease in the cost of genomic technologies, collaborative efforts were made to uncover the cellular and molecular mechanisms leading to this highly heterogeneous condition.

1.2.6 Autism susceptibility genes

Although autism is thought to be highly heritable (Abrahams and Geschwind, 2008, Beaudet, 2007), no gene has been labelled as 100% penetrant or causative and environmental influences contribute to the severity of the condition (Lyall *et al.,* 2016; Frazier, 2014). It has been shown that with continued support, previously withdrawn individuals can increase social communication skills often becoming high functioning members of society (Gilchrist *et al*., 2001, Field *et al*., 2001). It has recently been hypothesised that the environment can have an impact on the post-transcriptional modifications of autism susceptibility genes (Wong *et al*., 2013). There has also been speculation that exposure to external chemicals during development may be causative for example the chemical *p*-Cresol has been found to be increased in the urine of autistic children (Altieri *et al*., 2011). Previous links to the MMR vaccine have been widely discredited (Farrington *et al*., 2001, Halsey *et al*., 2001; Taylor *et al*., 1999).

Genetically, there are more than 500 identified autism susceptibility genes (ASGs; figure 1.5; Complete list available at the Simons Foundation database; https://gene.safari.org). No single gene is present in more than 1% of people with autism (Devlin and Scherer, 2012). These findings gave rise to the theory that the large number of identified susceptibility genes are part of convergent molecular pathways, that when perturbed, result in the ASD phenotype (Voineagu *et al*., 2011, Geschwind, 2008).

(Voineagu *et al*., 2011) compared microarray data that was generated using samples from post mortem brain tissue of autistic and control samples, to search for differentially expressed genes. There were 444 differentially expressed genes detected between the cortical samples of ASD patients and matched controls. Gene ontology enrichment analysis of this gene set identified multiple categories including those associated with synaptic function, cell adhesion, regulation of cell proliferation and immune and inflammatory response. Furthermore, (Parikshak *et al*., 2013) applied weighted gain co-expression network analysis (WGCNA) to microarray transcriptome data obtained from human brain samples collected throughout development from 8 PCW to 12 postnatal months to identify co-expression modules. Genes within these modules have predicted functional relationships. Using 155 ASD susceptibility genes from the Simons Foundation database (https://gene.safari.org), the group was able to conclude that ASD susceptibility genes are

highly co-expressed during development and map to modules which contain genes that fall within the gene ontology categories synaptic transmission, regulation of neuronal synaptic plasticity, cell adhesion, calcium dependant regulation of synaptic transmission, DNA binding, chromatin remodelling and transcriptional regulation (Parikshak *et al*., 2013). In addition to this finding, genes that link these co-expression modules have transcriptional and translational regulatory functions and include *E74 like factor 1* (*ELF1), Forkhead box protein O1 (FOXO1), Spermidine/spermine N1-acetyltransferase 1B (SAT1B)* and *Fragile X mental retardation 1* (*FMR1) (Parikshak et al., 2013)*.

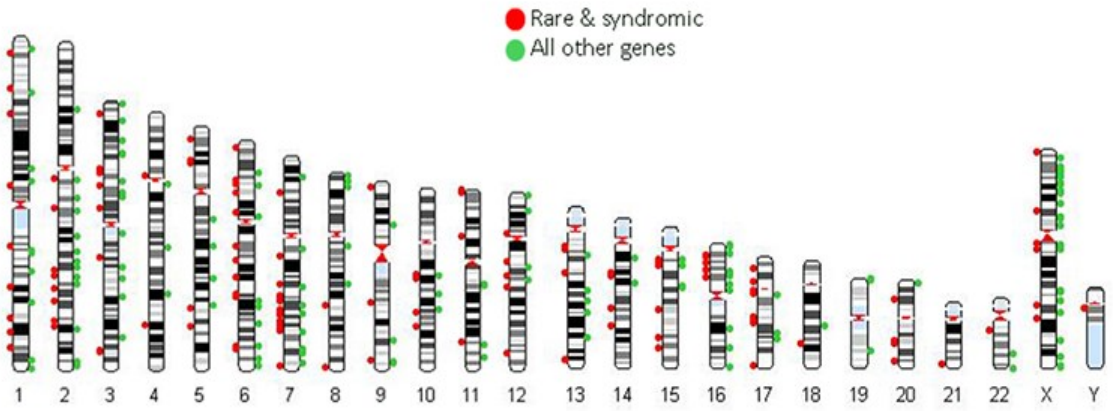


Figure 1.5 Susceptibility genes across chromosomes (Abrahams *et al*., 2013). There are over 500 ASD susceptibility genes identified that are situated throughout the human genome. Some are rare but mutations in these genes usually cause ASD. These are syndromic mutations. Mutations in other susceptibility genes may not be 100% penetrant but are more common.

1.2.4 Transcriptional and translational changes associated with ASD

(Parikshak *et al*., 2013), by mapping ASGs to human developmental co-expression modules, identified that many ASGs encode proteins that fall into the categories of DNA binding, chromatin remodelling and transcriptional and translational regulation. (De Rubeis *et al*., 2014) using exome sequencing to identify ASD risk genes also found an enrichment of transcriptional and chromatin remodelling pathways. Two genes associated with syndromic autism, *Methyl-CpG Binding Protein 2* (*MECP2*) and *FMR1,* which are responsible for transcriptional and translational changes respectively, are discussed further.

Epigenetic marks change the chromatin state and the availability of the DNA for transcription. These marks can be in the form of acetylation and methylation, which enhance and repress transcription respectively. Gene enhancer and promoter regions often contain CpG islands that are highly methylated (Saxonov *et al*., 2006). These epigenetic marks begin to occur in the womb during development and can be the products of both genetics and environment (Feil and Fraga, 2011). RNAs initiate the recruitment of proteins known as writers, which are responsible for adding the epigenetic mark to the DNA whilst readers bind the modified DNA and are able to change the chromatin structure. Many autism susceptibility genes are associated with epigenetic pathways (Zhubi *et al*., 2014b, Lasalle, 2013, Rangasamy *et al*., 2013).

MECP2 mutations are the genetic cause of Rett syndrome (RTT) (Percy, 2008, Amir *et al*., 1999). A large number of people with Rett syndrome are also diagnosed with ASD or show autistic tendencies (Wulffaert *et al*., 2009). The effects of *MECP2* mutations are therefore contributing to the autistic phenotype. MECP2 protein contains both a CPG binding domain (MBD) (Nan *et al*., 1997) and a transcription repression domain (TRD) (Lewis *et al*., 1992), which are essential for its function in binding methylated DNA and repressing transcription (Li *et al*., 2013). MECP2 recognises the methylation marks 5 methyl cytosine (5mC) and 5 hydroxy-methyl cytosine (5hmC) (Mellén *et al*., 2012) and is able to repress gene transcription via interaction with the SIN3A/ HDAC complex which leads to histone deacetylation (Jones *et al*., 1998). Many of the genes repressed have established neuronal functions including *Bone derived neurotropic factor* (*BDNF*)*, Insulin like growth factor binding protein 3* (*IGFBP3*)*, Glutamic acid decarboxylase* (*GAD1*)*, Reelin* (*RELN*)*, Engrailed homeobox 2* (*EN2*)*, Protocadherin* (*PCDH*) *B1* and *PCDH7* (James *et al*., 2014, Miyake *et al*., 2011, Itoh *et*

al., 2007, Chen *et al*., 2003, Martinowich *et al*., 2003). In addition to this, 5hmC is enriched in genes involved with synaptic function in mouse and human brain (Khare *et al*., 2012).

ASD susceptibility genes *GAD1* and *RELN* were shown to have increased 5hmc marks at their promoters in cells of the cerebellum of autistic patients. This resulted in increased binding to these regions by MECP2 and subsequently their repression (Zhubi *et al*., 2014a). GAD1 is an enzyme that catalyses the production of the inhibitory neurotransmitter GABA (Roberts and Frankel, 1950) and RELN protein is essential for neuronal migration, cell positioning and synaptic plasticity (Lakatosova and Ostatnikova, 2012). The receptor for the neurotransmitter oxytocin (*OXTR*) is another autism susceptibility gene that has roles in social recognition, anxiety and depression (LoParo and Waldman, 2015). Autistic individuals show higher *OXTR* promoter methylation compared to controls which corresponds with a decreased expression of oxytocin in the temporal lobe (Gregory *et al*., 2009).

Another monogenetic syndrome that shows a high prevalence of autism is Fragile X syndrome (Kaufmann *et al*., 2004, Brown *et al*., 1982). Fragile X syndrome arises from mutations in the *FMR1* gene which codes for FMRP. FMRP is an RNA binding protein and acts as a repressor of translation (Li *et al*., 2001, Siomi *et al*., 1993, Ashley *et al*., 1993). In the mouse brain, using immunoprecipitation followed by microarray analysis of mRNAs bound to the protein complex, FMRP was shown to bind about 4% of mRNA transcripts including that of the *post synaptic density protein 95* (*PSD95*) (Brown *et al*., 2001). Loss of function drosophila mutants present with enlarged synaptic terminals and altered neurotransmission (Zhang *et al*., 2001). In line with this synaptic malfunction, knockout mice and Fragile X patients exhibit delayed maturation of dendritic spines (Irwin *et al*., 2001, Nimchinsky *et al*., 2001, Comery *et al*., 1997).

1.2.4 Converging signalling pathways

In addition to shared molecular functions of ASGs, some of these susceptibility genes code for proteins that are key components of molecular signalling pathways. Multiple signalling pathways have been shown to be affected in ASD patients including the Canonical Wnt pathway and mTOR signalling. Wnts are lipid-based molecules that act locally to activate Frizzled receptors (FZR) (Wodarz and Nusse, 1998). The canonical Wnt pathway, also referred to as the Wnt/β-Catenin pathway, causes β-catenin to accumulate in the cytoplasm

before it is transferred to the nucleus to act as a coactivator of T-cell factor/ lymphoid enhancer factor (TCF/LEF) family transcription factors. If signalling is not activated, cytoplasmic β-catenin is broken down by a destruction complex (reviewed in Komiya and Habas, 2008). The destruction complex contains the proteins Axin, adenomatous polyposis coli (APC), GSK-3, CK1, protein phosphatase 2A (PP2A) and CK1α (See Stamos and Weis, 2013 for a review of β-catenin degradation via the destruction complex). Dishevelled (DVL) transfers the signal from the activated receptors to the destruction complex. Dickkopf related protein (DKK) is able to antagonise wnt signalling by inhibiting the low density lipoprotein co receptors (LRP) 5/6 (Pinson *et al*., 2000, Wehrli *et al*., 2000, Fedi *et al*., 1999, Glinka *et al*., 1998). A schematic of canonical Wnt signalling can be seen in figure 1.6.

Multiple genes within this pathway and upstream and downstream of this pathway, have been identified as ASGs including *WNT2*, *APC* of the destruction complex, the *hepatocyte growth factor receptor* (*MET*), *PTPRZ* and *engrailed 2* (*EN2*)(Wang *et al*., 2008, Zhou *et al*., 2007). These are shown in figure 1.6. Mutations in the 7q31-33 chromosome region, which contains the *WNT2* gene, have been identified as conferring a susceptibility to ASD (Chien *et al*., 2011). WNT2 mutations were also identified by sequencing the gene in 135 autistic individuals and 160 matched controls (Wassink *et al*., 2001). However, the association between *WNT2* and ASD was not observed in a later study from (McCoy *et al*., 2002) in which 217 families were tested for this genetic association suggesting that, like many of the other ASGs, *WNT2* mutations are rare in ASD.

A chromosome deletion and resultant deletion of the APC gene was found in a patient diagnosed with ASD (Barber *et al*., 1994) making APC deletion a potential risk factor for the condition. A more recent study, that screened for single nucleotide polymorphisms (SNPs) in the APC gene of 177 people with autism and 476 controls, identified an association between APC gene and ASD, with a significantly higher number of people with autism showing sequence variation in the gene compared to the control group (Zhou *et al.,* 2007). A further analysis of 75 individuals with ASD compared with the same control population as before revealed the same result, with a significantly higher number of people with autism carrying the 8636C>A variant compared to the control population (Zhou *et al.,* 2007).

Hepatocyte growth factor receptor (HGFR/ MET) associates with βcatenin at the cell membrane and acts to regulate cell proliferation. βcatenin phosphorylation by MET causes it translocation to the nucleus (Monga *et al*., 2002). An association study carried out in 204

autistic individuals and their families showed that variation in the MET gene promoter, which decreases functionality of the protein by 2 fold, confers a risk to autism (Campbell *et al*., 2006). Additional association studies have supported this finding (Thanseem *et al*., 2010, Campbell *et al*., 2009, Jackson *et al*., 2009). A decrease in the MET protein was also observed in the mothers of autistic children (Heuer *et al*., 2011). This decrease in MET protein was linked to the presence of maternal antibodies that react with proteins in the brain of the fetus, presumably causing the characteristics of ASD.

Downstream EN2, which plays multiple roles in neural development, is a direct target of Wnt signalling, containing three TCF/LEF sites in its promotor region (Sgado *et al*., 2006, Simon *et al*., 2001, McGrew *et al*., 1999). Screening for SNPs in 167 people with autism and their families identified that two of those investigated (rs1861972 and rs1861973) were significantly associated with the condition (Gharani *et al*., 2004). A follow up study, in which two additional datasets were screened, identified that the same SNPs in *EN2* were significantly associated with ASD (Benayed *et al*., 2005). Additional studies have confirmed an association between variation in the EN2 gene and the autistic phenotype (Yang *et al*., 2008, Wang *et al*., 2008). (Zhong *et al*., 2003) found no association between an SNP in exon 1 of EN2 and the ASD phenotype in the 204 families screened suggesting that not all EN2 mutations confer a risk to the condition. However, since most ASG mutations are rare, the possibility that there may be association at this locus should not be ruled out.

There are various links proposed between abnormal mTOR signalling and ASD. Knockout of *Tsc2* and *Fmr1* in mice leads to an overactive mTOR pathway (Sharma *et al*., 2010, Zeng *et al*., 2011). In humans, these mutations lead to tuberous sclerosis and Fragile X syndrome respectively - conditions that both show a high prevalence of ASD (Hagerman *et al*., 2010, Harrison and Bolton, 1997).

Mammalian target of rapamycin (mTOR) is a key protein in both the mammalian TORC1 and TORC2 complexes. These are key complexes in nutrient signalling that are rapamycin sensitive and insensitive respectively. The signalling pathways are activated by various cellular nutrients and stressors and regulate cell growth, proliferation, survival and cap dependant translation (reviewed in Sawicka and Zukin, 2012). The mTORC1 complex usually associated with autism, is able to sense and respond to insulin, growth factors, amino acids, oxidative stress and mechanical stimuli (reviewed in Bond, 2016) and its activation results in protein translation.

A downregulation of AKT/ mTOR signalling has been observed in idiopathic autism. Proteins that participate in mTOR signalling, including mTOR, P13K, AKT and eIF4B, are decreased in the fusiform gyrus of autistic individuals (Nicolini *et al*., 2015). Proteins downstream of mTOR signalling, PSD-95 and TrkB, are also reduced in these individuals. Rats, at age P35-P38, exposed to valproic acid show a decrease in expression of genes coding for mTOR signalling proteins as well as a decrease in the abundance of these proteins in the temporal neocortex (Nicolini *et al.,* 2015). Human fetal exposure to valproic acid increases the risk of developing autism (Christensen *et al*., 2013), potentially by reducing mTOR gene and protein expression levels.

The mTOR proteins affected in individuals with autism are part of the mTOR/P13K/AKT signalling pathway. In this pathway, the tyrosine kinase receptor (TKR), after binding its ligand, stimulates P13K to phosphorylate PIP2. PIP3 can then recruit AKT to the membrane, which is phosphorylated and activated by PDK1 (figure 1.7). Once activated, AKT can activate mTOR signalling (Rafalski and Brunet, 2011). The inhibition of TSC2 by AKT prevents the inhibition of Rheb. If active, Rheb can then stimulate the phosphorylation of mTOR (Inoki *et al*., 2003) which promotes translation via eIF4B (figure 1.7). EGF, shh, IGF1 and insulin have been shown to enhance this pathway (Ojeda *et al*., 2011, Rafalski and Brunet, 2011, Peltier *et al*., 2007) whereas PTEN and GSK3B have been shown to inhibit it (Wyatt *et al*., 2014, Ojeda *et al*., 2011, Peltier *et al*., 2007). P13K/Act/mTOR signalling is essential for the proliferation, growth and migration of neural stem cells (Zhou *et al*., 2011, Groszer *et al*., 2001). Tying in with the synaptic pathophysiology of autism, the mTOR/P13/AKT pathway is required for synaptogenesis and synaptic plasticity (Enríquez-Barreto *et al*., 2014, Martin-Pena *et al*., 2006).

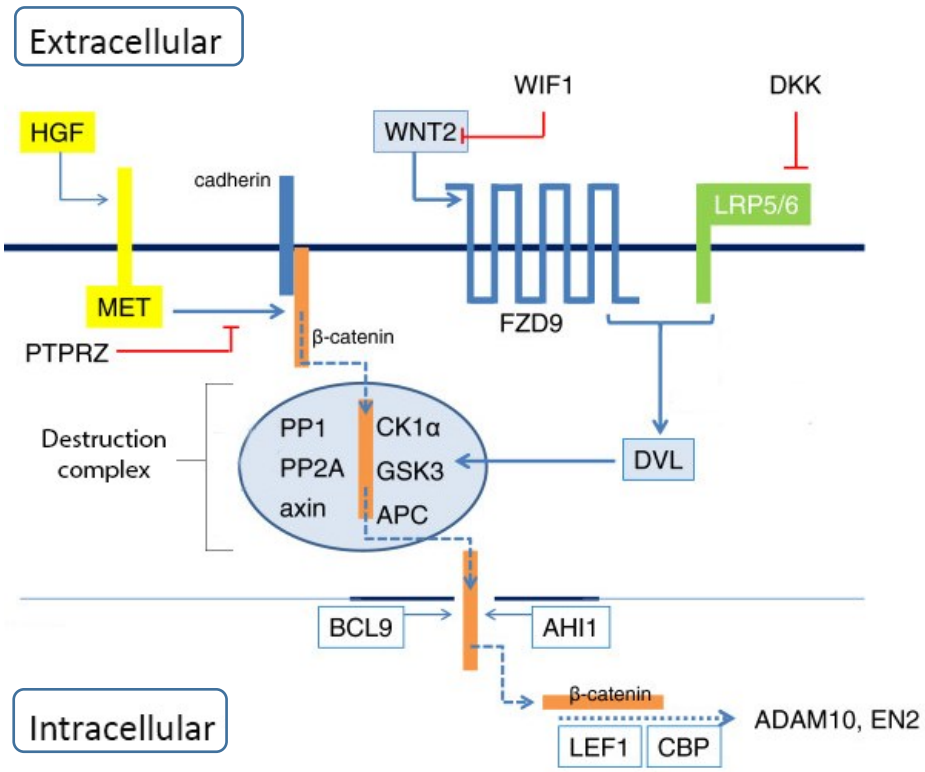


Figure 1.6 Canonical Wnt signalling (adapted from Kalkman *et al.*, 2012). WNT proteins are ligands for Frizzled (FZD) receptors. Dishevelled (DVL) protein relays signals from the receptor ligand complex, to downstream effectors. Wnt signalling can prevent the proteolysis of βcatenin by the destruction complex. The destruction complex is made up of PP1, PP2A, axin, CK1α, GSK3 and APC. βcatenin, once safe from the destruction complex, translocates to the nucleus to bind TCF/LEF proteins, which in turn activate the expression of target genes including EN2. βcatenin phosphorylation by MET *al*so causes its translocation to the nucleus.

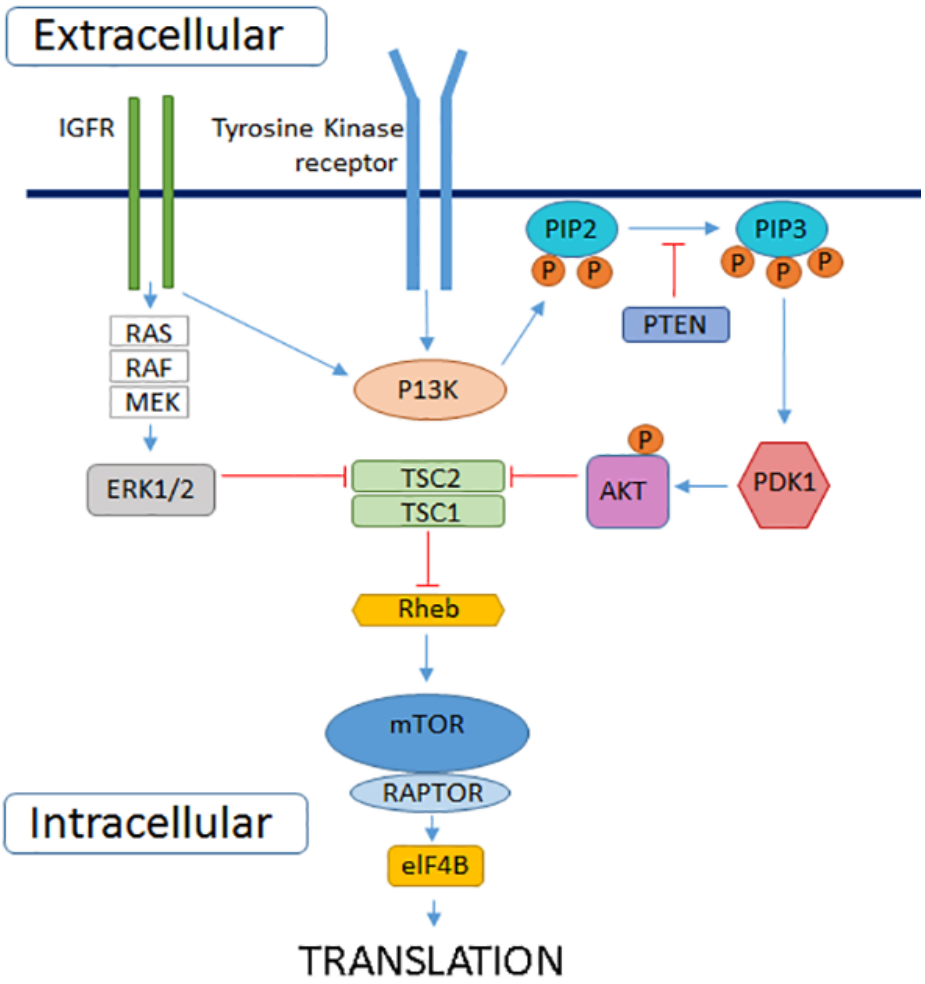


Figure 1.7 mTOR/P13K signalling. Activation of the IGF and tyrosine kinase receptor can stimulate P13K. P13K, via the phosphorylation of PIP2 at the cellular membrane, triggers PDK1 to phosphorylate AKT and inhibit TSC2. When TSC2 is inhibited, it cannot act to inhibit Rheb. Activated Rheb activates mTOR, which is part of the mTORC1 complex that includes the protein RAPTOR. This complex activates eIF4B, which initiates translation of target genes. ERK1/2 can also activate this pathway via the inhibition of TSC2. PTEN inhibits this pathway by dephosphorylating PIP3.

1.3 The synaptic pathophysiology of ASD

The transcriptome analysis studies mentioned in section 1.2.1 identified that genes involved in synaptic function, synaptic plasticity and synaptic transmission are greatly enriched in autism susceptibility gene lists. The synaptic pathophysiology of autism is complex as neuronal cells require specific combinations of synaptic proteins at specific time periods for neurite outgrowth, pathfinding, developing synaptic connections, correct neurotransmitter vesicle assembly and transport, correct neurotransmitter release the correct interpretation of the signal. Perturbations in any of these processes can disrupt cortical circuitry and interfere with the correct functioning of the human brain (Dawson *et al*., 2005).

Magnetic resonance imaging (MRI) can be used to compare the grey and white matter tracts in the brains of autistic individuals to the brains of neurotypical individuals. Functional MRI studies have been used extensively to monitor the level of neuronal pathway activation when performing various tasks. Currently, two contradictory theories regarding the connectivity of the autistic brain exist, the first being the underconnectivity theory (Just *et al.,* 2004; Just *et al.,* 2007). Multiple studies have shown a decrease in functional connectivity between anterior and posterior brain regions (Koshino *et al.,* 2008; Kana *et al.,* 2006). However, a more recent study has suggested that autistic brains may have an increased number of connections between the anterior and posterior regions (Keehn *et al.,* 2013). In addition to abnormal long-range connections, there has been reports of both decreased and increased local connectivity. Using diffusion tensor imaging (DTI) data Shukla *et al.,* (2011) showed abnormal short range connectivity in the frontal, parietal and temporal lobes of ASD children and fMRI studies have reported increased short range connectivity in the frontal and occipital lobes of children with ASD (Solso *et al.,* 2016; Keehn *et al.,* 2013; Shih *et al.,* 2010). Differences in the observations can be due to a number of things including methodology, technical equipment used, sample size, the task being measured and the variability of the symptoms within the group.

It is important that we understand how genetic mutations may affect brain connectivity and to do this we must first understand the functions of these synaptic autism susceptibility genes. A subset of ASGs present in the synaptic modules, and hypothesised to have an effect on the connectivity of the human brain, are discussed below in more detail. The *NRXNs, NLGNs* and *SHANK* genes have been at the forefront of the synaptic pathophysiology theory (reviewed in Baudouin, 2014).

1.3.1 NRXN and NLGN structure and function

There are three identified *NRXN* genes (*NRXN1, 2 & 3*) and five *NLGN* genes (*NLGN1, 2, 3, 4X &4Y*) in humans. Neurexin (NRXN) and neuroligin (NLGN) proteins are associated with the maturation and maintenance of synapses. Overexpression of *NRXN* and *NLGN* genes causes an increase in synapse number (Krueger *et al*., 2012). Expression of *NRXN*s or *NLGN*s alone is enough to provoke synapse formation in the pre and post synaptic membranes. (Graf *et al*., 2004, Scheiffele *et al*., 2000). Neurexins (NRXNs) were first identified as mediators of neurotransmitter release after being induced to do so by binding α latrotoxin, which is found in Black Widow spider venom (Ushkaryov *et al*., 1992). They are abundantly expressed in adult neural tissue and are not only involved in neurotransmitter release but also recruit pre-synaptic density proteins to the membrane and link the pre and post synaptic membranes via their interaction with neuroligins (NLGNs) which are situated in the post-synaptic cell membrane (figure 1.8). However, NRXN proteins have also been identified in the post-synaptic density, binding to NLGN1 to inhibit its binding to pre-synaptic NRXNs (Taniguchi *et al*., 2007). NLGN1 protein is localised to the post synaptic density of excitatory synapses in the hippocampus of rats (Song *et al*., 1999) while NLGN2 protein is found at the post synaptic membrane of mainly inhibitory synapses in rat retinal cell culture (Hoon *et al*., 2009). The other NLGN proteins have been found in both although their presence may differ across brain regions (Krueger *et al*., 2012). Therefore, NRXN-NLGN interactions may be more complex than we first thought.

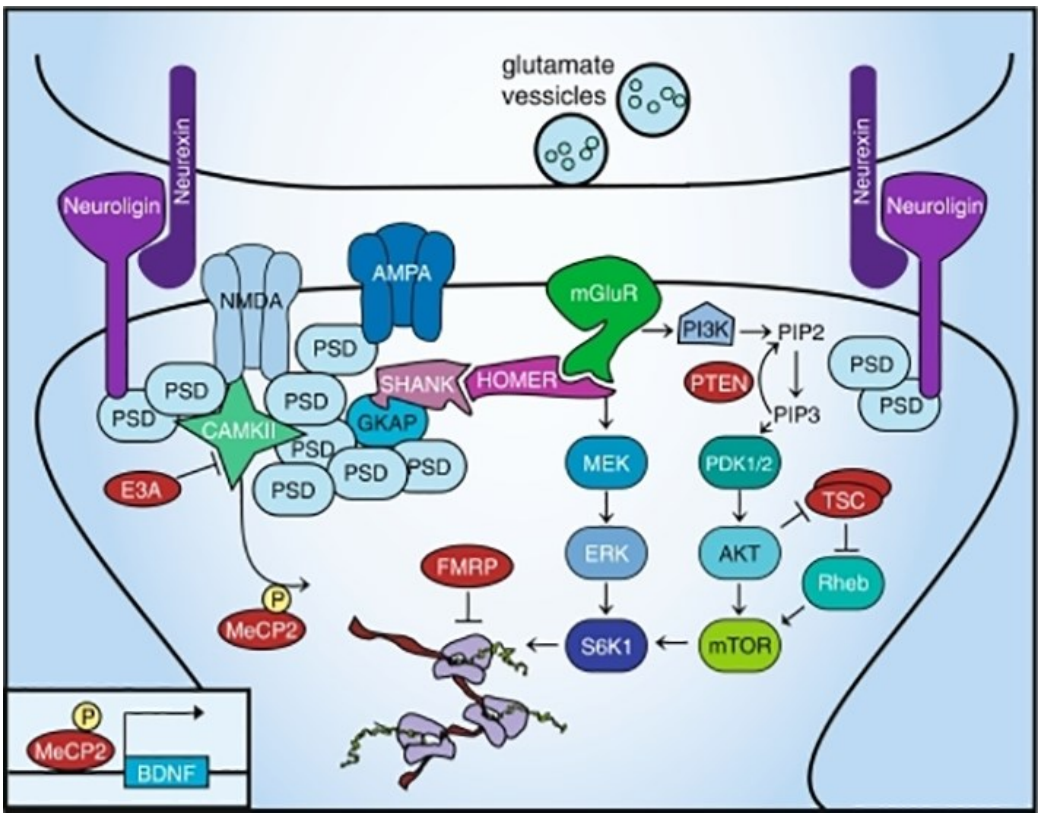


Figure 1.8 Neurexins and Neuroligins link the pre and postsynaptic membranes (Dölen and Bear, 2009). Neurexins are situated at the presynaptic membrane and binds neuroligins that are situated in the postsynaptic membrane. Neuroligins are able to bind post synaptic density proteins and recruit additional proteins such as SHANKs. Through this network of scaffold proteins, NLGNs make molecular links with neurotransmitter receptors such as NMDA, AMPA and mGluR. mGluR can activate the mTOR pathway.

1.3.2 Alternative splicing of NRXN genes in development and adults

The *neurexin* genes are some of the largest in the human genome reaching up to 1.62Mb (Tabuchi and Südhof, 2002). It is therefore, no surprise that these genes are differentially spliced into thousands of isoforms (Missler and Südhof, 1998). Each *neurexin* gene codes for a longer *α* and shorter *β* transcript (figure 1.9). The *α* transcript contains six laminin/neurexin/sex-hormone binding globulin (LNS) domains and three epidermal growth factor (EGF) - like domains. In comparison, the *β* transcripts contain no EGF- like domains and only the sixth LNS domain (figure 1.9). These transcripts are transcribed from separate promoters. There are six sites of differential splicing identified in *α neurexins* (SS#1-SS#6),

two of which are also identified in *β neurexins* (Sugita *et al*., 2001a, Serova *et al*., 2015). α-*NRXNs* seem to be conserved between invertebrates and vertebrates but this is not the case for *βNRXNs* and *NRXN* alterative splicing which are specific to vertebrates (Tabuchi and Südhof, 2002). As organisms became more complex, it is possible that the gene family evolved allowing the acquisition of more complex functions. The addition of a second promoter site into the gene, the generation of the βNRXNs, the resulting interactions with neuroligins and the thousands of isoforms generated from alternative splicing could have contributed to the complexity of the human brain (Tabuchi and Südhof, 2002). This information makes the *NRXN* family of genes, and those genes that regulate their splicing, interesting candidates for studying during human brain development.

α –neurexins are involved in Ca2+ dependent neurotransmitter release and are not essential for synapse formation (Missler *et al*., 2003). It is thought that mainly the βNRXNs bind to NLGN proteins via their extracellular domain to connect the pre and post synaptic membranes, however, certain splice variants of α NRXNs can bind NLGNs (Boucard *et al*., 2005). The intracellular PDZ domain of β NRXNs recruits PDZ domain proteins to the pre-synaptic membrane. These events are regulated by alternative splicing of the neurexin genes (Ichtchenko *et al*., 1995, Ichtchenko *et al*., 1996, Sugita *et al*., 2001a).

The STAR family of proteins includes KHDRBS1, KHDRBS2 and KHDRBS3, also referred to as SAM68, SLM1 and SLM2 respectively. They contain an SH3 binding domain as well as a KH domain that enables the proteins to bind RNA. *Neurexin* mRNA has been identified as a target of the STAR proteins and when bound to the *NRXN* mRNA, they regulate its alternative splicing.

KHDRBS1 has been shown to be widely expressed throughout the brain in a variety of cell types (Iijima *et al*., 2011). This protein includes an extra 96 amino acid sequence at its N terminal that is not found in KHDRBS2 and three (Iijima *et al*., 2011). KHDRBS2 protein can be found in a restricted number of cells in layers 2, 3 and 5 of the adult mouse cerebral cortex as well as in the Purkinje cells of the cerebellum (Iijima *et al*., 2014). KHDRBS3 is found in the majority of cortical cells but is absent from the neurons of the superior and inferior colliculus. It is also present in the inner granular and the molecular layers of the cerebellum (Iijima *et al*., 2011). KHDRBS1 and 2 can form protein complexes, however, there is no evidence to suggest that KHDRBS1 and 3 form a complex (Iijima *et al*., 2011).

In mice, all of the KHDBRS proteins interact with *Nrxns 1* and *3* and prevent the inclusion of the AS4 splice site corresponding to exon 20. SLM1 and 2 also cause the exclusion of exon 20 in *Nrxn2* mRNA, however, SAM68 has no effect on the alternative splicing of *Nrxn2* (Ehrmann *et al*., 2013, Iijima *et al*., 2011). There is also a higher level of exon skipping in the cerebral cortex compared to the rest of the brain (Ehrmann *et al*., 2013). The inclusion or exclusion of exon 20 of NRXNs can consequently affect what proteins they can bind. NRXN1 requires the exclusion of exon 20 in order to bind NLGN1 (Dean *et al*., 2003, Boucard *et al*., 2005, Chih *et al*., 2006) whereas, it requires the inclusion of exon 20 to be able to bind the CBLN1:GLUD2 complex (Uemura *et al*., 2010, Matsuda and Yuzaki, 2011).

Slm1 knockout mice are viable and fertile and show no behavioural abnormalities (Iijima *et al*., 2011). *Slm1/Sam68* knockout mice and *Sam68*$^{-/-}$ male mice are viable but not fertile (Iijima *et al*., 2011, Huot *et al*., 2012). No alterations in the distribution of synaptic markers was detected in the double knockout mice but their brains were slightly smaller than those of the wildtype mice. There was also a loss of a cerebellar fissure and a disorganised purkinje cell layer (Iijima *et al*., 2011). In E13.5 mice, SLM2 was shown to be present in the CP of the cortex and absent from the proliferative regions (Ehrmann *et al*., 2013). There is also evidence of SLM2 regulated exon skipping in NRXNs during mouse development (Ehrmann *et al*., 2013).

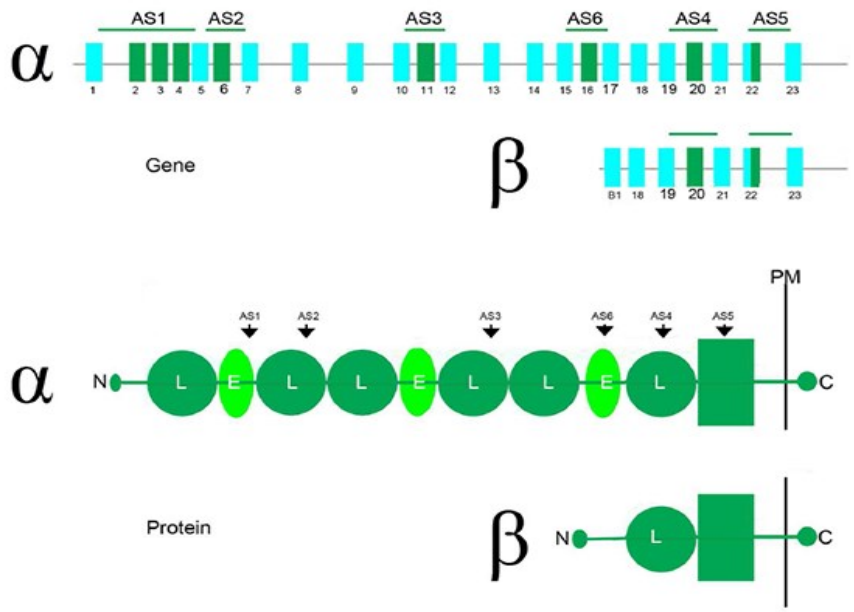


Figure 1.9. Structure of *α* and *βNRXN* genes and their protein products. The *α* and *βNRXN* genes are transcribed from two separate promoters. The longer α transcript contains six splice sites (AS1-6) whilst the β transcript contains only AS4 and AS5. The αNRXN protein contains six laminin G domains, three epidermal growth factor (EGF) domains and a transmembrane region at its C terminal end containing a PDZ recognition motif. The βNRXN protein is much shorter sharing only the last laminin G domain and the transmembrane region.

1.3.3 NRXN and NLGN mutations in ASD

As previously mentioned, autism is a heterogeneous condition in which there are many susceptibility genes. Mutations in these genes, although rare, include *NRXNs* and *NLGNs* (reviewed in Buxbaum, 2009). *NLGN3* and *4* mutations were identified in individuals with ASD before *NRXN* mutations. A screen for *NLGN3* and 4X/Y mutations was carried out on 158 individuals with ASD. Single strand conformation polymorphism (SSCP) scanning detected a single base change in the *NLGN3* gene in two brothers with ASD and a frameshift mutation in *NLGN4X* was identified in two unconnected brothers with ASD that were not identified in the 200 and 350 control subjects respectively (Jamain *et al*., 2003). Subsequent studies, however, have failed to identify mutations in these genes in people with autism

suggesting that their occurrence is rare (Gauthier *et al*., 2005, Vincent *et al*., 2004). A study searching for copy number variants (CNVs) that confer susceptibility to ASD genotyped 859 people with autism and 1409 control subjects. There was a significantly higher number of CNVs in NLGN1 of the people with autism compared with controls (Glessner *et al*., 2009). At present, there are no identified *NLGN2* mutations in individuals with ASD, although sequencing identified mutations in this gene in Schizophrenic patients (Sun *et al*., 2011).

After the detection of *NLGN3* and *4X* mutations in individuals with autism, Feng *et al.* (2006) used SSCP scanning to search for mutations within the *βNRXN* genes in 264 individuals with ASD in addition to 535 controls. Mutations were identified in the *NRXN1β* gene of four individuals with ASD compared to none of the controls. A later study using a single nucleotide polymorphism (SNP) array to look for linkage and CNVs in over 1000 families who had 2 or more members with ASD, identified two female siblings with a deletion in the coding region of *NRXN1* (Szatmari *et al*., 2007). Mutations in *NRXN1α* as well as β have been identified in autistic individuals (Yan *et al*., 2008, Kim *et al*., 2008). A study that sequenced the *NRXN* genes of 142 individuals with autism and 92 controls using the 3730XL DNA analyser system, found a frameshift mutation in exon 12 of *NRXN2* in 1 of the subjects with ASD and none of the controls (Gauthier *et al*., 2011). These previous studies had failed to identify *NRXN3* mutations in ASD cases. However, in 2012, mutations in exons 1 and 10-13 as well as missense mutations of the *NRXN3* gene in individuals with ASD were uncovered (Vaags *et al*., 2012). Although mutations in the NRXN and NLGN genes confer a susceptibility to autism, the mutations are not always fully penetrant and unaffected family members may carry the same mutations (Kim *et al*., 2008, Cukier *et al*., 2014, Vaags *et al*., 2012).

The closely related *CNTNAP2/ NRXN4* gene was found to contribute to the condition also (Alarcon *et al*., 2008, Strauss *et al*., 2006, Arking *et al*., 2008). A mutation in this gene causes the condition cortical dysplasia-focal epilepsy (CDFE) and is one of syndromes in which many of the individuals also exhibit autism. It is believed to be caused by abnormal neuronal migration with almost half of CDFE patients showing cortical dysplasia in MRI analysis. This syndrome, like autism, becomes apparent in early childhood when seizures, hyperactivity, language delay and mental retardation develop. Since its discovery, *CNTNAP2* has been linked to age at first word by association studies (Alarcon *et al*., 2008), and to language delay and stuttering in CNTNAP2 haploinsufficient people with autism carrying a deletion in the 7q

33-35 region (Petrin *et al*., 2010, Sehested *et al*., 2010). A summary of *NRXN* and *NLGN* mutations in humans can be seen in table 1.1.

1.3.4 SHANK structure and function

The *SHANK* gene family consists of three genes (*1, 2* and *3*) which function as scaffold proteins at the post synaptic density (PSD) of excitatory synapses in the brain (Naisbitt *et al*., 1999, Tu *et al*., 1999, Boeckers *et al*., 2002) (figure 1.8). These proteins consist of an SH3 domain, a PDZ domain, a SAM domain, ankyrin repeats and a proline rich area (Sheng and Kim, 2000). In rats, *SHANK1* and *SHANK2* are expressed throughout the brain whereas *SHANK3* is more highly expressed in the heart with lower levels in the brain (Lim *et al*., 1999). In humans, *SHANK* expression has been observed in the brain, digestive tract and liver (www.proteinatlas.org).

Before accumulating at synaptic junctions, SHANK can be found in developing neurites (Du *et al*., 1998, Naisbitt *et al*., 1999). SHANK was first identified by the binding of its PDZ domain to the scaffold protein GKAP.This interaction is thought to be important in the localisation of SHANK to the postsynaptic membrane (Sheng and Kim, 2000). The presence of SHANK in developing neurons and tissue other than brain suggests that it may also additional functions for example in cell migration, cytoskeletal organisation and neurite outgrowth in non-neuronal and neuronal cells (Du *et al*., 1998, Naisbitt *et al*., 1999, Sheng and Kim, 2000).

The proline rich region of the SHANK proteins has been shown to bind the scaffold proteins Homer and contactin (figure 1.8). The SAM domains at the C terminus of each protein may allow SHANKS to bind one another creating an increased number of available domains for PSD and scaffold protein binding (Sheng and Kim, 2000). The SHANK transcripts have also been shown to be alternatively spliced during development (Lim *et al*., 1999, Boeckers *et al*., 2002). This likely affects the binding of other proteins to SHANK proteins and therefore, shapes the structure of the PSD complex.

1.3.5 SHANK mutations in ASD

SHANK1 mutations in autistic cases have only been identified recently (Sato *et al.,* 2012). Deletions in the SHANK1 gene affecting the first 20 exons were common between three related individuals of different generations and one un-related subject. The same *SHANK1* mutations were found in two females of the first family but they did not present with ASD characteristics (Sato *et al*., 2012). Further genotyping of 509 ASD and Intellectual disability (ID) subjects identified 23 and 7 missense variants respectively. Some of the fathers of the autistic individuals also shared the SHANK1 mutations without consequence. Although these mutations are found in non-autistic individuals, they are only present in families in which some members suffer from ASD. In both experiments, none of the controls have SHANK1 mutations. This suggests that *SHANK1* is an autism susceptibility gene but, like *NRXN* and *NLGN* genes, is not completely penetrant.

A study looking for links between ASDs and mental retardation identified deletions affecting the PDZ domain of *SHANK2* as well as several variants in the gene that are not observed in non-affected control individuals (Berkel *et al*., 2010). Individuals with deletions of the 22q13 chromosomal region, also known as Phelan McDermid syndrome, present with social deficits and many are diagnosed with ASD (Oberman *et al*., 2015). Durand *et al*., (2007) and Waga *et al*., (2014) directly sequenced *SHANK3* in ASD patients and identified multiple mutations including a deletion breakpoint in intron 8, an insertion in exon 21 a deletion upstream of the SH3 domain, a missense mutation of the PDZ domain and a mutation downstream of exon 11. In addition, a 22q deletion was observed that resulted in a chromosomal rearrangement that affected 25 genes including *SHANK3*. These mutations were not present in the control group. Since not all of the 22q13 genomic region was sequenced, additional mutations may contribute to the autistic phenotype in these individuals (Durand *et al.,* 2006; Waga *et al.,* 2011). Despite this evidence for *SHANK2* and *3* mutations conferring a risk to ASD, one study found no association between mutations in this gene and the condition (Liu *et al*., 2013). A summary of *SHANK* mutations in humans can be seen in table 1.1.

Table 1.1 Human *NRXN, NLGN* and *SHANK* gene mutations in ASD cases

Gene	Mutations	References
NRXN1	*NRXN1β* mutations in 4 individuals with ASD Deletion in the coding region of *NRXN1* in two female siblings	(Szatmari *et al*., 2007)
NRXN2	*NRXN2* frameshift mutation in exon 12 in one individual with ASD	(Gauthier *et al.,* 2011)
NRXN3	*NRXN3* mutations in exons 1 and 10-13 as well as missense mutations of the *NRXN3* gene in individuals with ASD	(Vaags *et al.,* 2012)
NLGN1	CNVs in *NLGN1* in people with autism	(Glessner *et al*., 2009)
NLGN2	No known mutations in ASD	N/A
NLGN3	Single base change in the *NLGN3* gene in two brothers with ASD	Jamain *et al.,* 2003
NLGN4X	Frameshift mutation in *NLGN4X* was identified in two unconnected brothers with ASD	Jamain *et al.,* 2003
SHANK1	Deletions in the SHANK1 gene affecting the first 20 exons found in three related individuals of different generations and one un-related subject	(Sato *et al.,* 2012)
SHANK2	Deletions affecting the PDZ domain of *SHANK2*	(Berkel *et al.,* 2010)
SHANK3	Deletions of the 22q13 chromosomal region Deletion breakpoint in intron 8 An insertion in exon 21 a deletion upstream of the SH3 domain A missense mutation of the PDZ domain A mutation downstream of exon 11.	(Oberman *et al.,* 2015) (Durand *et al.,* 2007) (Waga *et al.,* 2014)

1.4 Rodent and cellular models of ASD

Mouse models can be viewed as a high throughput system as they breed well in captivity and are relatively easily genetically manipulated. Models of single gene mutations or specific genetic loci changes can be produced to monitor the effects of these mutations in vitro (Halladay *et al.,* 2009). Both behavioural and molecular analysis can be carried out which is rarely possible in humans, unless high quality post mortem tissue is obtained. The genetic variability of mouse models can be decreased by using particular strains. However, different strains of mice exhibit differences in behaviours and so for behavioural analysis it is important to utilize the appropriate strain (Moy & Nadler, 2008).

Mouse models of autism display core symptoms of the disorder, which are impaired social interaction and repetitive and stereotyped behaviour. Mice exhibit complex social behaviours, which enables detailed behavioural phenotyping. Some of the behaviours analysed in testing include aggressive behaviours, juvenile play, social approach, response to a strange versus a familiar mouse, reciprocal social interactions, anxiety, fear, mating and rearing of young. Repetitive behaviours analysed include marble burying and stereotyped behaviours such as self-grooming, circling and back flipping (Silverman *et al.,* 2010).

Following the identification of *NRXN*, *NLGN* and *SHANK* mutations in ASD patients, multiple mouse models have been created. Here, the molecular and behavioural phenotypes of these mice will be discussed as well as the generation of cellular models using human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hIPSCs).

1.4.1 NRXN knockout mice as a model of ASD

Triple *α-NRXN* knockout mice and double knockouts have severely reduced survival rates with many unable to survive past day one despite apparently normal prenatal development (Missler *et al*., 2003). Triple knockouts were anatomically identical to wildtype with no differences in brain weight, lamination or synaptic protein distribution. The density of GABAergic synapses was decreased in the brainstem of triple knockout mice and the neocortex of double *NRXN1α/ 2α* knockout mice. It was also shown that αNRXNs are essential for calcium triggered neurotransmitter release. Cultured hippocampal neurons show a decrease in presynaptic Ca^{2+} currents but the expression of Ca^{2+} channels at the cell surface was comparable to controls (Missler *et al.,* 2003). Missler *et al.,* (2003) suggests that

αNRXNs function to link the organisation of presynaptic neurotransmitter secretion to extracellular synaptic events.

A vector designed to recognise the first exon and part of the promoter of *NRXN1α* was inserted into embryonic stem cells to knock down the gene by homologous recombination. Positive clones were then injected into blastocysts and resultant mice were bred to produce homozygous genotypes. *αNRXN1*$^{-/-}$ mice were viable and fertile with no anatomical or phenotype abnormalities and minimal behavioural abnormalities (Geppert *et al*., 1998). The only behavioural effect observed was neglect of their litters causing increased offspring mortality. These findings suggest functional redundancy of the *NRXN* genes, however, the *NRXN1β* transcript may also have been functional (Geppert *et al*., 1998). A later study examining the behavioural phenotype of *αNRXN1*$^{-/-}$ showed there was an increased acoustic startle response, decreased prepulse inhibition (PPI), increased repetitive grooming and impaired nest building. This study found no social impairments of the mice and they performed better than wildtype in the rotarod test exhibiting enhanced motor learning (Etherton *et al*., 2009). Using hippocampal slices and CA1 cell cultures to examine the molecular phenotype they observed a decrease in spontaneous excitatory synaptic transmission and basal excitatory synaptic strength compared with controls. Whole cell voltage-clamp recordings revealed that this was due to a reduced mEPSC frequency that affected the inhibitory/ excitatory balance (Etherton *et al.,* 2009).

NRXN2$^{-/-}$ mice display defects in social behaviour. In the three-chamber test, knockout mice show no preference for the chamber containing an unknown mouse (stranger 1) rather than an empty chamber. When the empty chamber was replaced with a different unfamiliar mouse (stranger 2), wildtype mice spent an increased amount of time with stranger 2 over stranger 1. This was not observed in the *NRNX2* knockout mice (Dachtler *et al*., 2014). Knockout mice also show increased anxiety in the open maze test spending an increased period of time at the periphery compared to wildtype mice (Dachtler *et al.,* 2014). In the elevated plus maze test, knockout mice spend significantly less time in the open arms compared to wildtype (Born *et al*., 2015, Dachtler *et al*., 2014). An emergence test places mice into a dark enclosure before giving them the option of emerging into a well-lit open space. Wildtype mice took significantly less time to emerge into the open space compared to the *NRXN2* knockout mice and spent significantly less time in the open space (Dachtler *et al*., 2014*)*. Molecularly, in contrast to the *NRXN1*$^{-/-}$ mice, *NRXN2* knockout mice have normal

PPI. There is a decrease in the expression of *Dlg4, Grin2a, NMDA receptor subunit 2a and Munc18-1* in the cortex and hippocampus. In a separate study from the same group examining *NRXN2* heterozygous mutant mice, many of the behavioural phenotypes observed in the homozygous mutants were abolished (Dachtler *et al*., 2015). Interestingly, they identified social defects in a *NRXN1* heterozygous mutant mouse.

Homozygous *NRXN3α* and *β* knockout mice have been generated (Aoto *et al.,* 2015) as have *NRXN3* SS#4 inclusion mice (Aoto *et al*., 2013). No detailed behavioural analysis has yet been carried out on these mice. (Aoto *et al*., 2015) generated the *Nrxn3α* and *β* knockout mice by targeting exons 1 and 18. Homozygous mutants mostly died at birth and any surviving mutants were smaller than their littermates and displayed ataxia and hyperactivity. A summary of the physical, behavioural and molecular consequences of *NRXN* mutations can be found in table 1.2.

Table 1.2 *NRXN* knockout mice and their physical, behavioural and molecular consequences.

	Physical	**Behavioural**	**Molecular**
NRXN1	None observed	Neglect of pups (Geppert *et al.,* 1998) Increased acoustic startle response Increased repetitive grooming Impaired nest building Enhanced motor learning (Etherton *et al.,* 2009)	Decreased prepulse inhibition (PPI) Reduced mEPSC frequency (Etherton *et al.,* 2009)

NRXN2	None observed	Social impairments in 3 chamber test (Dachtler *et al.,* 2014) Increased anxiety in open maze, elevated plus maze test and emergence test (Born *et al.,* 2015; Dachtler *et al.,* 2014)	Decrease in the expression of synaptic genes (Dachtler *et al.,* 2014)
NRXN3	Severely reduced survival rates of homozygous mutants Decreased size Ataxia (Aoto *et al.,* 2015)	Hyperactivity (Aoto *et al.,* 2015)	Decrease in mEPSC amplitude Decrease in AMPA mediated synaptic transmission Decrease in mIPSC frequency and the evoked mIPSC amplitude (Aoto *et al.,* 2013)
Triple *NRXN knockout*	Reduced survival rates (Missler *et al.,* 2003)		Decrease in GABAergic synapse density (Missler *et al.,* 2003) Decrease in presynaptic Ca^{2+} currents (Missler *et al.,* 2003)

This is the first generated mouse model that has abolished the functions of both the α and β NRXNs and the effect is more severe than any single knockouts (Born *et al*., 2015, Dachtler *et al*., 2015, Dachtler *et al*., 2014, Etherton *et al*., 2009, Missler *et al*., 2003, Geppert *et al*., 1998). Hippocampal cultures from these homozygous knockout mice and observed a decrease in amplitude of mEPSCs, which was also observed in a *Nrxn3* mouse that was engineered to include SS#4 (Aoto *et al*., 2013). Post synaptic AMPAR mediated synaptic transmission is decreased as are the surface levels of GlUA1 and GLUA2 in hippocampal cell cultures and in adult hippocampus of homozygous mutants. This study also observed that different brain regions can have different molecular phenotypes caused by the mutation.

Olfactory bulb cell cultures showed no change in the mEPSCs or AMPAR mediated synaptic transmission of homozygous knockouts compared to wildtype cultures. Instead mIPSC frequency is decreased as is the evoked mIPSC amplitude and this phenotype is independent of SS#4 exclusion.

1.4.2 NLGN knockout mice as a model of ASD

Single *NLGN* knockout, double *NLGN* knockout combinations and triple knockout mice have been generated by (Varoqueaux *et al*., 2006). All mutants, except homozygous triple knockouts, were viable and fertile but showed reduced rates of reproduction suggesting social deficits. Homozygous triple knockout mice died soon after birth. Synaptic protein levels are reduced in triple *NLGN* knockout mice but this does not appear to effect the formation of synapses. Triple *NLGN* knockout mice show reduced GABAergic and glutamatergic synaptic transmission. (Blundell *et al*., 2010) studied the single *NLGN1* mice in more detail and found that there was an increase in *NLGN3* expression and a decrease in both *α* and *β NRXN*s. There is a reduced NDMA/AMPA ratio in the dorsal striatum of these mice and a reduced magnitude of LTP in the hippocampus. Behaviourally, these mice performed the same as wildtype mice in anxiety testing but did show slight social deficits and repetitive grooming. The Morris water maze task revealed that *NLGN1* knockout mice show deficits in spatial learning and memory. There is also evidence for reduced excitatory synaptic transmission due to a decrease in glutamate receptor proteins in *NLGN1* KO mice and deficits in LTP and synaptic plasticity (Jedlicka *et al*., 2015).

NLGN2 knockout mice experienced both developmental delays and physical differences when compared with wildtype and heterozygous mice. These included reduced body and tail length and delayed eye opening and grasping reflex (Wohr *et al.,* 2013). Isolating pups from their mothers prompted vocalisations, which were reduced in *NLGN2* mice compared to wildtype. *NLGN2* null mice spent less time exploring and in the centre of the open field arena and generally were less mobile in other tests compared to wildtype. Sociability of the null mice appeared to be unaffected. Anxiety testing proved inconclusive as although the null mice appeared to exhibit anxiety like behaviours in the light→dark exploration task, this could have been due to the reduced mobility of the mutants (Wohr *et al*., 2013).

Interestingly, it has been observed that a substitution mutation in *NLGN3* in mice has a different effect to *NLGN3* knockdown in mice. The substitution, which is modelled on an observed mutation in the human genome (R451C; (Jamain *et al*., 2003), results in an increase in inhibitory synaptic transmission in pyramidal neurons of the somatosensory cortex as determined by whole cell recordings. There were also observed social impairments in the substitution mutants that were not observed in the knockout or wildtype mice. The substitution also appeared to enhance spatial learning as shown by the Morris water maze task (Tabuchi *et al*., 2007). Recapitulating this genotype in another study with mice found no ASD associated behavioural abnormalities (Chadman *et al*., 2008). Also, in contrast to the lack of behavioural abnormalities observed in the *NLGN3* knockout mice by Tabuchi *et al.* (2007), the knockout of *NLGN3* exon 5 in rats reduced juvenile play compared to wildtype (Hamilton *et al*., 2014). Mutant rats also exhibited repetitive behaviour in the form of chewing through their water bottles.

Knocking out *NLGN4* in mice did not lead to a compensatory increase of other *NLGN* genes or associated binding partners (Jamain *et al*., 2008). In a separate study, a *NLGN4* mutation results in an increase in the NMDAR subunit, NR1, mGluR5 and the cell adhesion molecule NCAM as revealed by Western blot analysis. It is hypothesised that the upregulation of these proteins is a compensatory mechanism for the loss of *NLGN4.* Patch clamp recordings in single cells from the brains of *NLGN4* knockout mice also reveal effects in both inhibitory and excitatory synapses (Delattre *et al*., 2013). A summary of the physical, behavioural and molecular consequences of *NLGN* mutations can be found in table 1.2

Table 1.3 *NLGN* knockout mice and their physical, behavioural and molecular consequences.

	Physical	**Behavioural**	**Molecular**
NLGN1	Not studied	Deficits in spatial learning and memory shown in Morris water maze task Repetitive grooming (Jedlicka *et al.,* 2015)	Increase in *NLGN3* expression and a decrease in both *α* and *β NRXNs* Reduced NDMA/ AMPA ratio Reduced LTP magnitude (Blundell *et al.,* 2010) Decrease in glutamate receptor proteins (Jedlicka *et al.,* 2015)
NLGN2	Reduced body and tail length Delayed eye opening and grasping reflex Reduced pup vocalisations (Wohr *et al.,* 2013)	Reduced mobility in anxiety testing (Wohr *et al.,*2013)	Not studied
NLGN3	Not studied	Reduced social interaction with unfamiliar mouse Enhanced spatial learning in Morris water maze task (Tabuchi *et al.,* 2007)	Increased inhibitory synaptic transmission (Jamain *et al.,* 2003)
NLGN4	Not studied	Not studied	Increase in synaptic proteins and cell adhesion molecule NCAM (Delattre *et al.,* 2013)

>1 *NLGN* mutation **(double and triple knockouts)**	Fatal / severely reduced survival rates Respiratory failure (Varoqueaux *et al.,* 2006)	Not studied due to severely reduced survival	Decrease in synaptic proteins Reduced GABAergic and glutamatergic synaptic transmission (Varoqueaux *et al.,* 2006)

1.4.3 SHANK knockout mice as a model of ASD

Shank1 mutant mice have been generated using homologous recombination of a targeting vector to exons 14 and 15 that disrupted the PDZ domain of the gene (Hung *et al.,* 2008). These mice have been used in multiple studies to look for structural, molecular and behavioural differences between mutant and wildtype animals (Silverman *et al*., 2011, Wohr *et al*., 2011, Hung *et al*., 2008). *Shank1*$^{-/-}$ knockout mice show no obvious structural differences in the brain compared to wildtype (Hung *et al*., 2008). Quantitative immunoblotting of PSD fractions obtained from the forebrain of *Shank1*$^{-/-}$ mice revealed a decrease in the PSD proteins GKAP and Homer1b/c compared to wildtype (Hung *et al*., 2008). Immunohistochemistry performed on dissociated hippocampal cell cultures confirmed a decrease in the density of GKAP positive puncta and a more diffuse staining pattern of Homer1b/c (Hung *et al*., 2008). Electron micrographs of hippocampal neurons from *Shank1*$^{-/-}$ knockout mice revealed that they had thinner PSDs and shorter dendritic spines compared to wildtype (Hung *et al*., 2008). Reduced basal synaptic transmission was observed in acute hippocampal slices from *Shank1*$^{-/-}$ mice aged 3-5 weeks presumably due to a reduced number of functional synapses rather than a reduction in synapse number (Hung *et al*., 2008).

Shank1 knockout mice were less active in an open field environment and spent less time in the more open central regions of the field compared to wild type mice (Silverman *et al*., 2011, Hung *et al*., 2008). They were also more reluctant to move between light and dark chambers in the light/ dark exploration test suggesting anxiety like behaviours of *Shank1*$^{-/-}$

mice (Silverman *et al*., 2011, Hung *et al*., 2008). *Shank1*$^{-/-}$ mice bred less and were less nurturing (Hung *et al*., 2008).

Tone shock fear conditioning showed that *Shank1*$^{-/-}$ mice froze less than wildtype mice 24 hours after fear conditioning suggesting a contextual fear memory impairment (Hung *et al*., 2008). However, mutant mice showed enhanced spatial learning uncovered using the eight arm radial maze task in which two of the arms contain food. *Shank1*$^{-/-}$ mice made less mistakes when remembering which of the eight arms to acquire the food from, however, after a 4 week rest period, they performed at the same level as the wildtype mice.

The three-chambered test in which the mouse has access to both a foreign object and a foreign mouse revealed that homozygous and heterozygous *Shank1* mutant mice spent equal time sniffing the object and the mouse compared to wildtype mice who spent more time sniffing the novel mouse (Silverman *et al*., 2011). The inverted wire hang test in which the time a mouse can hang onto an inverted metal grid using all four limbs is measured as an indicator of muscle strength. Shank1-/- mice fell off faster than wildtype mice (Silverman *et al*., 2011). There was also reduced ultrasonic vocalisation in *Shank1*$^{-/-}$ miceand pups took longer to flip from their backs to an upright position compared to heterozygous or wildtype pups (Wohr *et al*., 2011).

Schmeisser *et al.* (2012) generated *SHANK2* knockout mice, with deletions in exons 6 and 7 corresponding to the PDZ domain that had reduced body weight, normal brain morphology and reduced survival compared to wildtype. However, another study using mice with deletions in the same exons showed them to be phenotypically and reproductively normal (Won *et al*., 2012). Won *et al.,* (2012) observed no increase in *SHANKs 1* or *3* expression in response to the *SHANK2* deletion, however, an increase in *SHANK3* expression was observed in the cortex, hippocampus and striatum of P70 mutant mice in the Schmeisser study.

Homozygous mutant mice were shown, using the three-chambered test, to exhibit a reduction in social interaction compared to wildtype (Won *et al*., 2012). The mutant mice also decreased the number of ultrasonic vocalisations when interacting with a novel female and were less efficient in the pup retrieval assay. The Morris water maze assay detected an impairment in spatial learning and memory (Won *et al*., 2012) and mutants showed extended grooming periods. In terms of motor skills, *SHANK2*$^{-/-}$ mice showed hyperactivity in the open field test and show increased anxiety behaviours as identified from the elevated plus maze test and the light and dark test (Schmeisser *et al*., 2012, Won *et al*., 2012).

In the hippocampus of *SHANK2* mutant mice, electron microscopy showed that these mutations in *SHANK2* mice had no effect on synapse number, PSD morphology or on basal synaptic transmission in Schaffer-collateral-CA1 pyramidal (SC-CA1) synapses. However, high and low frequency stimulation revealed that long-term potentiation (LTP) was impaired and long-term depression (LTD) was abolished. The NDMA/ AMPA ratio was reduced in SC-CA1 synapses but not in the medial prefrontal cortex (Won *et al*., 2012). (Schmeisser *et al*., 2012) did not identify any defects in LTD but also identified an increase in the NMDA/ AMPA ratio and a small decrease in basal synaptic transmission. Extracellular field and whole patch clamp recordings of hippocampal pyramidal cells in homozygous and heterozygous mutants show a decrease in field EPSPs and a reduction in mEPSC frequency. There is also a small decrease in spine density and number, and similarly to (Won *et al*., 2012), a decrease in basal transmission.

Multiple SHANK3 mutant mice have been generated with mutations in the Ankyrin repeat domain, mutations in exons 4-9 and mutations in the PDZ domain. Mutant mice are viable and fertile with normal brain morphology but are smaller than wildtype mice and develop lesions on their skin due to excessive grooming (Wang *et al*., 2011, Bozdagi *et al*., 2010, Peça *et al*., 2011). Molecularly, mice show a reduction in basal transmission and a reduced amplitude and frequency of mEPSCs in hippocampal CA1 pyramidal neurons and striatal medium spiny neurons (Peça *et al*., 2011). Immunocytochemistry also shows a decrease in AMPA receptor proteins and impaired spine extension after inducing LTP (Bozdagi *et al*., 2010). Analysis of the PSD extracted from the striatum of *SHANK3* mutants revealed a decrease in PSD associated proteins compared to wildtype (Peca *et al.,* 2010). Mutants have longer dendrites, with a larger surface area although dendritic density is decreased (Peca *et al.,* 2010). Longer dendritic spines were observed in hippocampal slices from *SHANK3* mutants by (Wang *et al*., 2011), although spine density was indistinguishable from wildtype mice.

Behaviourally, *SHANK3* mice show impaired social interaction. Heterozygous males demonstrated less social sniffing and a decrease in the number of ultrasonic vocalisations when interacting with oestrus female mice (Bozdagi *et al*., 2010). In the three-chambered test, *SHANK3* mutants spent more time in the chamber with the empty cage rather than the chamber containing the novel mouse. In the open field test, mutants exhibited less social sniffing. In some cases, there is a difference in the behaviour of male and female mutant

mice. Male mutants are more vocal than wildtype mice when presented with a novel mouse whereas female mutants are less vocal than wildtype mice (Wang *et al*., 2011). A summary of the physical, behavioural and molecular consequences of *SHANK* mutations can be found in table 1.4.

Table 1.4 *SHANK* knockout mice and their physical, behavioural and molecular consequences.

	Physical	**Behavioural**	**Molecular**
SHANK1	None observed	Increased anxiety in open maze, light → dark tests Contextual fear memory impairment in tone shock fear conditioning Enhanced spatial learning in radial maze task (Silverman *et al.,* 2011; Hung *et al.,* 2008) Less active in open field test Decreased muscle strength in wire hang test Reduced social interaction in 3 chambered test (Silverman *et al.,* 2008) Reduced ultrasonic vocalisation (Wohr *et al.,* 2011)	Decrease in the PSD proteins Thinner PSDs and shorter dendritic spines Reduced basal synaptic transmission (Hung *et al.,* 2008)

SHANK2	Reduced body weight Reduced survival (Schmeisser *et al.,* 2012)	Reduced social interaction in 3 chambered test Decreased ultrasonic vocalisations Less efficient in the pup retrieval assay Impairment in spatial learning in Morris water maze task Increased grooming (Won *et al.,* 2012) Hyperactivity in the open field test Increased anxiety behaviours in the elevated plus maze test and the light → dark test (Schmeisser *et al.,* 2012; Won *et al.,* 2012)	Increase in *SHANK3* expression (Schmeisser *et al.,* 2012) Impaired LTP Abolished LTD Both reduced and increased NDMA/ AMPA ratio Decrease in basal transmission (Won *et al.,* 2012; Schmeisser *et al.,* 2012) Decrease in field EPSPs and a reduction in mEPSC frequency Decrease in spine density and number (Schmeisser *et al.,* 2012)
SHANK3	Reduced size Skin lesions	Excessive grooming Impaired social interaction as shown by interactions with oestrous female mouse (Bozdagi *et al.,* 2010) Reduced social interaction in 3 chambered test and open field test (X. Wang *et al*., 2011)	Decrease in AMPA receptor proteins (Bozdagi *et al.,* 2010) Decrease in PSD proteins Reduced basal transmission Reduced mEPSC amplitude and frequency hippocampal CA1 pyramidal neurons and striatal medium spiny neurons Longer dendritic spines (Peca *et al.,* 2011)

1.4.4 Cellular models of ASD

Human embryonic stem cells (hESCs) have the potential to become any cell type in the body making them an exciting therapeutic agent for a multitude of diseases. They are derived from the inner cell mass of the blastula (Thomson *et al*., 1998) and their use has been controversial due to the ethical implications surrounding the disposal of the remaining embryonic cells. Induced pluripotent stem cells (iPSCs) have managed to bypass these ethical implications as they are the reprogrammed versions of somatic cells. The forced expression of the transcription factors Klf-4, Oct4, Sox2 and c-Myc reprograms these cells to a pluripotent state (Takahashi *et al*., 2007, Takahashi and Yamanaka, 2006). Characteristic of stem cells, they express pluripotent markers such as NANOG, OCT4 and SOX2 and can differentiate into multiple lineages. Both hESCs and iPSCs have been differentiated into neurons, oligodendrocytes and glial cells although the generation of one of these cell types, without the contamination of other neural lineages, has proved problematic.

Attempts have been made to generate regional specific neuronal progenitor cells and these can be characterised by the expression of specific genes. Forebrain cells are known to express OTX1, OTX2 and BF1 and the removal of LIF and inhibition of Wnt signalling can drive cells towards this fate (Ying *et al*., 2003, Watanabe *et al*., 2005). Midbrain progenitors express TH, RAX, NURR1, PTX3 and LMX1a/b, and PAX2 and can be induced using FGF8 signalling (Perrier *et al*., 2004). Progenitor hindbrain neurons have been shown to express the HOX genes and FGF2, SHH and RA signalling can all contribute to their phenotype (Tailor *et al*., 2013, Lee *et al*., 2000). Region specific neurons have been further differentiated to produce human dopaminergic, cortical, motor and inter-neurons by the addition of numerous transcription factors and growth factors and the manipulation of specific signalling pathways (Corti *et al*., 2015).

Similarly to the mouse models, iPSC lines can be created that contain single gene disruptions leading to a reduced level of or absent protein. Both *MECP2* and *FMR1* knockout lines have been developed. RTT mutations in iPS cells cause a reduction in spine density and soma size, a reduction in synapse number and alterations in both calcium signalling and electrophysiological properties (Cheung *et al*., 2012, Ananiev *et al*., 2011, Marchetto *et al*., 2010). FMR1 mutations cause a decrease in neurite number and length, alterations in calcium signalling and a decrease in synaptic protein expression (Liu *et al*., 2012a, Sheridan *et al*., 2011). These cellular models can be used to begin to examine the molecular

architecture of ASD, however, these mutations in patients are responsible for additional symptoms not present in all cases of ASD.

Specific ASG gene knockouts would allow a platform to study molecular events present only in autistic individuals. (Zeng *et al*., 2013) used shRNAmir targeted to knockdown *NRXN1* during the differentiation of hIPSCs into neural stem cells. RNA sequencing and qPCR was used to look at gene expression changes at different time points during differentiation in both the *NRXN1* knockdown and control cultures. The time period examined (4 weeks) is thought to correspond to 13-16PCW in human development. *NRXN1* knockdown causes changes in the cell adhesion and neuronal differentiation pathway. Knockdown also reduced the level of GFAP in the cells suggesting a link with astrocyte differentiation. Interestingly, the expression of *NLGNs, PSD95, FMR1, SHANKs and NMDARs* were not significantly different to their expression in control cultures.

Pak *et al*.(2016) created an embryonic stem cell line with conditional *NRXN1* mutations using recombinant adeno-associated viruses. After differentiation into excitatory cortical neurons, it was found that the release of neurotransmitters was impaired in mutant cells as shown by a decrease in mEPSC frequency but not amplitude. Neuronal differentiation and synapse formation was unaffected. An increase in calcium/calmodulin-dependent serine protein kinase (CASK), which is a synaptic scaffold protein found at both the pre and post synaptic membranes (Kim and Sheng, 2004), was observed in the mutants.

However, due to each ASG occurring in less than 2% of autistic cases and the fact that there are hundreds of ASGs means that information from single gene knockout cultures may not be applicable to the majority of ASD cases. A grant has been provided to the research group of Joachim Hallmayer at Stanford University to reprogram fibroblasts from 200 children with ASD and 100 controls and differentiate them into neurons. The iPSC lines developed from autistic patients will be made available to the wider research community (cirm.ca.gov).

1.5. The importance of autism genetics in translational research

Now that a large number of autism susceptibility genes have been identified using multiple approaches (see section 1.2 & 1.3), the focus is switching to identifying the effect that these gene mutations have on cellular processes and how these effects can lead to the development of autism. Many research studies have focused on the molecular convergence

of these susceptibility genes (Hormozdiari *et al.,* 2015; Willsey *et al.,* 2013; Parikshak *et al.,* 2013; O'Roak *et al.,* 2012). We have an incomplete understanding of the roles of many of these genes and their expression patterns and functions during human development remain poorly researched. Much of the current understanding of function has been achieved through research in animal models and, although valuable, animal models cannot fully recapitulate the human phenotype. Additionally, the response of humans and animals to targeted drug treatments differs (Shanks *et al.,* 2009), emphasising the need to develop human specific models for drug screening. Induced pluripotent stem cell models have been created from patients who harbour ASD gene mutations, however, studying the function of these genes during development can be problematic due to inefficient reprogramming, differentiation and the persistent epigenetic marks (McNeish *et al.,* 2015; Vaskova *et al.,* 2013). Knowing at what periods of development these genes are expressed will provide information as to when specific treatments should be administered. In addition, gene expression information from neurotypical brains will provide a benchmark for comparison of abnormal tissue that could aid our understanding of the molecular prodrome. This study utilizes human embryonic and fetal material as a model to explore these questions and to propose possible roles of a subset of ASD genes during early cortical development.

1.6 Aims

The aims of this project were to: -

1) Use RNA sequencing to 1) Identify AS genes expressed between 9 and 12 PCW, 2) Any genes differentially between 9 and 12 PCW and 3) Genes differentially expressed between the anterior and posterior cortex at these ages.

2) Carry out a more detailed examination of the cortical gene and protein expression patterns of a subset of ASGs (NRXNs, NLGNs and SHANKs) between 8 and 12 PCW.

3) Examine the expression patterns of genes that affect *NRXN* transcription and splicing.

4) Find out if topoisomerase inhibitor, ICRF-193, is able to reduce the expression of ASDs in human cortical cultures

CHAPTER 2 MATERIAL & METHODS

2.1 Dissection and Ethical Approvals

2.1.1 Human brain material and Ethical Approval

The Human Developmental Biology Resource (HDBR), based in Newcastle, UK, has provided the embryonic and fetal material used in this study. The material was collected from pregnancy terminations, complying with the highest ethical standards. Funding for the resource comes from the Medical Research Council and Wellcome Trust and it has obtained ethical approvals from the NRES Committee North East - Newcastle and North Tyneside 1 (REC reference 08/H0906/21+5) and also holds a UK Human Tissue Authority license (licence number 12534). Material is distributed to research project throughout the UK without the need for individual project ethical review (Gerelli *et al.,* 2015). The material collected by the resource ranges in age from 3-20 post conceptional weeks (PCW). All material is karyotyped and about 4% of the samples have an abnormal karyotype, usually an extra chromosome 21 or monosomy X. Additionally, 9% of the samples collected display some form of phenotypic abnormality (Gerrelli *et al.,* 2015). The material is not genotyped or molecularly examined before being distributed to research projects.

Adult brain tissue used in this study was provided by the Newcastle Brain Tissue Resource which is funded in part by a grant from the UK Medical Research Council (G0400074), by NIHR Newcastle Biomedical Research Centre and Unit awarded to the Newcastle upon Tyne NHS Foundation Trust and Newcastle University, and as part of the Brains for Dementia Research Programme jointly funded by Alzheimer's Research UK and Alzheimer's Society.

2.1.2 Embryonic & Fetal Brain Dissection

For the RNA sequencing study, 192 prenatal human brains were collected by the Human Developmental Biology Resource (HDBR) with maternal and ethical consents (http://www.hdbr.org). Age PCWs was determined using the foetal staging guide as described by [261]Hern (1984). The brains ranged in age from just over 4 PCW (CS13) to 20 PCW. From these brains, 637 samples were dissected from the forebrain, midbrain, hindbrain and choroid plexus regions. From some of the embryos and fetuses spinal cord samples were also collected. Depending on the age of the embryo or fetus, the forebrain tissues were further dissected into basal ganglia, telencephalon, diencephalon and cortex and from 9 PCW the cortex was dissected into strips as described below.

For the qPCR study, samples between 8-12 PCW were collected using the above ethical and maternal consents. Age PCWs was determined using the foetal staging guide as described by Hern (1984). Whole brains were isolated from the skull and meninges were removed. The hemispheres were split apart (figure 2.1A) allowing the removal of the choroid plexus,

and subcortical structures to leave only the cerebral cortex. The temporal lobe, including lateral and medial walls was removed and the remaining cortex was divided into sections of equal width from the anterior (A) to the posterior (P) pole of the cortex including lateral and medial cortical walls (figure 2.1B). When possible, there were five equal cortical sections, however, this varied from three to ten cortical sections. The most anterior (A), central (C) and posterior (P) sections as well as the temporal (T) cortex were used in the RNA sequencing and the qPCR analysis. Once dissected, sections were immediately frozen and stored at -80^{o}C and subsequently used to extract RNA.

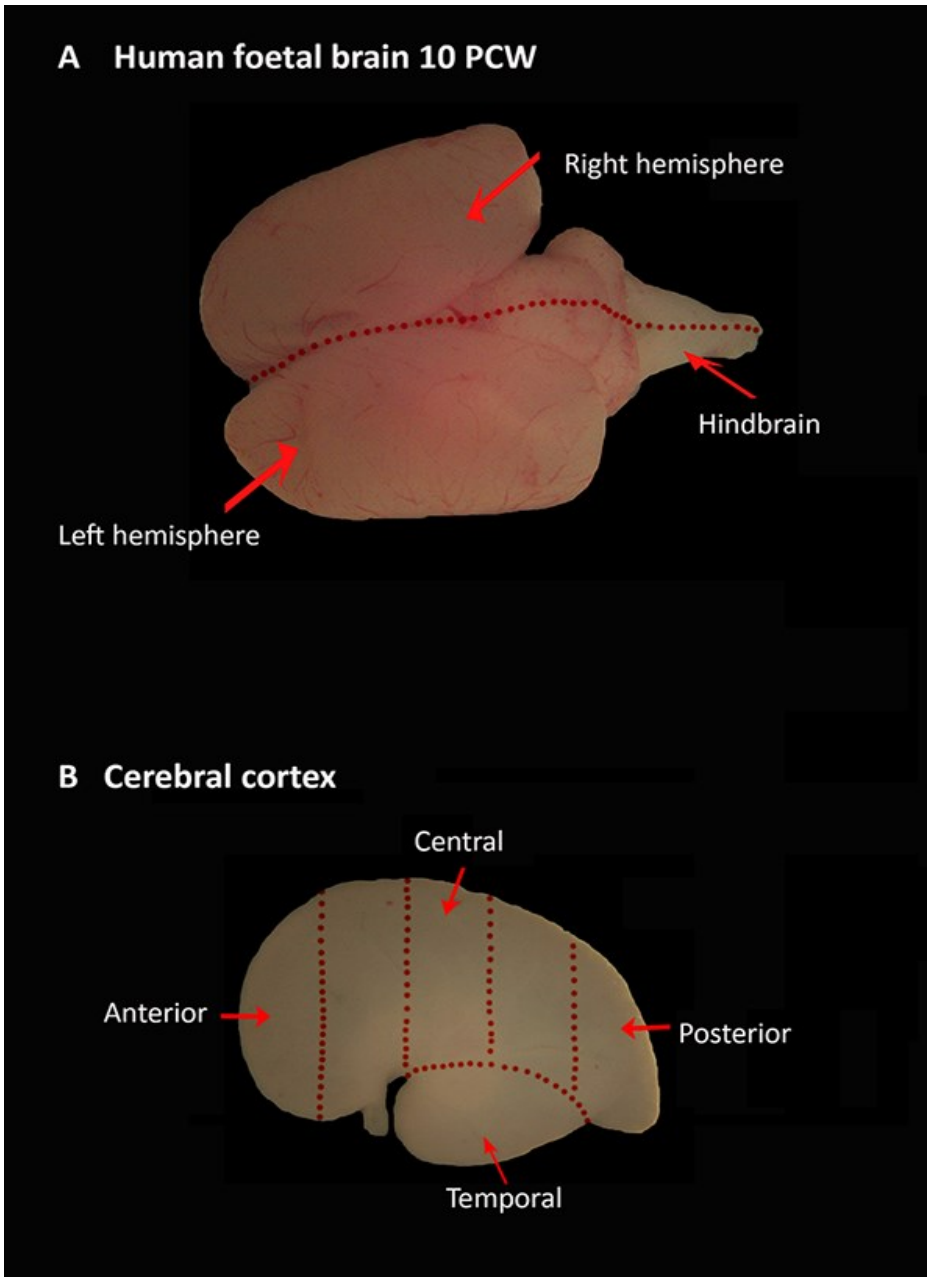


Figure 2.1 Human brain dissection. Human foetal brain was severed from the spinal cord and removed from the head (A). Removal of meninges, hindbrain and sub-cortical structures exposed the cerebral cortex (B) which was further divided into six sections including the anterior, central, posterior and temporal cortex.

2.2 RNA Isolation & Reverse Transcription

2.2.1 RNA Isolation from brain tissue for qPCR

RNA was extracted from the anterior, central, posterior and temporal cortical sections using the QIAgen RNeasy mini kit according to the manufacturer's protocol. The concentration and quality of RNA was measured using the Nanodrop 8000 (Thermo Fisher Scientific, Paisley, UK) to detect spectrophotometric absorbance at 280nm. To control for RNA purity and degradation, 260/230 and 260/280 spectrophotometric ratios were only accepted between the ranges of 1.8 and 2.1. A selection of the RNA was run on the Bioanalyser 2100 (Agilent, Stockport, UK) and all RNA integrity number (RIN) values measured were above 7.

2.2.2 RNA Isolation from cell cultures

The media was removed from all wells of a 12 well plate (Thermo Fisher Scientific) and was substituted with 500µl of 0.5% Trypsin: EDTA (Thermo Fisher Scientific) for 5 minutes at 37°C. Trypsin: EDTA was removed by aspiration and 500µl media (MEM: F12; section 2.7) was added to each well and used to manually remove cells from the bottom of the plates by pipetting up and down. Cells were collected and spun down at 8000 rcf for 5 minutes. The supernatant was discarded, lysis solution was added to the pellet (QIAgen RNeasy kit, Manchester, UK) and the RNA was extracted according to the manufacturer's protocol. The concentration and quality of RNA was measured as described previously (section 2.2.1).

2.2.3 Reverse Transcription

To reverse transcribe the RNA to cDNA, 5µg of RNA, 5mM $MgCl_2$, 0.2µg of random primers (Promega, Southampton, UK) and 0.5mM dNTPs (Biolabs ® Inc) were combined and the volume made up to 13µl using RNase free water. The mixture was heated at 65^{o}C for 5 minutes and cooled on ice for 1 minute. 4µL 5X first strand buffer (10mM Tris-HCl [pH 9.0 at 25°C]; 50mM KCl; 0.1% Triton® X-100), 1µl 0.1M DTT, 1µl (200 U/µl), Superscript™ Reverse Transcriptase and 1µl (40U/µl) RNaseOut™ Recombinant Ribonuclease Inhibitor (all Invitrogen, Thermo Fisher Scientific, Paisley, UK) were added to the same tube and incubated at room temperature for 5 minutes before being heated to 50^{o}C for 60 minutes. The reaction was inactivated by heating to 70°C for 15 minutes. One unit incorporates 1

nmol of dTTP into acid-precipitable material in 10 min at 37°C using poly (A) oligo (dT)25 as template-primer. Total cDNA concentration was measured using the Nanodrop 8000 (Thermo Fisher Scientific) to detect spectrophotometric absorbance at 280nm and 260/230, 260/280 spectrophotometric ratios were between the accepted range of 1.7-2.1. cDNA samples were then diluted to a concentration of 100ng/µl for use in PCR and qPCR methods.

2.3 Polymerase Chain Reaction (PCR)

2.3.1 Primer Design

Sequence specific primer sets were designed using Primer3 software (http://frodo.wi.mit.edu/) and obtained from Eurofins MWG Operon (http://www.eurofins.co.uk/). Table 2.1 shows the list of primer sequences used in this study.

2.3.2 Non Quantitative Polymerase Chain Reaction (PCR) & Gel Electrophoresis

The thermal cycle conditions are outlined in table 2.2. A positive βACTIN control was included in each PCR in addition to a 100 bp DNA ladder (Thermo Fisher Scientific, Invitrogen™). PCR products were run by gel electrophoresis on a 1.5% agarose gel (Sigma Aldrich, Dorset, UK). Bands were extracted from the gel using the Qiagen gel extraction kit (Qiagen, Crawley, UK) according to the manufacturer's protocol and sent for direct sequencing with their forward primers (Eurofins MWG Operon, Ebersberg, Germany) to ensure the correct product had been amplified by each primer pair.

Table 2.1 List of Primers for PCR and qPCR. *NRXN, Neurexin; NLGN, Neuroligin; SHANK, SH3 and multiple ankyrin domains; SYP, Synaptophysin; *Reference genes βActin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SDHA, Succinate dehydrogenase complex subunit A.*

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Amplicon Size (bp)
NRXN1	aggacattgacccctgtgag	ccttcatcccggtttctgta	205
NRXN2	catcctcctctacgccatgt	ttgttcttcttggccttgct	165
NRXN3	gctgagaacaaccccaata	atgctggctgtagagcgatt	179
NLGN1	tgcaaaggggaactatggac	ttgctccaacggttaccttc	177
NLGN2	cagaagggctgttccagaag	gcagacactccacagcttca	150
NLGN3	agagccatcatccaaagtgg	ggatctcagggtcatcagga	244
NLGN4X	tcgctcctcttcctcaacat	acactcgtgatcgtgttcca	180
SHANK1	actctcagcacctggagcat	catcctgttctcggtggttt	243
SHANK2	gtgtacgaacgccaaggaat	tgccgctcttcctctgttat	152
SHANK3	cttcacacaaaggcgaacct	ccaccattcttcagcacctt	191
SYP	tctggccacctacatcttcc	tcagctccttgcatgtgttc	218
*βACTIN	ctacaatgagctgcgtgtggc	caggtccagacgcaggatggc	271
*GAPDH	tgcaccaccaactgcttagc	ggcatggactgtggtcatgag	87
*SDHA	tgggaacaagagggcatctg	ccaccactgcatcaaattcatg	86

Table 2.2 Standard PCR protocol used for cDNA amplification.

Temperature °C	Time	No. of cycles
95	3 minutes	1
94	15 seconds	35
60	40 seconds	35
72	50 seconds	35
72	5 minutes	1

2.3.3 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR reactions were set up in MicroAmp® Optical 384 well reaction plates (Invitrogen) and run on the 7900 HT Fast Real-Time PCR System (Applied Biosystems). A reaction volume of 10µl was prepared in each well consisting of 0.5µl of 10 pmol/µl forward primer, 0.5µl of 10pmol/µl reverse primer, 5µl SYBR® Green JumpStart Taq Ready Mix (Sigma-Aldrich), 1µl 100ng/µl cDNA and 3µl RNase free water.

Triplicates of each primer pair were run each time along with triplicates of three reference genes, *βACTIN, GAPDH* and *SDHA*, to which the data were normalised during analysis.

The thermal cycle protocol was as follows; 95°C for 15 minutes, (95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 74°C for 10 seconds) x40 cycles. Dissociation curve analysis (68°C for 10 seconds, 99°C for 10 seconds) at the end of the 40 cycles confirmed the absence of non-specific amplification and primer dimers.

2.3.4 Statistical analysis of differential expression

Raw fluorescent SYBR readings were taken from the 7900 HT Fast machine and analysed in the program data miner (http://www.miner.ewindup.info/) created by Zhao & Fernald (2005). The threshold at which the signal crosses the background noise was set manually by the program and cycle threshold (ct) and reaction efficiency (E) values were taken for each reaction. An average efficiency value was calculated for each primer pair and used to obtain an accurate CT value for each gene. We can then calculated the relative amount of gene

expression from the gene of interest (GOI) by dividing by the geometric mean expression of the reference genes, *βACTIN, GAPDH* and *SDHA*. The equation used to produce the relative amount of expression for each gene of interest was as follows;

$$\text{Relative expression} = \frac{T_{GOI}}{T_R} = \frac{2^{ctR \times ER}}{2^{ctGOI \times EGOI}}$$

Where,

T - Amount of original template

GOI - Gene of Interest

R - Geometric mean of three reference genes

Significance ($p<0.05$) across ages and between areas of the cortex was calculated using a one way Analysis of Variance (ANOVA) test which included significant factors being subject to a post-Hoc Tukey test. SPSS statistics program was used to carry out these tests. Correlation coefficient

2.4 Tissue Processing & Sectioning (carried out by the Human Developmental Biology Resource)

Processing and sectioning of embryonic and foetal material for paraffin sections was performed by the staff of the Human Developmental Biology Resource (HDBR) in Newcastle. After removal, brain tissue was fixed for a minimum of 24 hours in 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffered saline (PBS). Tissue processing consisted of immersing tissue to be sectioned in 70% alcohol for 15 minutes, 100% ethanol for 45 minutes, 2 x 100% ethanol for 1 hour, xylene for 45 minutes, 2 x xylene for 1 hour, wax for 1.5 hours, 2 x wax for 1 hour, 45 minutes. (Shandon Pathcentre Tissue Processor, Thermo Scientific, Epsom, UK) before paraffin embedding. Sagittal or coronal brain sections were cut at 8um thick and mounted on slides for use in IHC or ISH.

2.5 Haematoxylin & Eosin (H & E) Histological Staining

Paraffin sections were immersed in xylene for 5 minutes to remove wax and rehydrated in a series of ethanol dilutions (100%, 100%, 95%, 70%). After rinsing in tap water, sections were placed into Harris' Haematoxylin solution (Raymond A Lamb Ltd., Eastbourne, UK) for 30 seconds-1 minute and then again rinsed in tap water. 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%, 100%, and 100%), immersed in two changes of xylene and mounted using DPX (Merck).

2.6 Immunohistochemistry (IHC)

Paraffin sections were dewaxed by 2 x 10 minute xylene treatment and rinsed in 2 x 100% ethanol for 5 minutes. Endogenous peroxidase activity was blocked by immersion in methanol peroxide solution (3ml hydrogen peroxide, Sigma Aldrich, 180ml methanol) for 10 minutes. Slides were rinsed in tap water and antigen retrieval was carried out by boiling in citrate buffer (tri-sodium citrate, (C6H5Na3O7)2H2O) for 10 minutes. Sections were incubated in 10% normal serum (species in which secondary antibody was raised) diluted in TBS (tris buffered saline, Sigma) for 10 minutes at room temperature. Sections were then incubated with the primary antibody diluted in 10% normal serum: TBS overnight at 4^{o}C. The following day, the primary was removed by 2 x 5 minute washes in TBS. Biotinylated secondary antibody appropriate to the primary antibody, diluted in 10% normal serum: TBS was then applied to the sections for 30 minutes at room temperature. To remove the secondary there were a further 2 x 5 minute washes in TBS before the tertiary ABC-HRP complex was applied for 30m minutes (Vector Laboratories). Following final 2 x 5 minute washes, the sections were reacted with DAB (Vector Laboratories) for 10 minutes and washed thoroughly in running tap water for 10 minutes. The slides were put through a series of ethanol dehydrations (70%, 95%, 100%, and 100%) and mounted using DPX.

Table 2.3 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

2.7 Immunocytochemistry (ICC)

Cells were fixed for 20 minutes in 4% PFA/ PBS and then washed 3 times in TBS before overnight incubation at 4°C with a specific primary antibody diluted in 0.1% Triton X/ TBS (See table 2.4 for details of primary antibodies).

Cells were then washed 3 times with 0.1% Triton X/ TBS and incubated with the appropriate biotinylated secondary antibody (table 2.4) for 2 hours at 4°C. Cells were washed 3 times in Triton X/ TBS and incubated with Streptavidin-HRP (Vector Laboratories) diluted 1/50 in TBS for 1 hour at 4°C before a further three washes in Triton X/ TBS. The cell cultures were then incubated with ImmPACT DAB (3, 3'-diaminobenzidine) Peroxidase (HRP) Substrate (Vector Laboratories) for up to 10 minutes and rinsed in three TBS washes. The cell cultures were finally stained with DAPI nucleic acid stain (Invitrogen) diluted 1/10,000 TBS, washed two times in TBS and kept in TBS in the dark at 4°C until images were obtained (section 2.8).

2.8 Immunohistochemistry- Fluorescence

As for non-fluorescent IHC, paraffin sections were dewaxed in xylene, rinsed in 100% ethanol and endogenous peroxidase activity was blocked with methanol peroxide solution. Slides were rinsed in tap water and antigen retrieval was carried out by boiling in citrate buffer before incubation in 10% normal serum. Sections were then incubated with the primary antibody overnight at 4^{o}C. The following day, the primary was removed by 2 x 5 minute

washes in TBS and the appropriate secondary antibody, HRP conjugate (ImmPRESS™ HRP IgG (Peroxidase) Polymer, made in horse/ goat, Vector Labs) applied for 30 minutes. 2 x 5 minute washes in TBS removed the secondary antibody and the slides were incubated with Tyramide signal amplification (TSA™) fluorescein Plus system reagent (Perkin Elmer, London, UK) at a concentration of 1/500 in the dark for 10 minutes. Slides were washed twice in TBS before being subjected to microwave antigen retrieval in citrate buffer for a second time. Normal serum was applied to the slides for 10 minutes before incubating them with the second primary antibody for 2 hours at room temperature. The primary was removed by 2 x 5 minute washes in TBS before incubation for 30 minutes with the secondary HRP conjugated antibody. An additional 2 x 5 minute washes in TBS removed the secondary before incubation with Tyramide signal amplification (TSA™) CY3 Plus system reagent (Perkin Elmer) in the dark for 10 minutes. Sections were washed twice for 5 minutes in TSA before applying 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Thermo Fisher Scientific) for 5 minutes. Slides were then rinsed twice in TSA and mounted using Vectashield Hardset Mounting Medium (Vector Laboratories).

Table 2.4 Primary antibodies used for chromogen (DAB), Fluorescence (F) immunohistochemistry (IHC) on Paraffin and Immuno cytochemistry (ICC)

Primary antibodies	**Host species & Company**	**Antibody dilution**	**Secondary antibodies**	**References**
GAP43	Mouse monoclonal, Sigma G9264	1/10,000	Horse anti mouse Vector Laboratories	Bayatti *et al.,* 2008a Eyre *et al*. 2000 Koutcherov *et al*. 2003 Clowry *et al.* 2005
Ki67	Mouse	1/150	Horse anti mouse Vector Laboratories	Meyer *et al*. 2002 Chan *et al*. 2006
Pax6	Rabbit polyclonal, Covance PRB-278P	1/1500	Goat anti rabbit Vector Laboratories	Bayatti *et al.,* 2008

NKX2.1	Dako, Cambridgeshire, UK	1/150	Horse anti mouse Vector Laboratories	Harkin *et al*., 2016
TOP2A	Rabbit polyclonal Prof. Austin	1/800	Goat anti rabbit Vector Laboratories	Harkin *et al.,* 2016
TOP2B	Rabbit polyclonal Prof. Austin	1/800	Goat anti rabbit Vector Laboratories	Harkin *et al.,* 2016
NRXN1	Rabbit polyclonal, Santa Cruz Sc14334	1/300	Goat anti rabbit Vector Laboratories	Kim *et al.,* 2008
NRXN2	Rabbit polyclonal, Abcam Ab34245	1/2000	Goat anti rabbit Vector Laboratories	Borsics *et al.,* 2010
NRXN3	Rabbit polyclonal, Sigma Aldrich Prestige HPA002727	1/300	Goat anti rabbit Vector Laboratories	Human protein atlas
SYP	Mouse monoclonal Sigma Aldrich	1/1000	Horse anti mouse Vector Laboratories	Bayatti *et al.,* 2008
TBR1	Rabbit Polyclonal, Abcam ab31940	1/1500	Goat anti rabbit Vector Laboratories	Bayatti *et al.,* 2008
KHDBRS2	Rabbit polyclonal	1/5000	Goat anti rabbit Vector Laboratories	Iijima *et al.,* 2014

KHDBRS3	Guinea pig polyclonal	1/10,000	Anti guinea pig Vector Laboratories	Iijima *et al.,* 2014

2.9 Human Cortical Cell Culture -8-Day In-vitro characterisation

The brain was isolated from a 10-12 PCW foetus and the meninges and subcortical structures were removed. The anterior 50% of the cerebral cortex, excluding temporal lobe, was detached, cut into smaller pieces and immersed in 0.05% trypsin: ethylene diamine tetra-acetic acid (EDTA) (Life Technologies Ltd, Paisley, UK) and 10% foetal calf serum (Sigma Aldrich) diluted in Ca^{2+}, Mg^{2+} free Hank's balanced salt solution (HBSS)(Life Technologies)for 20 minutes. The cells were then pelleted at 1400 rpm for 5 minutes and re-suspended in fresh 10% FCS: HBSS solution. The cells were gently dissociated by trituration through a 10ml and 5 ml pipette and transferred through a 70µm pore cell strainer (VWR, Leicestershire, UK). The cells were pelleted at 1400 rpm for 5 minutes and re-suspended in Minimal Essential Media (MEM) (Life Technologies) supplemented with 10% FCS. Cells were plated onto Poly-L-Lysine (Sigma Aldrich, Poole, UK) coated 12 well plates (VWR) at a density of 200,000 cells/ cm2. The media was changed after 24 hours to MEM/ 1:1Ham's F12 Nutrient Mix with L glutamine (Life Technologies) supplemented with 1/50 B27 (Life Technologies) and Gentamicin solution (Sigma Aldrich). Cells were grown in this media for a further 6 days for cell characterisation and media was changed every 2 days. On days 1, 3, 5 and 7 of culture in the above conditions, cells were fixed in 4% PFA: PBS for 20 minutes and subjected to imaging (section 2.11) and Immunocytochemistry (section 2.7).

2.10 Knockdown of *Neurexin* genes in Cell Culture using ICRF-193

Cells were isolated, dissociated and pelleted as outlined in section 2.9.1. Cells were then re-suspended in MEM/ 1:1Ham's F12 Nutrient Mix with L glutamine (Life Technologies) supplemented with 1/50 B27 (Life Technologies) that also contained a concentration of either 5, 50 or 100µM ICRF-193 topoisomerase 2 inhibitor (biomol, Hamburg, Germany) dissolved in DMSO. Control sample media contained 1% DMSO. Cells were plated at a density of 200,000 cells/ cm^2 onto Poly-L-Lysine (Sigma Aldrich, Poole, UK) coated 12 well

plates (VWR) and cultured for 48 hours. Cells were either fixed in 4% PFA: PBS for 20 minutes and images were captured (section 2.11) or RNA was extracted (section2.2.2) and subjected to reverse transcription (section 2.2.3) and qPCR (section 2.3.3).

2.11 Image Acquisition

2.11.1 Light Microscopy

Images were captured using a Zeiss Axioimager Z2 Apotome and Axioimager Z2 4.8.1 software (IHC) and the Axiovert 200 (ICC) using the Axioplan Z2 software. Objectives 1.25x, 5x, 10x, 20x, 40x Oil. Both microscopes were attached with Axioplan microscope digital camera. Adobe Photoshop was used to prepare figures with resolution set at 300 pixels per inch.

2.11.2 Fluorescent Microscopy

Images were captured using either the Zeiss Axioplan 2 or the Zeiss Axio Imager Z1. The fluorophores used were DAPI, Fluorescein and CY3 and their emission values can be seen in table 2.5.

2.11.3 Slide scanning

Images were scanned using the Leica SCN400 Slide Scanner (Newcastle Biomedicine Biobank Imaging facility). Final images for publication were produced using Adobe Photoshop CS6 software, resolution 300 pixels per inch.

Table 2.5 Excitation/ emission values (nm) for DAPI and fluorophore signal amplification (TSA™) Plus system reagents (Perkin Elmer).

Fluorophore	Excitation/Emission (nm)
DAPI	358/461
TSA Plus Fluorescein	494/517
TSA Plus CY3	550/570

2.12 Quantification - Cell counts

For each primary antibody, three separate wells of cultured cells from one foetal cortex were immunoperoxidase stained and then counter-stained with DAPI (section 2.8). For each well, both bright field (DAB stain) and 405nm laser line (DAPI) images were captured for three random fields of vision using a Plan NeoFluar 20x /0.50 optical lens. Images were uploaded into Image J (https://imagej.nih.gov/ij/) and the number of cells stained by DAB and DAPI were counted and recorded. DAB stained cells were calculated as a percentage of the DAB plus DAPI stained cells, as DAPI could not penetrate cells stained with DAB. This was repeated for three separate brains.

2.13 RNA Sequencing

2.13.1 RNA extraction, library preparation and sequencing performed by AROS Applied Biotechnology (Aarhus, Denmark)

RNA was extracted from the tissue samples according to the company's protocol and the quality was assured using the Agilent Bioanalyzer. Poly (A) was performed on the samples to pull out the polyadenylated transcripts from the sample. This ensures that only the coding transcripts are sequenced and not the ribosomal RNA.

The RNA library was prepared using the guidelines produced for the TruSeq Stranded mRNA LT sample prep kit (Illumina part # 15031047 Rev. E). RNA was reverse transcribed to cDNA and was fragmented, before PCR amplification. Libraries 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.

Library pools were loaded onto a paired-end flow cell of the Illumina HighSeq 2000 platform and each pool was loaded onto one lane of the flow cell. At least 80% of the bases in a 101 Paired End run had Q30 or above. The data output corresponded to an average of 90 M reads and a minimum of 63M reads.

2.13.2 Quality control, mapping and differential expression analysis was performed by Yaobo Xu at the Newcastle University Bio-informatics unit

The quality of sequencing reads was firstly checked with FastQC (Andrews). The 12 bp on the left ends and 4 bp of the right ends of all reads were clipped off with Seqtk (Li) to remove GC-content biased bases. Autoadapt (Shuttleworth 2013) was then used to remove low quality bases (Q < 20) and contaminations from standard Illumina (Illumina, Inc. California, U.S.) paired-end sequencing adaptors on 3' ends of reads. Autoadapt uses FastQC to identify the exact sources of contaminations and uses cutadapt (Martin, 2011) to remove them automatically. Poly-N tails were trimmed off from reads with an in house perl script. 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 & Huber, 2014). Differentially expressed genes were then identified with Bionconductor Gentleman *et al.,* 2004) package DESeq2 (Love *et al.,* 2014). RPKMs of genes were calculated and normalized using cqn (Hansen *et al.,* 2013).

2.14 Venn diagrams

Venn diagrams developed from an interactive tool (Oliveros, 2007) were used to compare overlapping genes between two lists of differentially expressed genes. The tool was made available through the following website: - https://www.stefanjol.nl/venny.

2.15 Gene Ontology Analysis

Gene ontology analysis was carried out using the tool provided on the Gene ontology consortium website (http://geneontology.org/). Assigning GO terms to genes is based on annotations contributed to the GO consortium, which are subject to guidelines and standard operating procedures and are being continually updated.

Chapter 3 High throughput sequencing analysis of the developing human cerebral cortex from 9-12 PCW

3.1 Aim of study

The aim of this study was to compare the transcriptomes, generated by RNA sequencing methodology, of 9, 11 and 12 PCW human cerebral cortex samples and in doing so to identify spatial and temporal changes in gene expression associated with this developmental period.

3.2 Results

3.2.1 Brain samples cluster according to anatomical location

Human embryonic and fetal brains were collected by the Human Developmental Biology Resource (HDBR) and dissected as described in chapter 2.1.2. These samples were sent to AROS Applied Biotechnology where whole genome sequencing on the Illumina HighSeq 2000 platform. RNA extraction, library preparation and RNA sequencing on the Illumina HighSeq 2000 platform was performed by AROS Applied Biotechnology as previously described (chapter 2.13.1). The mapped data was sorted according to its anatomical location. Samples labelled 'brain' were neural tissue but were not identifiable by anatomical location. PCA analysis was carried out by Dr Yaobo Xu (IGM, Newcastle University) on the 637 resulting RNA datasets to look for underlying structure within the data. In PCA analysis, datasets with more similar gene expression profiles appear closer together on the resulting plot whereas samples with less similar gene expression profiles are further apart as components are separated by the variance. Two variables; brain region and expression value (RPKM) were examined using PCA analysis (figure 3.1). Samples from the same brain region would be expected to cluster together if this was the variable that was having the largest effect on the expression levels. Each sample was given an identifying colour. Forebrain, midbrain, hindbrain and spinal cord, choroid plexus and unidentified brain samples were assigned the colours dark green, purple, blue, light green and red respectively to make them easier to visualise (figure 3.1). Choroid Plexus samples very clearly separated out from the rest of the samples forming a light green cluster (figure 3.1). The expression profiles of hindbrain and

forebrain samples were more similar to each other than they were to choroid plexus samples, however, they clearly formed separate blue and dark green clusters (figure 3.1). A small number of hindbrain samples appeared intermingled with the forebrain samples and vice versa, but, in general, forebrain samples had identifiably different gene expression profiles compared with hindbrain samples. Midbrain samples were dispersed mostly within the hindbrain cluster but midbrain samples can also be found in the forebrain cluster. The unidentified neural tissue samples labelled 'brain' were dispersed throughout the other four clusters, suggesting that they are derived from neural tissue across all of these regions (figure 3.1).

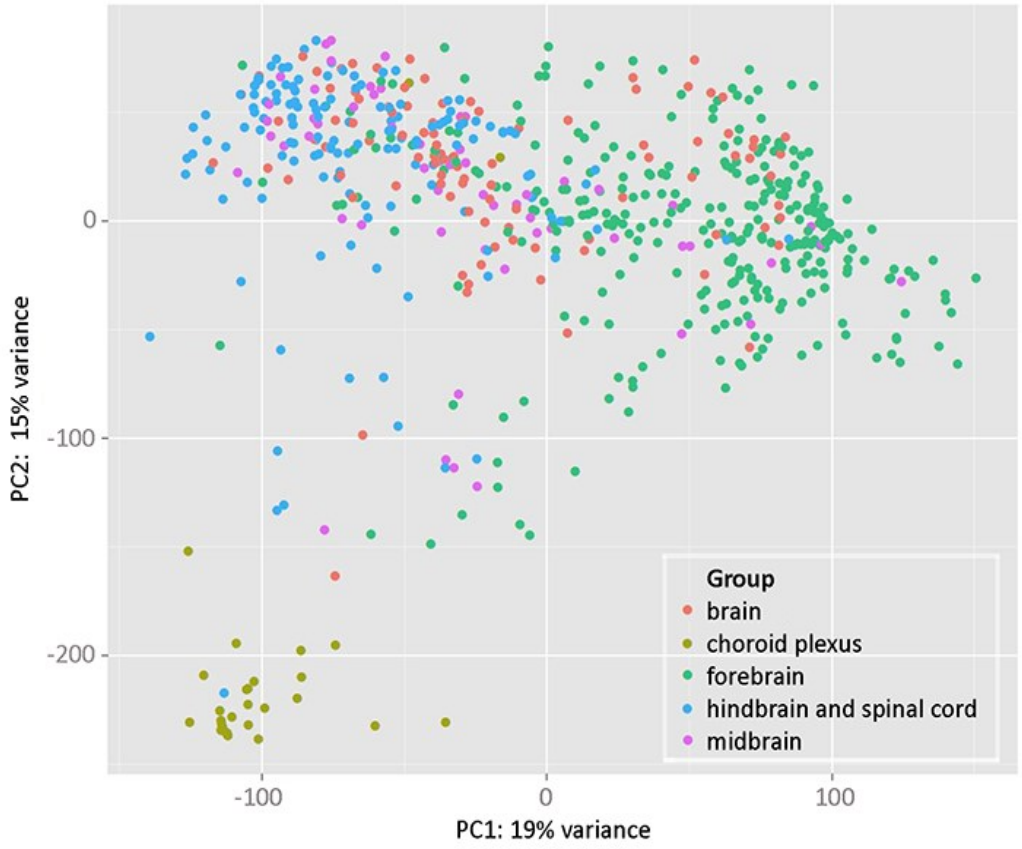


Figure 3.1 PCA analysis of all sequenced human brain samples (Dr Yaobo Xu, Newcastle University). Samples were separated based on variance. Two variables, brain region and expression value (RPKM) were examined. Choroid plexus samples (khaki green) were the most distinct set of samples. Forebrain (green) and hindbrain (blue) samples separated out with some slight overlap. Midbrain samples (purple) and unidentified brain samples (red) fell within the forebrain and hindbrain clusters.

3.2.2 Variation between fetuses was greater than variation between cortical regions

As my project focussed on cortical development only, the RNAseq datasets from the cortex were analysed further. Many of the cortical samples had been further dissected (as described in section 1.2.2) and the analyses below were carried out on RNAseq datasets from the anterior, central, posterior and temporal cortex tissue samples from 9 and 12 PCW fetal brains. The brain identification numbers, age, sex and hemisphere used can be seen in table 3.1.

Hierarchal clustering was carried out by Dr Yaobo Xu on the RNAseq datasets from the 64 dissected cortical samples. Samples were assigned colours based on their cortical region, age and sex to visualise whether these factors contributed to a similarity in expression profiles. None of these factors caused clustering of the samples into expected groups (figure 3.2). Although age and sex showed a higher degree of clustering than cortical region, the samples clustered according mainly to which individual they were collected from. This implied that variation between individuals was greater than the variation between either cortical regions or between males and females. Human variation between individuals is known to be very high (The Genomes Project, 2015) and so this finding was expected.

PCA analysis, carried out by Dr Yaobo Xu, was used to look more specifically at the RNAseq datasets from the anterior and posterior cortical samples at both 9 and 12 PCW. At 9 PCW, there was a clear separation of anterior and posterior samples suggesting that there are regionally imposed transcriptional profiles (figure 3.3A). At 12 PCW there was some separation between anterior and posterior samples (figure 3.3B) again suggesting that at 12 PCW, regionally imposed transcriptional differences between the anterior and posterior cortex exist.

Table 3.1 List of brains dissected and used for RNA Sequencing analysis of cortex

Identification no.	Age (PCW)	Sex F – Female, M - Male	Hemispheres used L – Left, R - Right
11845	9	M	L & R
11873	9	F	L & R
11851	9	F	L & R
11930	11	F	L & R
11942	11	F	L & R
11833	11	F	R
1123	11	Unknown	L
1111	11	Unknown	Unknown
1110	11	Unknown	Unknown
11834	12	M	L & R
11885	12	M	L & R
12007	12	M	L & R
1118	12	Unknown	Unknown

Table 3.2 Total number of genes differentially expressed between different ages and regions

Adjusted P value <0.05

Differential expression analysis comparisons	Total number of genes that are differentially expressed	Number of genes significantly upregulated	Number of genes significantly downregulated
9 - 12 PCW	4153	1819	2334
Anterior- Posterior at 9 PCW	146	36	110
Anterior- Posterior at 12 PCW	185	168	17

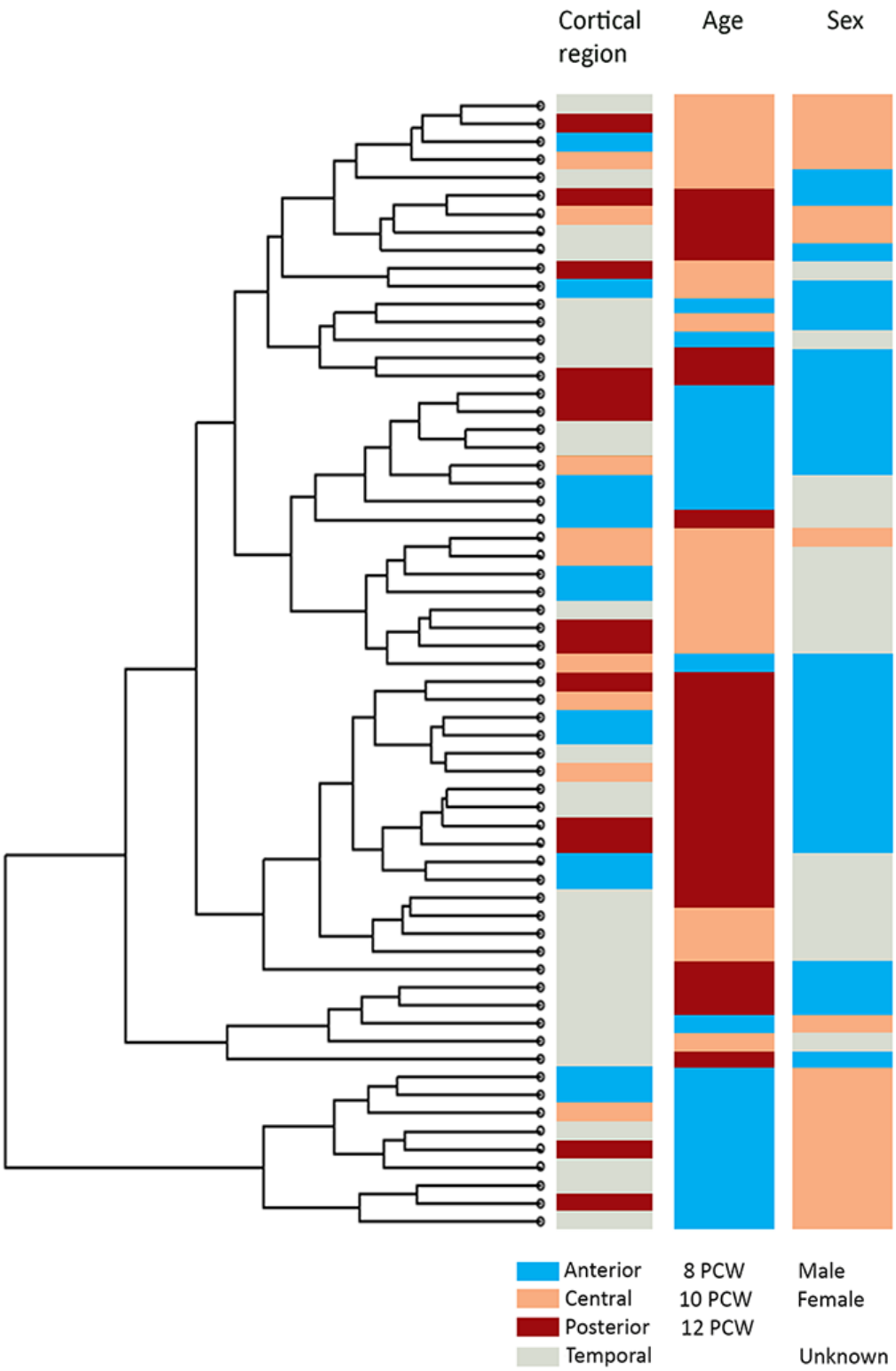


Figure 3.2 Hierarchical clustering of human cortical samples from anterior, central, posterior and temporal cortex (carried out by Dr Yaobo Xu). Samples that are from the same cortical region, from the same age brain or are the same sex do not have largely similar gene expression profiles.

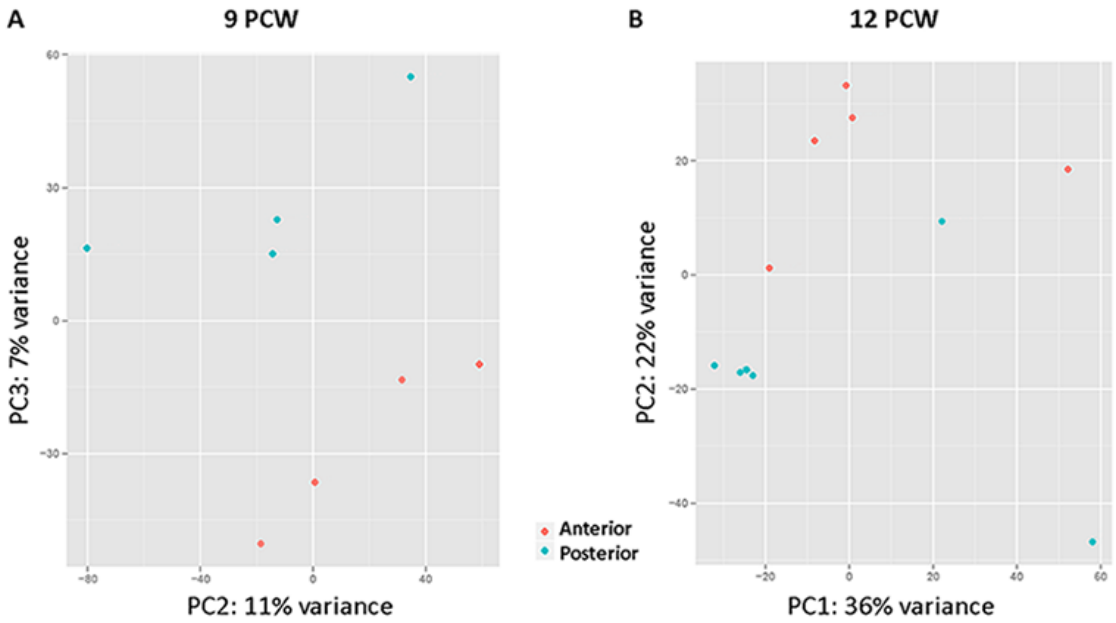


Figure 3.3 PCA analysis of anterior and posterior samples from 9 and 12 PCW brain samples (carried out by Dr Yaobo Xu). PCA analysis revealed that there was a transcriptional separation of anterior from posterior samples at both (A) 9 and (B) 12 PCW, although the separation required a larger variance and a third principal component for 9 PCW.

3.2.3 A higher number of protein coding genes are upregulated compared to downregulated with age

Despite the highest genetic variability being between individuals, it was still possible to isolate genes that were differentially expressed between ages and between cortical regions. Firstly, differential expression analysis was carried out (as described in section 2.14.2) to identify genes that differ in expression significantly ($p<0.05$) between 9 and 12 PCW. For this analysis, the RPKM values for each gene from the anterior, central, posterior and temporal regions of the cortex were combined for each age. This analysis yielded a total of 1819 genes that were upregulated and 2334 genes that were downregulated (table 3.2). The gene list included protein coding genes, pseudogenes, long non-coding (linc) RNA, micro (mi) RNA, ribosomal (r) RNA, small nuclear (sn) RNA, small nucleolar (sno) RNA and antisense transcripts (table 3.3).

Table 3.3. Categories of genes that are differentially expressed between 9 and 12 PCW and between anterior and posterior cortex. Differential analysis and mapping reads to the human genome carried out by Dr Yaobo Xu, Newcastle University.

Gene name	% of differentially expressed genes between 9-12 PCW	% of differentially expressed genes between anterior-posterior at 9 PCW	% of differentially expressed genes between anterior-posterior at 12 PCW
Protein Coding	39	75	82
Processed pseudogene	13	<1	3
Long non-coding (linc) RNA	13	12	9
Antisense	9	5	2
Micro (mi) RNA	4	0	<1
Sense intronic	4	0	1
TEC	3	<1	<1
Unprocessed pseudogene	3	<1	<1
Small nuclear (sn) RNA	3	0	0
Miscellaneous (misc) RNA	3	0	0
Small nucleolar (sno) RNA	2	0	<1
Processed transcript	1	2	1
Transcribed processed pseudogene	1	0	<1
Transcribed unprocessed pseudogene	1	2	0
Ribosomal (r) RNA	1	0	0
Sense overlapping	<1	0	0
Unitary pseudogene	<1	1	0

Human mitochondrial transfer (Mt t) RNA	<1	0	0
pseudogene	<1	0	0
Translated unprocessed pseudogene	<1	0	0
IG C Gene	<1	0	0
3 prime overlapping nc RNA	<1	0	0
TRC gene	<1	<1	0

Table 3.4 Total number of protein coding genes differentially expressed between different ages and regions. Differential analysis and mapping reads to the human genome carried out by Dr Yaobo Xu, Newcastle University.

Differential expression analysis comparisons	**Total number of genes that are differentially expressed**	**Number of genes significantly upregulated**	**Number of genes significantly downregulated**
9 - 12 PCW	1637	1013	624
Anterior- Posterior at 9 PCW	109	29	80
Anterior- Posterior at 12 PCW	151	144	7

Due to the very large number of differentially expressed genes, only the 1637 protein coding genes were examined further. There were a larger number of protein coding genes upregulated (1013) with age compared to downregulated (624) with age (table 3.4). This was not the case when considering all categories of reads, which gave a higher fraction of downregulated compared to upregulated genes (table 3.2). P value and fold change can both be used as indicators as to how likely the change in expression seen is significant. We decided on a cut off adjusted P value of less than 0.05 for assessing biologically significant changes. The top 200 protein coding genes with the highest fold change and an adjusted p value <0.05 can be seen in supplementary table 3.1 and the top 200 protein coding genes with the most significant P value can be seen in supplementary table 3.2. Of these two datasets generated, 81 genes (40%) were present in both suggesting that less than half of the genes with the most significant P values, also had the largest fold changes (figure 3.4A; Supplementary table 3.3).

3.2.4 Gene ontology analysis for differentially expressed genes between 9 and 12 PCW reveals an enrichment in cellular differentiation

Gene ontology analysis (chapter 2.14.4) was carried out on the top 200 protein coding genes with the highest fold change from 9 to 12 PCW as well as the top 200 with the lowest p value. Both the molecular function and the biological processes were examined in the enrichment analysis. For molecular function, the list of genes with the highest fold change contained a higher than expected number of genes whose proteins had G-protein coupled amine receptor activity (figure 3.5). In terms of biological processes, the most enriched terms included tissue development and cell differentiation (figure 3.5). The top 200 protein coding genes with the most significant p values were also subjected to GO analysis. There were fewer suggested biological processes in this analysis with the enriched processes being multicellular organismal development and tissue development (figure 3.6). No molecular function categories could be distinguished from this gene set.

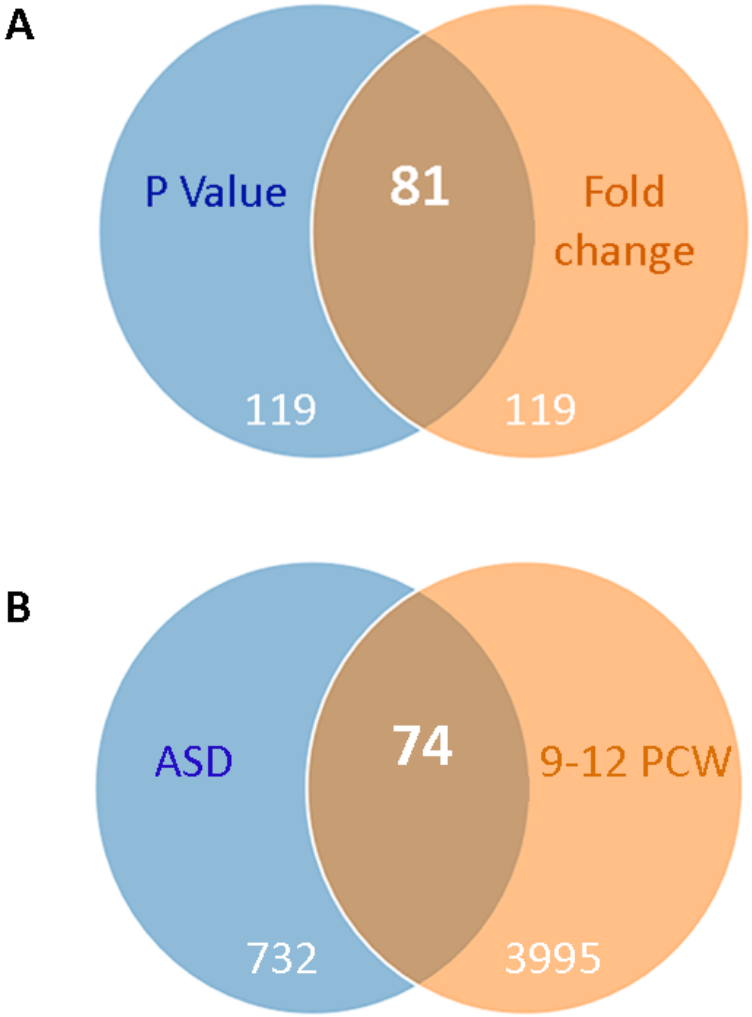


Figure 3.4 (A) Venn diagram comparing differentially expressed genes identified using p value <0.05 and fold change >2. The top 200 differentially expressed genes with the lowest p values compared with the top 200 differentially expressed genes with the largest fold changes. There were 81 genes that were identified as differentially expressed between 9 and 12 PCW that had both the lowest p values and the highest fold change. All 200 fold genes had a p value <0.05. **(B) Venn diagram showing the number of ASD susceptibility genes that were differentially expressed between 9 and 12 PCW.** 74 identified ASD susceptibility genes were differentially expressed between 9 and 12 PCW in the developing human cortex.

Molecular Function

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO molecular function complete	#	#	expected	Fold Enrichment	+/-	P value
G-protein coupled amine receptor activity (GO:0008227)	52	7	.48	> 5	+	1.70E-03
Unclassified	4166	28	38.43	.73	-	0.00E00

Biological Process

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO biological process complete	#	#	expected	Fold Enrichment	+/-	P value
tissue development (GO:0009888)	1601	37	14.77	2.51	+	1.56E-03
cell differentiation (GO:0030154)	3372	57	31.11	1.83	+	1.73E-02
cellular developmental process (GO:0048869)	3543	58	32.68	1.77	+	3.76E-02
multicellular organismal development (GO:0007275)	4571	73	42.17	1.73	+	2.67E-03
system development (GO:0048731)	4011	64	37.00	1.73	+	2.26E-02
single-multicellular organism process (GO:0044707)	6193	95	57.13	1.66	+	6.18E-05
anatomical structure development (GO:0048856)	4699	72	43.35	1.66	+	1.71E-02
developmental process (GO:0032502)	5291	81	48.81	1.66	+	2.37E-03
single-organism developmental process (GO:0044767)	5209	78	48.05	1.62	+	1.19E-02
multicellular organismal process (GO:0032501)	6444	96	59.44	1.61	+	2.33E-04
response to stimulus (GO:0050896)	7621	103	70.30	1.47	+	8.90E-03
single-organism process (GO:0044699)	12755	158	117.66	1.34	+	1.84E-06
single-organism cellular process (GO:0044763)	11415	140	105.30	1.33	+	1.51E-03
biological_process (GO:0008150)	16542	178	152.59	1.17	+	2.95E-03
Unclassified	4272	15	39.41	.38	-	0.00E00

Figure 3.5 **Gene ontology analysis of differentially expressed genes with the largest fold change and an adjusted p value of <0.05 showing both molecular function and biological process.** The identified molecular function category identified from this gene list was G protein coupled amine receptor activity. Biological process categories identified included but were not limited to tissue development, cell differentiation and multicellular organismal development. The 'Homo sapiens' column states the total number of human genes that are present in this category. The '#' column shows the number of genes from this category that were present in the gene list. The 'expected' column states the number of genes you would expect to find by chance in a gene list of this size for that category. 'Fold enrichment' is the

number of genes present in the list divided by the expected number of genes in each category. P value shows if the fold enrichment is significant. P<0.05 showed that there was a significantly higher number of genes in that particular category identified in the gene list.

Molecular Function

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO molecular function complete	#	#	expected	Fold Enrichment	+/-	P value
Unclassified	4166	26	39.43	.66	-	0.00E00

Biological Process

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO biological process complete	#	#	expected	Fold Enrichment	+/-	P value
tissue development (GO:0009888)	1601	35	15.15	2.31	+	2.33E-02
regulation of multicellular organismal process (GO:0051239)	2381	45	22.54	2.00	+	3.47E-02
Unclassified	4272	23	40.43	.57	-	0.00E00

Figure 3.6 Gene ontology analysis of differentially expressed genes with the lowest p values (<0.01) showing both molecular function and biological process. There were no identified molecular function categories identified from this gene list. Biological process categories identified were tissue development and regulation of multicellular organismal process. The 'Homo sapiens' column states the total number of human genes that are present in this category. The '#' column shows the number of genes from this category that were present in the gene list. The 'expected' column states the number of genes you would expect to find by chance in a gene list of this size for that category. 'Fold enrichment' is the number of genes present in the list divided by the expected number of genes in each category. P value shows if the fold enrichment is significant. P<0.05 showed that there was a significantly higher number of genes in that particular category identified in the gene list.

3.2.5 Differentially expressed genes between 9-12 PCW include cell adhesion molecules and ASD susceptibility genes

Genes encoding cell adhesion molecules were among those identified as differentially expressed between 9 and 12 PCW (figure 3.7A). Cadherins and protocadherins mediate cell-cell recognition and adhesion (reviewed in Harris and Tepass, 2010). Integrins function to connect the cytoskeleton to the extra-cellular matrix and mediate signalling (Juliano, 2002). The majority of these genes were upregulated between 9 and 12 PCW with only *CDH19* and *ITGBL1* being downregulated (figure 3.7A). The highest fold increases were observed with PCDH11Y and ITGB5 increasing more than 2 fold from 9 to 12 PCW.

Genes whose protein products are associated with the autism susceptibility genes *neurexins* were identified in this data set (table 3.5). Neurexophilin 1 (NXPH3), Leucine rich repeat transmembrane 1 (LRRTM1) and contactin associated protein like 2 (CNTNAP2) have been shown to either interact with NRXN or, in the case of CNTNAP2, is a member of the NRXN superfamily acting as a cell adhesion molecule, containing epidermal growth factor repeats and laminin G domains (Rodenas-Cuadrado *et al*., 2014). Both *NXPH3* and *LRRTM1* were upregulated with age whereas *CNTNAP2* decreased in expression with age (figure 3.7B). Within the 1635 genes identified as differentially expressed between 9 and 12 PCW, almost 5% (74 genes) were autism susceptibility genes (figure 3.4B). The list of ASGs that were differentially expressed between 9 and 12 PCW can be seen in table 3.5. Of these ASD susceptibility genes, the majority increase in expression with age (supplementary table 3.4). *Semaphorin 7A* (*SEMA7A*) increased in expression from 9-12 PCW (figure 3.7C). In neurons it has been shown to promote axon growth (Jeroen Pasterkamp *et al*., 2003) and is required for the maturation of cortical circuits (Carcea *et al*., 2014).

Transcription factors *special AT sequence binding protein 2* (*SAT2B*) and *NK2 homeobox 2* (*NKX2.2*) were both upregulated in the developing cortex from 9-12 PCW (figure 3.7C). SATB2 has been previously identified as a layer V marker (Ip *et al*., 2011) and can regulate transcription of genes such as *CTIP2* by altering the chromosomal structure. It is required for the correct extension of corticocortical fibres through the corpus callosum (Alcamo *et al*., 2008). It was upregulated between 9 and 12 PCW (figureE 3.7C) which is expected due to the formation of layer V in the cortical plate. NKX2.2 protein is required for the switch in progenitor cell differentiation from neurons to produce oligodendrocytes (Zhou *et al*., 2001).

3.2.6 A higher proportion of genes were upregulated in the anterior cortex at 9 PCW

Differential expression analysis was then carried out to identify genes that significantly differ in expression between the anterior and posterior cortex at both 9 and 12 PCW. The number of genes that were differentially expressed between these cortical regions was significantly less than those differentially expressed between ages (table 3.2). At 9 PCW, 146 genes were differentially expressed between anterior and posterior cortex, of which 75% of them were protein coding (table 3.3). 110 genes were significantly more highly expressed in the anterior cortex compared to the posterior cortex whereas only 36 genes were significantly more highly expressed in the posterior compared to the anterior cortex (table 3.2, supplementary table 3.4).

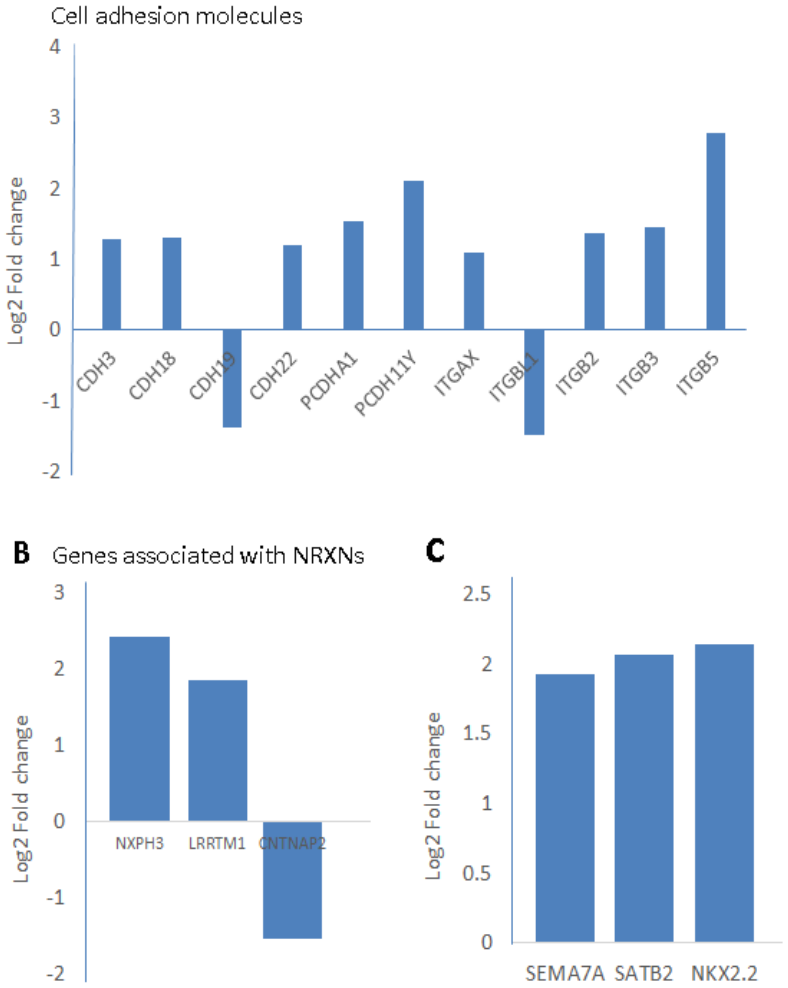


Figure 3.7 Genes that were differentially expressed between 9 and 12 PCW. (A) Cell adhesion molecules cadherins, protocadherins and integrins appeared in the list (table) of differentially expressed genes between 9 and 12 PCW. Of the cell adhesion genes, only CDH19 and ITGB11 are downregulated with age, being higher in expression at 9 PCW compared to 12 PCW. **(B)** Genes associated with NRXNs were differentially expressed between 9 and 12 PCW. NXPH3 and LRRTM1 were both upregulated at 12 PCW compared to 9 PCW and CNTNAP2 was downregulated. **(C)** SEMA7, SATB2 and NKX2.2 were upregulated with age. SEMA7 plays a role in integrin mediated signalling and cell migration. Transcription factors SATB2 and NKX2.2 were involved in chromatin remodelling and axon guidance respectively.

Table 3.5 ASD susceptibility genes differentially expressed between 9 and 12 PCW and the anterior and posterior cortex at 9 and 12 PCW

9-12 PCW			Anterior- Posterior 9 PCW	Anterior – Posterior 12 PCW
ACHE	GALNT14	NXF5	CDH9	CACNA1D
ADAMTS18	GAS2	PACS1	FOLH1	CNTN6
AGAP1	GLRA2	PCDHA1	FRK	DLX1
ALDH1A3	GRID2	PER1	HTR2A	DNAH10
ALOX5AP	GRID2IP	POU3F2	IL1RAPL2	ERBB4
ASTN2	GRIN2A	PRICKLE2	PCDH8	GABRA1
AVPR1A	GRIN2B	PTGER3	PDE1C	GABRQ
BCAS1	GRM4	PTGS2	POU3F2	GAD1
BRCA2	GRM7	PVALB	ROBO1	GRM4
C12orf57	GSTM1	RBMS3	SYN2	GRM7
CD38	HEPACAM	SATB2		KIRREL3
CD44	HLA-B	SERPINE1		NRXN3
CDH22	HTR3A	SEZ6L2		PCDH19
CNTNAP2	IL1RAPL2	SH3KBP1		PTGS2
DEAF1	ITGB3	SIK1		PTPRT
DLGAP3	KCNJ12	SLC16A3		SLIT3
DNAH10	LMX1B	SLC6A4		SYN3
DPP10	LPL	SLIT3		
DSCAM	LRRC1	SNTG2		
EEF1A2	MEF2C	STX1A		
EPHB6	MOCOS	THBS1		
F13A1	MYH4	TRPC6		
FABP3	NFIX	USP45		
FRMPD4	NPAS2	WNT2		
GADD45B	NRG1			

Figure 3.8 Genes differentially expressed between the anterior and posterior cortex contain ASGs. **(A)** Venn diagram comparing differentially expressed genes between the anterior and posterior cortex at 9 PCW and 12 PCW. At 9 PCW, 146 genes were differentially expressed between the anterior and posterior cortex. At 12 PCW, 185 genes were differentially expressed between the anterior and posterior cortex. 17 of these genes were differentially expressed between the anterior and posterior cortex at both 9 and 12 PCW. **(B)** Venn diagram showing the number of ASD susceptibility genes that were differentially expressed between the anterior and posterior cortex at 9 PCW and **(C)** 12 PCW. 10 and 17 identified ASD susceptibility genes are differentially expressed between the anterior and posterior cortex at 9 PCW and 12 PCW respectively.

Table 3.6 List of 17 differentially expressed genes that overlap between anterior and posterior cortex

Gene Name	Gene Type	9 PCW		12 PCW	
		Log 2 fold	P adjusted	Log 2 fold	P adjusted
CYP26A1	Protein coding	**3.03**	3.75E-12	**1.14**	0.042762
LAMP5	Protein coding	**2.49**	2.16E-07	**1.51**	0.002314
AE000661.37	Processed transcript	**2.29**	6.61E-07	**1.23**	0.025233
LRRTM3	Protein coding	**2.25**	1.07E-07	**1.06**	0.017628
PIP5K1B	Protein coding	**1.69**	4.78E-08	**1.37**	0.001322
CYP26B1	Protein coding	**1.69**	3.67E-05	**1.12**	0.019668
GIPR	Protein coding	**1.67**	0.007744	**1.80**	0.0002
CNIH3	Protein coding	**1.64**	2.68E-05	**1.20**	0.019277
ARAP2	Protein coding	**1.61**	0.003659	**1.36**	0.001466
RP11-517P14.7	Unprocessed pseudogene	**1.41**	0.00345	**1.07**	0.013836
LRRC9	Protein coding	**1.41**	0.008442	**1.04**	0.00747
C11orf63	Protein coding	**1.35**	0.028916	**1.32**	0.005681
SYNJ2	Protein coding	**1.24**	0.005558	**1.36**	0.000432
CA8	Protein coding	**1.20**	0.016209	**1.75**	0.000125
SYNM	Protein coding	**1.19**	0.029353	**1.15**	0.003113
NPY5R	Protein coding	**1.13**	0.033269	**1.53**	0.001127
CHRNA3	Protein coding	**1.54**	8.82E-06	**1.02**	0.017992

*Red = higher expression in the anterior cortex, blue = higher expression in the posterior cortex

3.2.7 The majority of differentially expressed genes at 12 PCW were upregulated in the posterior cortex

At 12 PCW, 168 genes were significantly more highly expressed in the posterior cortex compared to the anterior cortex, whilst only 17 genes were significantly more highly expressed in the anterior cortex compared to the posterior cortex (table 3.2, supplementary table 3.5). Looking at only the protein coding genes showed the same pattern with a higher number of genes significantly higher in expression in the posterior compared to anterior cortex at 12 PCW (table 3.4).

To compare if genes involved in anterior-posterior arealisation at 9 PCW were overlapping with the genes involved in anterior-posterior arealisation at 12 PCW, the two data sets were compared. This yielded only 17 genes that were differentially expressed to a significant degree between the anterior and posterior cortex at both 9 and 12 PCW (figure 3.8A, table 3.5). This suggests that not only are the gene expression patterns of the anterior and posterior different at both 9 and 12 PCW, but that the gene expression profiles of both the anterior and posterior cortex change from 9-12 PCW. Of those 17 genes that changed significantly in expression at both 9 and 12 PCW, the majority of them show a transient wave of gene expression, being higher in expression in the anterior cortex at 9 PCW and then higher in the posterior cortex at 12 PCW.

3.2.8 Analysis of the gene set identified as differentially expressed between the anterior and posterior cortex at 9 PCW

Gene ontology analysis was carried out on the differentially expressed genes between anterior and posterior at 9 PCW (figure 3.9). The molecular function analysis at 9 PCW showed an enrichment for ion binding. The biological processes analysis showed an enrichment of genes involved in tissue development, anatomical structure morphogenesis, organ development and developmental process (figure 3.9).

Genes involved in cortical arealisation could code for proteins that act morphogenetically, for example Bone morphogenic proteins (BMPs), Wingless-type MMTV integration site family (Wnts) and fibroblast growth factors (FGFs) (see chapter 1.1). Alternatively, they may be genes that are up and down-regulated as a result of morphogen gradients and act to further define cortical regions by controlling the cellular processes occurring in that region

either directly or indirectly for example transcription factors such as SP8 (see chapter 1.1). At 9 PCW, *FGFR3* and the Wnt receptor *Frizzled class receptor 7* (*FZD7*) were significantly upregulated in the posterior cortex, whereas, *WNT3* was significantly upregulated in the anterior cortex (figure 3.10A). FGF3 has been identified as a marker of the posterior cortex in both rodents and humans (Moldrich *et al*., 2011, Bayatti *et al*., 2008a). WNTs have distinct roles in cell-cell signalling during morphogenesis of the developing neural tube (Dorsky *et al*., 1998, Ikeya *et al*., 1997) and in the patterning of the developing cortex of mice (Shimogori *et al*., 2004), but their roles as morphogens in the human cortex at these ages is largely unexplored.

A previous microarray study investigating expression gradients in the developing human brain (Bayatti *et al*., 2008a) identified cell adhesion molecules as being upregulated anteriorly. Members of the cadherin and protocadherin superfamilies *CDH7, CDH9, PCDH8*, *PCDH17* appear in the differentially expressed genes from anterior-posterior cortex at 9 PCW (figure 3.11B). CDH7, PCDH8 and PCDH17 are more highly expressed in the anterior cortex whereas CDH9 is more highly expressed in the posterior cortex (figure 3.10A). *PCDH17* was identified previously as an anterior marker (Ip *et al.,* 2010) but the study did not identify the three other adhesion molecules. LRRTM3, a neurexin binding protein, is also higher in expression in the anterior cortex (figure 3.10A).

Due to previous studies suggesting that autism susceptibility genes are expressed at these ages in the human cortex (Ip *et al*., 2010), we wanted to see if any ASGs changes in expression between the anterior and posterior cortex. If this was the case, these genes may be contributing to cortical arealisation. It was found that there were 10 ASD susceptibility genes differentially expressed between the anterior and posterior cortex (figure 3.8A, table 3.5). The majority of these are higher in expression in the anterior cortex with only *CDH9* and the synaptic vesicle protein, synapsin II (*SYN2*) being higher in expression in the posterior cortex (figure 3.10B). ASD susceptibility genes that are upregulated in the anterior cortex include but are not limited to *roundabout axon guidance receptor homolog 1* (*ROBO1*) which is involved in axon guidance and cell migration (Kidd *et al*., 1998), *serotonin receptor 5 hydroxytryptamine receptor 2A* (*HTR2A*) and the *transcription factor pou class 3 homeobox 2* (*POU3F2*) which labels neural progeny switching from deep-layer Ctip2 (+) identity to Satb2 (+) upper-layer fate (Dominguez *et al*., 2013) (figure 3.10B).

Molecular Function

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO molecular function complete	#	#	expected	Fold Enrichment	+/-	P value
ion binding (GO:0043167)	6075	57	32.98	1.73	+	4.11E-03
Unclassified	4166	16	22.62	.71	-	0.00E00

Biological Process

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO biological process complete	#	#	expected	Fold Enrichment	+/-	P value
tissue development (GO:0009888)	1601	27	8.69	3.11	+	7.55E-04
anatomical structure morphogenesis (GO:0009653)	2341	33	12.71	2.60	+	1.37E-03
organ development (GO:0048513)	2784	34	15.11	2.25	+	2.33E-02
anatomical structure development (GO:0048856)	4699	50	25.51	1.96	+	2.14E-03
developmental process (GO:0032502)	5291	53	28.73	1.85	+	5.27E-03
single-organism developmental process (GO:0044767)	5209	52	28.28	1.84	+	8.29E-03
Unclassified	4272	10	23.19	.43	-	0.00E00

Figure 3.9 Gene ontology analysis of differentially expressed (adjusted p<0.05) genes between anterior and posterior cortex at nine PCW. The molecular function category identified in this gene list was 'ion binding'. The biological process categories identified from the gene list in order of most to least significant were 'tissue development', 'anatomical structure morphogenesis', 'organ development', 'anatomical structure development', developmental process' and 'single organism developmental process'.

'Homo sapiens' column states the total number of human genes that are present in this category. '#' column shows the number of genes from this category that were present in the gene list. 'expected' column states the number of genes you would expect to find by chance in a gene list of this size for that category. 'Fold enrichment' is the number of genes present in the list divided by the expected number of genes in each category. P<0.05 shows that there was a significantly higher number of genes in that particular category identified in the gene list than would be identified by chance.

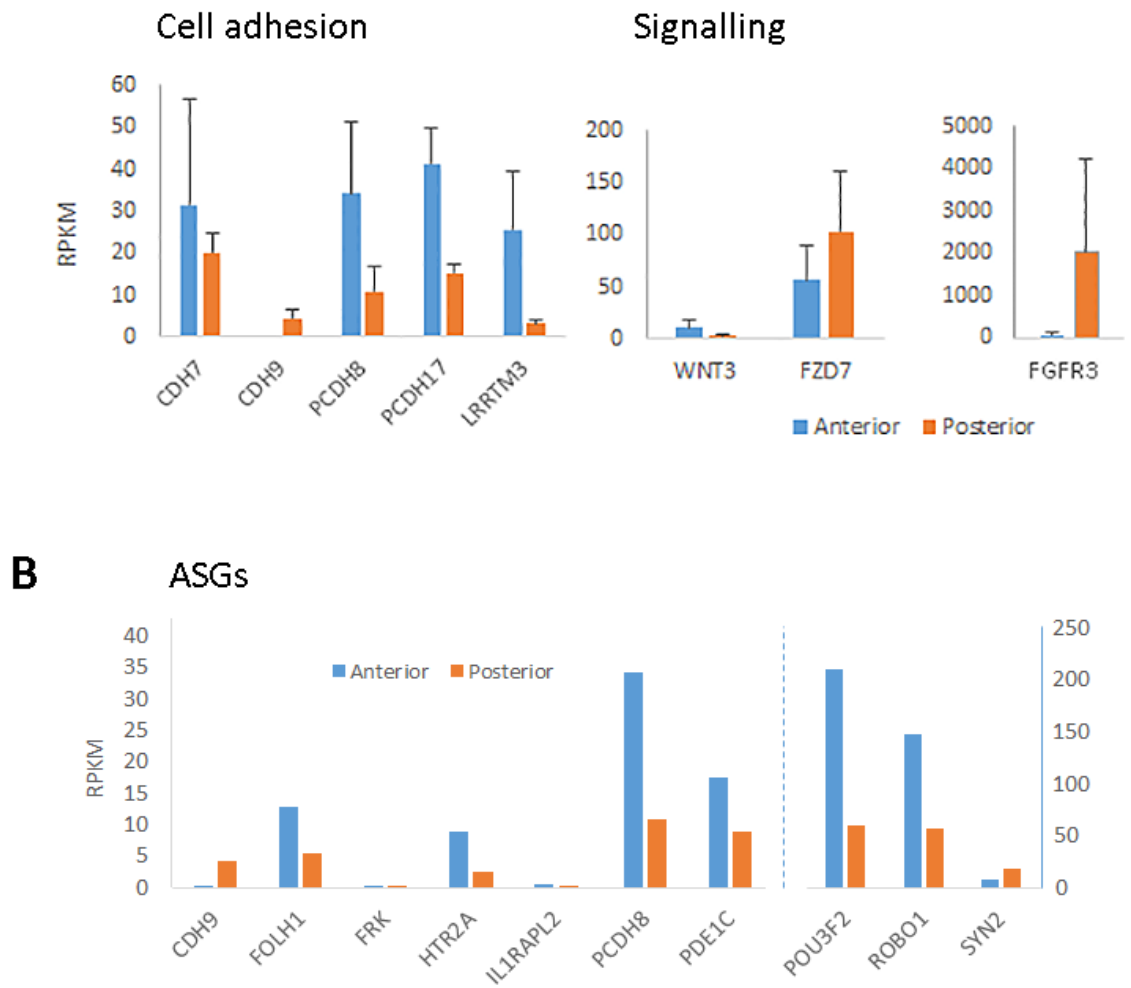


Figure 3.10 Genes that were differentially expressed between the anterior and posterior cortex at 9 PCW. (A) Genes that were differentially expressed between anterior and posterior cortex include the cell adhesion molecules *CDH7, CDH9, PCDH8, PCDH17* and *LRRTM3.* Signalling molecules *WNT3, FZD7* and *FGFR3* were also differentially expressed between the anterior and posterior. Of these genes *CDH9, FZD7* and *FGFR3* had a significantly higher expression level in the posterior cortex. **(B)** A number of autism susceptibility genes were differentially expressed between 9 and 12 PCW, the majority of which were significantly higher in expression in the anterior cortex. *CDH9* and *SYN2* were significantly higher in the posterior cortex.

*Significance adjusted p<0.05.

3.2.9 Identified gene expression gradients at 12 PCW

Gene ontology analysis was carried out on the genes identified as differentially expressed between the anterior and posterior cortex at 12 PCW (figure 3.11). The molecular function analysis yielded an enrichment of genes in the categories of neurotransmitter receptor activity, signalling receptor activity, signal transducer activity and molecular transducer activity. Molecular function analysis included an enrichment in the functions of neurotransmitter transport, synaptic transmission, cell-cell signalling, neuron differentiation and the regulation of signalling (figure 3.10).

Since GO analysis revealed that a large number of differentially expressed genes code for proteins that function in neurotransmitter production, uptake and release and these are also highly represented in the list of ASGs, we examined these in more detail. The list includes *solute carrier family 32 member 1* (*SLC32A1*), *glutamate decarboxylase 1* (*GAD1*) and *synapsin 3* (*SYN3*). SLC32A1 is involved in the uptake of GABA and glycine into synaptic vesicles (McIntire *et al*., 1997), GAD1 catalyses the production of GABA (Fenalti *et al*., 2007) and SYN3 has been associated with neurotransmitter release (Feng *et al*., 2002). The glutamate transporter solute carrier family member 17 member 8 (SLC17A8), synaptogamin like 5 (SYTL5) and rabphillin 3A (RPH3A) also appear in the list of differentially expressed genes and are predicted to have roles in synaptic vesicle trafficking (Colvin *et al*., 2010, Seal *et al*., 2008, Li *et al*., 1994). All are significantly ($p<0.05$) more highly expressed in the posterior cortex compared to the anterior cortex (figure 3.12A).

Both inhibitory and excitatory neurotransmitter receptors appear in the list of differentially expressed genes. The GABA receptor subunits, gamma amino butyric acid A receptor theta (GABRQ) and (GABRA1) and the glutamate receptors, glutamate receptor metabotropic (GRM) 4, GRM7 and GRIN2C were identified as being significantly more highly expressed in the posterior cortex compared with the anterior cortex at 12 PCW (figure 3.12B). Glutamate Receptor Interacting Protein 2 (GRIP2) is also more highly expressed in the posterior cortex at 12 PCW (figure 3.12B). Genes encoding cell adhesion molecules *Neurexin 3* (*NRXN3*), *cadherin 6* (*CDH6*), *protocadherin 19* (*PCDH19*) also appears to be significantly ($p<0.05$) more highly expressed in the posterior cortex at 12 PCW (figure 3.12C). In total, there were 17 genes that have been identified as differentially expressed between anterior and posterior cortex at 12 PCW that are autism susceptibility genes (figure 3.8C, table 3.5).

Molecular Function

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO molecular function complete	#	#	expected	▼ Fold Enrichment	+/-	P value
neurotransmitter receptor activity (GO:0030594)	86	7	.63	> 5	+	1.01E-02
signaling receptor activity (GO:0038023)	1360	28	10.00	2.80	+	1.74E-03
signal transducer activity (GO:0004871)	1644	31	12.08	2.57	+	2.60E-03
receptor activity (GO:0004872)	1567	29	11.52	2.52	+	8.92E-03
molecular transducer activity (GO:0060089)	1848	32	13.58	2.36	+	1.03E-02
Unclassified	4166	22	30.62	.72	-	0.00E00

Biological Process

Export results

Displaying only results with P<0.05; click here to display all results

	Homo sapiens (REF)	upload_1				
GO biological process complete	#	#	expected	▼ Fold Enrichment	+/-	P value
neurotransmitter transport (GO:0006836)	160	9	1.18	> 5	+	2.69E-02
bone development (GO:0060348)	165	9	1.21	> 5	+	3.43E-02
synaptic transmission (GO:0007268)	560	23	4.12	> 5	+	2.43E-07
behavior (GO:0007610)	527	19	3.87	4.90	+	1.16E-04
skeletal system development (GO:0001501)	474	16	3.48	4.59	+	4.07E-03
cell-cell signaling (GO:0007267)	911	28	6.70	4.18	+	1.13E-06
neuron differentiation (GO:0030182)	1168	24	8.59	2.80	+	4.00E-02
generation of neurons (GO:0048699)	1551	29	11.40	2.54	+	2.32E-02
anatomical structure morphogenesis (GO:0009653)	2341	42	17.21	2.44	+	2.29E-04
nervous system development (GO:0007399)	2271	37	16.69	2.22	+	2.01E-02
regulation of signaling (GO:0023051)	2870	45	21.10	2.13	+	3.26E-03
regulation of biological quality (GO:0065008)	3134	49	23.04	2.13	+	8.52E-04
regulation of cell communication (GO:0010646)	3019	46	22.19	2.07	+	5.23E-03
organ development (GO:0048513)	2784	42	20.46	2.05	+	2.50E-02
system development (GO:0048731)	4011	60	29.48	2.03	+	7.09E-05
multicellular organismal development (GO:0007275)	4571	61	33.60	1.82	+	3.60E-03
anatomical structure development (GO:0048856)	4699	61	34.54	1.77	+	9.60E-03
single organism signaling (GO:0044700)	5192	66	38.17	1.73	+	5.39E-03
signaling (GO:0023052)	5195	66	38.19	1.73	+	5.51E-03
cell communication (GO:0007154)	5325	67	39.14	1.71	+	6.10E-03
single-organism developmental process (GO:0044767)	5209	64	38.29	1.67	+	3.12E-02
single-multicellular organism process (GO:0044707)	6193	76	45.52	1.67	+	1.48E-03
multicellular organismal process (GO:0032501)	6444	77	47.37	1.63	+	3.65E-03
single-organism cellular process (GO:0044763)	11415	114	83.91	1.36	+	3.13E-03
Unclassified	4272	18	31.40	.57	-	0.00E00

Figure 3.11 Gene ontology analysis of differentially expressed genes between anterior and posterior cortex at 12 PCW. See page 93 for figure legend.

Figure 3.11 Gene ontology analysis of differentially expressed genes between anterior and posterior cortex at 12 PCW. The molecular function categories identified in this gene list in order of most to least significant was 'neurotransmitter receptor activity', 'receptor signalling activity', 'signal transducer activity', receptor activity' and 'molecular transducer activity'. The biological process categories identified from the gene list in order of most to least significant included but were not limited to 'neurotransmitter transport', 'synaptic transmission', 'cell-cell signalling', 'neuron differentiation', 'generation of neurons' and 'cell communication'.

'Homo sapiens' column states the total number of human genes that are present in this category. '#' column shows the number of genes from this category that were present in the gene list. 'expected' column states the number of genes you would expect to find by chance in a gene list of this size for that category. 'Fold enrichment' is the number of genes present in the list divided by the expected number of genes in each category. $P<0.05$ shows that there was a significantly higher number of genes in that particular category identified in the gene list than would be identified by chance.

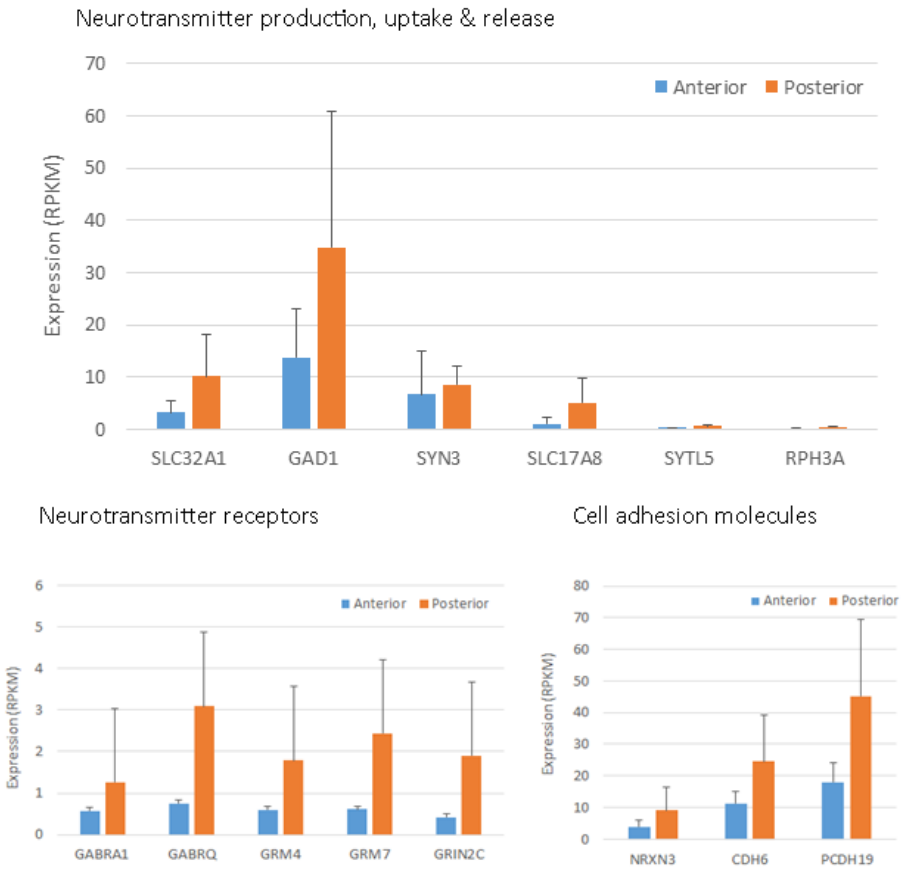


Figure 3.12 Genes that were differentially expressed between the anterior and posterior cortex at 12 PCW. (A) Genes whose protein products have functions in neurotransmitter production, uptake and release were significantly more highly expressed in the posterior cortex compared to the anterior cortex. **(B)** Genes that encode neurotransmitter receptors were significantly more highly expressed in the posterior cortex compared to the anterior cortex. **(C)** Genes that encode cell adhesion molecules were significantly more highly expressed in the posterior cortex compared to the anterior cortex.

Significance = adjusted $p<0.05$

3.3 Discussion

3.3.1 Choroid plexus samples are transcriptionally unique compared to other brain regions

High throughput sequencing of cDNA has increased in popularity over the past decade and has significantly reduced in cost. It has overtaken microarray studies to become the most accurate method for detecting changes in expression across the whole genome (Robinson *et al*., 2015, Naumova *et al*., 2013, Mortazavi *et al*., 2008).

By sequencing 557 samples taken from different brain regions as well as samples from the cerebral choroid plexus and spinal cord, we were able to see that the gene expression profiles of a number of these samples, in particular from the forebrain, hindbrain and choroid plexus, correlated with the region of the brain that they were taken from. This suggests that these parts of the developing brain have distinct patterns of gene expression that are contributing to regional identity. Distinct gene expression profiles and clustering for gross anatomical brain structures has been shown in both the adult (Roth *et al*., 2006) and the developing brain (Oldham *et al*., 2006).

Choroid plexus samples were found to be substantially different in their expression profiles compared to forebrain, midbrain and hindbrain. The choroid plexus is a network of blood vessels surrounded by epithelial cells that are responsible for the production of cerebral spinal fluid. This structure is found within the ventricles of the brain (Lun *et al*., 2015). The epithelial cells of the choroid plexus secrete growth factors, neuropeptides and cytokines including BDNF, TGFα, TGFβ and IGF-II (Emerich *et al*., 2004, Chodobski and Szmydynger-Chodobska, 2001). These molecules have important roles in brain development and abnormal production can be pathological (Stopa *et al*., 2001). The choroid plexus also secretes neuroprotective factors, essential for neuronal survival and repair (Emerich *et al*., 2004) functions to remove waste substances and it has been shown more recently, influences neural progenitors and may have roles in brain regionalisation during development (reviewed in Lun *et al*., 2015) . The telencephalic, diencephalic and hindbrain choroid plexus emerge as distinct structures before the telencephalic and diencephalic choroid plexus merge (reviewed in Dziegielewska *et al*., 2001). Lineage specification involves the repression of neural genes in the neuroepithelial cells and the upregulation of basic helix-loop-helix transcription factors (Imayoshi *et al*., 2008). Given that the choroid plexus consists of non-neural cells, some of which are mesodermal in origin, it is expected that it would have the most divergent transcriptome.

The human brain develops from the neural progenitor cells of the ectodermal layer that is specified during gastrulation. These progenitor cells lie along the rostral axis of the developing embryo forming, what is referred to as, the neural plate. In the third gestational week (GW) the neural tube arises from the folding of the neural plate and its subsequent closure (reviewed in (Copp *et al*., 2003). The cells located caudally will give rise to the hindbrain and spinal cord whilst the cells located rostrally will give rise to the forebrain (Stiles and Jernigan, 2010a). Given that the cells of the hindbrain and forebrain are separated in the neural tube at 3 GW, by 8 PCW they will have been subjected to distinct signalling environments (chapter 1.1) and the difference in the transcriptional profiles of the hindbrain and forebrain samples reflects this. The midbrain progenitor cells, which have been positioned between the forebrain and hindbrain during development, are likely to have been partly subjected to the signalling environments of both the forebrain and hindbrain cells and so it is unsurprising that they are the least distinct population of cells in the RNA seq data.

3.3.2 Factors affecting the ability to detect significant temporal and regional differences in cortical gene expression

Looking more specifically at the cerebral cortex samples between 9 and 12 PCW, it was shown that they do not cluster according to age (PCW), sex or regional identity. Previously, Kang *et al.* (2011) found that age contributed more to variability than sex, ethnicity and inter sample variation and PCA analysis by the group showed that 8-10 PCW samples clustered separately from 11-13 PCW samples. However, this PCA analysis was carried out on sequencing data from all brain regions rather than just cortical samples at these ages.

It is hard to assign functional identity to particular cortical region at this age as many structures are undefined and relating developing regions to post-natal structures would be inaccurate. This was overcome by referring to the regions as anterior, central, posterior and temporal. It has been observed that gradients of gene expression are developing across the cortex in order to specify cell fate (Ip *et al*., 2011, Bayatti *et al*., 2008a) and, ultimately, to produce the distinct cortical structures that we recognise in the adult brain. However, differences in gene expression between the regions were not apparent from the hierarchal clustering. Rather, variation between samples is higher than variation within the samples. This was expected firstly because neurons, in particular, of the different cortical regions are

more similar in function to each other than they are to neurons of sub cortical brain regions and secondly, they have had less time to become transcriptionally different, having all originated from a small group of rostrally located neural tube cells. It is also more difficult to control for post mortem delay and external environmental influences when working with human samples. These factors will have an effect on the signalling environment that the cells of the cortex are subjected to and they could mask smaller transcriptional differences that could exist between cortical regions, or between samples of different sex, or age. Although PCA analysis managed to separate anterior cortical samples from posterior cortical samples at both 9 and 12 PCW, cluster analysis suggests that individual variation is the main contributing factor to this. (Kang *et al*., 2011) did not look at transcriptional patterns within the cortex but instead grouped all cerebral cortex samples together.

Multiple software packages exist to aid in the identification of differentially expressed genes between two sample groups. These packages assess not only the average change in expression values between the two groups but also the variability in expression values between replicates within a group (Seyednasrollah *et al*., 2015). As RNA sequencing technology has advanced, it is now common practice to have a minimum of 3 replicates per sample group. An increased number of replicates makes it easier to identify significant differences in expression between two sample groups. This makes any gene expression differences detected more robust.

The DESeq software package used in this study is based on a negative binomial model (Anders and Huber, 2010). It takes into account existing biological inter-sample group variation when creating lists of differentially expressed genes. However, these software packages can seem conservative due to their requirements for identifying differences when producing the gene lists. A conservative approach will minimise the number of false positives yet may fail to identify a number of differentially expressed genes that become lost in the noise. Compared to other well-known differential expression software packages such as Cuffdiff 2 and edgeR, DESeq falls between the two, being more conservative than edgeR but less conservative than Cuffdiff 2. Increasing the number of sample replicates will usually increase the number of genes that are differentially expressed as well as decreasing the number of false positives (Seyednasrollah *et al*., 2015). Another problem with this type of differential expression analysis is that many of the genes identified as differentially expressed will have extremely low levels of expression. Often, a cut-off value for expression

is decided but studies have detected protein for genes with RPKM values below this threshold value (Nagaraj *et al*., 2011, Mortazavi *et al*., 2008). An increased read length in addition to increased sequencing depth enables a more accurate RPKM value to be obtained and subsequently differential expression analysis should yield fewer false positive results.

3.3.3 There were fewer differentially expressed genes between cortical regions compared to between ages

Despite the lack of clustering by age/ cortical region, differential expression analysis between 9 and 12 PCW and between the anterior and posterior cortex was able to identify genes that changed in expression across the conditions. It was revealed that a higher number of genes differ in expression temporally compared to spatially. In agreement with this finding, (Miller *et al*., 2014) analysed the transcriptional profiles of 15-21 PCW human brains using an Agilent microarray and found that there were major differences between proliferative cells and post-mitotic cell types; however, the areal differences were more subtle. The number of proliferative cells decreases with age whereas, the number of post-mitotic cells increases with age. The majority of these differentially expressed genes between 9 and 12 PCW increased in expression at 12 PCW and GO analysis revealed that they fell into the categories of ion binding and cell differentiation. Kang *et al.,* (2011) found that in the developing human brain, gene expression profiles differ between developmental milestones and that between 10 and 13 PCW, there is an increase in the expression of genes involved in neuronal differentiation, proliferation and cell migration. Therefore, between 9 and 12 PCW, the changes in gene expression may reflect a switch in the neurodevelopmental trajectory.

3.3.4 Fold change analysis can aid in the identification of biologically relevant genes

Just over 40% of the top 200 genes with the most significant (lowest) p value obtained from the differential expression analysis also had the highest fold change between 9 and 12 PCW. Despite this high overlap, GO analysis of these two data sets identified many more biological processes for the data set with the highest fold change. (McCarthy and Smyth, 2009) have suggested that applying fold change parameters to a set of significantly differentially expressed genes in a process known as TREAT will reduce the false discovery rate and

identify more biologically relevant genes. Although TREAT was not used here, it is clear that the genes identified as significantly different ($p<0.05$) that also had the largest fold change, were the most biologically interesting as shown by the gene ontology analysis (discussed further in sections 3.3.5 and 3.3.6). The GO analysis using the genes with the highest fold change identified cell differentiation as an enrichment term. Between the ages of 9 and 12 PCW, the post mitotic cortical plate is expanding with an increasing number of cortical cells exiting the cell cycle and beginning differentiation.

3.3.5 The expression of growth factors between 9 and 12 PCW

Growth factors and morphogens are essential in mammalian development (Sansom and Livesey, 2009b). Just as select combinations of these molecules are able to pattern the developing embryo and induce the formation of different anatomical structures, the temporal and spatial differential expression of such molecules in the brain contribute to its regionalisation and maturation (chapter 1.1).

FGFs 1, 10 and *16* were upregulated with age, whereas *FGF2* expression decreased. In mice, FGF10 is involved in regulating the differentiation of radial glia and thereby controls the expansion of the progenitor pool (Sahara and O'Leary, 2009). At the beginning of cerebral cortex formation, neural stem cells of the ventricular zone (VZ) divide symmetrically to expand the cortical cell population. These stem cells eventually differentiate into radial glial cells that divide asymmetrically, to produce a proliferative RG cell and one that will cease division and migrate out of the VZ to reside in the SVZ or the CP (Lui *et al*., 2011). The knock down of *Fgf10* in mice causes an increase in the size of the anterior cerebral cortex (Sahara and O'Leary, 2009), presumably due to a failure of neural stem cells to differentiate into radial glial cells and switch their mode of cell division from symmetric to asymmetric. An increase in *FGF10* with age would suggest that there are fewer neural stem cells dividing symmetrically in the 12 PCW cortex. *FGF2* is expressed in the VZ of the cerebral cortex and is hypothesised to play a role in the expansion of the neural stem cell population as *FGF2* null mice have a reduced number of cortical neurons in adults (Vaccarino *et al*., 1999). FGF2 is expressed in the VZ of the cerebral cortex and is hypothesised to play a role in the expansion of the neural stem cell population as *Fgf2* null mice have a reduced number of cortical neurons in adults (Vaccarino *et al*., 1999). The decrease in its expression observed in

this study correlates with the increased expression of *FGF16* and these two growth factors may act antagonistically to control the number of proliferating cells in the VZ.

FGF16 is required for cell proliferation in the forebrain of zebrafish embryos but also GABAergic neuron specification (Miyake *et al*., 2014). FGF1 binds to FGFR1 to induce neuronal differentiation and neurite outgrowth in PC12 cells (Lin *et al*., 1997). It is also produced by astrocytes (Ito *et al*., 2005) including radial glial cells. We have observed an increase in genes that are associated with cell differentiation between 9 and 12 PCW (figure 3.5) and the increase in the expression of *FGF16* and *FGF1,* if their functions are similar to those in mice, may induce neuronal differentiation in the human brain. It is plausible that these morphogens act to upregulate the expression of genes involved in specific aspects of neuronal differentiation for example neurite outgrowth and the production and organisation of synaptic proteins.

The expression patterns of these growth factors suggests that 9-12 PCW is a critical period of human cortical development that sees an increase in asymmetric division in line with a reduction of progenitor cell division. During this period, growth factors may act to upregulate genes required for neuronal differentiation.

3.3.6 Morphogens are contributing to the arealisation of the 9 PCW cortex

Two theories, the protomap and the protocortex hypothesis, likely both contribute to the phenomenon of cortical patterning. The protocortex hypothesis states that patterning relies on extrinsic signalling input from the innervating thalamic fibres (O'Leary, 1989, Van der Loos and Woolsey, 1973) whereas the protomap hypothesis suggests intrinsic cortical signalling is responsible (Rakic, 1999). Thalamic afferents have not yet innervated the cortex at the period of development in this study and so gene expression gradients can only be established in accordance with the protomap hypothesis. The protomap theory of arealisation predicts that signalling centres lie along the telencephalic vesicles and release morphogens such as FGFs, Wnts and BMPs (chapter 1.1; Fukuchi-Shimogori and Grove, 2001). FGF8 and EMX2 have previously been shown to produce rostral and caudal cortical cell fates respectively (Cholfin and Rubenstein, 2008) however; these signalling molecules were not identified from the differential expression analysis between the anterior and posterior cortex in this study. We have identified SP8 as an anteriorly expressed gene at 9

PCW. In mice SP8 has been shown to bind regulatory elements of *Fgf8* and also to induce downstream targets of FGF8. This regulatory function of Sp8 is inhibited by Emx2 (Sahara *et al*., 2007). Morphogen gradients tend to disappear with age, leaving behind gradients of downstream targets (Bayatti *et al*., 2008b). Although an *EMX2* mRNA gradient was detected previously at this developmental stage using in situ hybridisation (Bayatti *et al*., 2008b), the difference in *EMX2* gene expression may not have been large enough to be detected by the differential expression analysis software. This study, however, was able to identify SP8 as being upregulated anteriorly in humans, confirming the expression pattern shown in mice.

At 9 PCW, a number of additional genes encoding signalling molecules and receptors were identified as differentially expressed between the anterior and posterior cortex. Anteriorly, *WNT3* was upregulated. The Wnt family of genes are usually associated with a posterior neural identity (McGrew *et al*., 1995) but Wnt3 has been shown to be required for the formation of the mouse hippocampus (Lie *et al*., 2005) and also for the development of the posterior forebrain (Braun *et al*., 2003). However, little is known about its role in cortical arealisation. Dlx2 expression can be induced by blocking Wnt expression (Braun *et al.,* 2003), however, *DLX2* was also anteriorly upregulated at 9 PCW as has been shown by (Al-Jaberi *et al*., 2015) suggesting that the increased WNT3 expression in the anterior cortex at 9 PCW does not reduce the expression of DLX2 in humans.

Caudal markers included FGFR3, which had previously been identified as contributing to a caudal cell fate (Moldrich *et al*., 2011), and FZD7. Frizzled receptors are bound by Wnt proteins to initiate the canonical Wnt pathway (chapter 1.2.3). The differential expression of WNT receptors at 9 PCW could enable controlled WNT signalling without the need for gradients of the protein. It is expected that WNT proteins, whose mRNAs were not identified in this differential expression analysis, will be able to bind FZD7 to promote the differentiation of caudal cell types.

At 12 PCW, only FGF1 was identified as differentially expressed between the anterior and posterior cortex. FGF1 expression is higher in the anterior cortex and is also upregulated with age. It has been shown to induce neuronal differentiation and neurite outgrowth in PC12 cells (Lin *et al*., 1997). Cortical Wnt signalling decreases with age (Harrison-Uy and Pleasure, 2012) and this decrease in expression is expected for FGFs and BMPs also as the increased surface area of the cortex and the signalling centres of the brain are less influential on overall expression gradients. It is likely that at these later developmental stages, the

main arealisation markers are the downstream targets of morphogens that further differentiate cortical regions as diffusion of a signal through a large mass of cells would be difficult.

3.3.7 A number of autism susceptibility genes were differentially expressed regionally and temporally

During this early period of cortical development, we have shown that a number of ASD genes were present and that they were differentially expressed between the anterior and posterior cortex at both 9 and 12 PCW in addition to changing in expression with age (table 3.5). These ASD susceptibility genes code for proteins that have a range functions in the adult brain including cell adhesion, synaptogenesis and synaptic stabilisation, neurotransmitter release, synaptic vesicle transport, neurotransmitter receptors and neuronal differentiation. However, at these early stages of development, synapses are either absent or immature (de Graaf-Peters and Hadders-Algra, 2006) suggesting that these genes are expressed prior to synapse formation.

In addition to synaptic proteins, ASD susceptibility genes include those that encode cell adhesion molecules. Cadherins and protocadherins are involved in tissue morphogenesis (Halbleib and Nelson, 2006), and their functions expand beyond mechanical adhesion between cells. Our analysis comparing gene expression in the anterior and posterior cortex at 9 and 12 PCW yielded a number of cadherin and protocadherin family genes. CDH7 forms transient adhesions with contacting cells (Dufour *et al*., 1999), regulates unbranched axonal outgrowth in cranial motor cells (Barnes *et al*., 2010) and is present in migratory neural crest cells (Nakagawa and Takeichi, 1995). Mutations in *PCDH8* and *PCDH11Y*, identified as differentially expressed between anterior and posterior cortex, have been identified as susceptibility genes for multiple neurological disorders (Durand *et al*., 2006, Giouzeli *et al*., 2004, Bray *et al*., 2002). Cell adhesion genes have previously been associated with arealisation. CDH6, identified as having a higher expression in the posterior cortex at 12 PCW, is suggested to be involved in establishing the protomap in mice (Terakawa *et al*., 2013) whereas, *PCDH19* knockout causes a disruption in columnar organisation of the zebrafish optic tectum (Cooper *et al.,* 2015).

Potentially, as ASGs appear to be expressed between 9 and 12 PCW, ASD symptoms could arise as a result of perturbations in the expression of these genes at this developmental period. The patterning of the human cortex is a process that occurs throughout development, however, during 9-12 PCW, before thalamic axons arrive, the cortex is largely under its own control and deviations from normal gene expression patterns could have substantial effects on neuronal differentiation, migration, circuitry and function.

3.3.8 The posterior cortex is transcriptionally more mature than the anterior cortex

In the adult brain, Roth *et al.* (2006) showed that there were no gene expression differences identified between the different regions of the cerebral cortex, however, multiple studies have identified genes that are differentially expressed across cortical regions during development (Al-Jaberi *et al*., 2015, Ip *et al*., 2010, Bayatti *et al*., 2008a). The transcriptome of the prenatal cortex is more complex than that of the adult brain and a higher percentage of the genome is expressed (Kang *et al.,* 2011). A smaller number of genes were identified as significantly different between the anterior and posterior cortex at 9 and 12 PCW in comparison to the large number of genes differentially expressed temporally. At 9 PCW, the majority of the differentially expressed genes are more highly expressed in the anterior cortex than the posterior cortex as in previous studies (Ip *et al*. 2010). At 12 PCW, there was a shift in this pattern and the majority of differentially expressed genes were more highly expressed in the posterior cortex compared to the anterior cortex. A number of the genes identified as being more highly expressed in the anterior cortex also appeared in the list of genes upregulated in the anterior cortex in a previous microarray study (Ip *et al*., 2010).

The set of genes identified as differentially expressed between the anterior and posterior cortex at 9 and 12 PCW are largely independent. As mentioned, 10-12 PCW represents a new developmental milestone (Kang *et al*., 2011). Only a very small proportion of these genes are differentially expressed at both 9 and 12 PCW adding weight to the theory that these two developmental ages are transcriptionally separate. It is therefore apparent that cortical patterning at 9 PCW requires one set of genes to be anteriorly upregulated whilst patterning at 12 PCW requires a completely different set of genes to be posteriorly upregulated. GO analysis of the differentially expressed genes at 9 PCW failed to identify any biological processes that are neuron specific, whereas at 12 PCW there were many biological processes identified specifically related to neurons. These genes involved with neuron specific

processes are posteriorly upregulated, suggesting that the posterior cortex is maturing faster than the anterior cortex. The prefrontal cortex is evolutionarily more recent and is present in primates (Teffer and Semendeferi, 2012, Smaers *et al*., 2011). Its existence in non-human primates is controversial (Uylings *et al*., 2003) but it is clearly expanded in humans and its maturation is also much slower than the maturation of the visual and auditory cortex (Thompson-Schill *et al*., 2009).

The small number of genes that are differentially expressed at both time points appear to reverse their direction of expression from being upregulated in the anterior cortex at 9 PCW to being upregulated in the posterior cortex at 12 PCW. This graded expression is unlikely to be in response to morphogen gradients and instead may be due to a domino effect. The upregulation of these genes in the anterior at 9 PCW may cause the production of diffusible signals that signal to adjacent cells to upregulate the expression of the same gene. By 12 PCW, this upregulation signal could have propagated to posterior of the cortex whilst the expression at the anterior has been subsequently reduced/ silenced. This theory is similar to those proposed for the temporally progressive BMP patterning in mouse embryos (Tucker *et al*., 2008) and the segmentation clock mechanism (reviewed in Gibb *et al*., 2010).

CHAPTER 4

Investigating the expression patterns of autism susceptibility genes *NRXNs, NLGNs* and *SHANKs,* in the developing human cerebral cortex

4.1 Aim of study

The aim of this study was to decipher the spatial and temporal gene expression patterns of a subset of autism susceptibility genes across the cortex from 8-12 PCW.

Autism susceptibility genes from the *NRXN, NLGN* and *SHANK* families, implicated in synapse development and maintenance, were investigated in this study. Microarray and RNA sequencing studies have previously suggested that members of these gene families may be expressed in the developing cortex from as early as 8 PCW (Ip *et al*., 2011); brainspan.org).

4.2 Results

4.2.1 NRXNs, NLGNs and SHANKs were expressed in the cortex between 8 and 12 PCW

Samples were collected with maternal and ethical consents by the HDBR resource at Newcastle University (http://www.hdbr.org). The identifying number, age, sex and cerebral hemispheres used for each sample can be seen in table 3.1 for RNA sequencing and table 4.1 for qPCR analysis respectively. At least three brains were used per age, with both hemispheres included in as many cases as possible. The cerebral cortex was dissected as previously described into anterior, central, posterior and temporal regions (2.1.2). mRNA was extracted from the dissected cortical regions of 8-12 PCW brains, was reverse transcribed and either sequenced (2.13) or used for non-quantitative and quantitative PCR reactions (2.3). The primer sets used for control genes and genes of interest in both non-quantitative and quantitative PCR can be seen in table 4.2.

A standard PCR reaction was used to ensure, firstly, that the genes of interest were detectable in the developing cerebral cortex from 8-12 PCW, using the designed primer sets and secondly that the primers amplified the correct section of cDNA. Following gel extraction, fluorescent bands were sequenced. *NRXNs 1-3*, *NLGNs 1-4X* and *SHANKs 1-3* were detectable using the designed primer sets at 8, 10 and 12 PCW (figure 4.1). All amplification products had the correct band size (figure 4.1) and the correct DNA sequence

according to sequencing results (not shown). This demonstrates that they were all expressed in the developing human cortex between 8 and 12 PCW. The region of each gene that these amplification products map to can be visualised in figure 4.2.

Table 4.1 List of sample numbers dissected and used for non-quantitative and quantitative PCR

Sample no.	Age (PCW)	Sex F – Female M - Male	Hemispheres used L – Left R - Right
11688	8	F	L & R
1511	8	M	L
11691	8	M	L & R
11653	10	M	R
11703	10	M	L & R
1649	10	M	R
1484	10	M	R
11652	12	F	L & R
11853	12	M	L
11683	12	F	R

***Table 4.2** List **of Primers for PCR and qPCR.** NRXN, Neurexin; NLGN, Neuroligin; SHANK, SH3 and multiple ankyrin domains; SYP, Synaptophysin;* *Reference genes *BACTIN, β-Actin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SDHA, Succinate dehydrogenase complex subunit A.*

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Amplicon Size (bp)
NRXN1	aggacattgacccctgtgag	ccttcatcccggtttctgta	205
NRXN2	catcctcctctacgccatgt	ttgttcttcttggccttgct	165
NRXN3	ggctgagaacaaccccaata	atgctggctgtagagcgatt	179
NLGN1	tgcaaaggggaactatggac	ttgctccaacggttaccttc	177
NLGN2	cagaagggctgttccagaag	gcagacactccacagcttca	150
NLGN3	agagccatcatccaaagtgg	ggatctcagggtcatcagga	244
NLGN4X	tcgctcctcttcctcaacat	acactcgtgatcgtgttcca	180
SHANK1	actctcagcacctggagcat	catcctgttctcggtggttt	243
SHANK2	gtgtacgaacgccaaggaat	tgccgctcttcctctgttat	152
SHANK3	cttcacacaaaggcgaacct	ccaccattcttcagcacctt	191
SYP	tctggccacctacatcttcc	tcagctccttgcatgtgttc	218
*BACTIN	ctacaatgagctgcgtgtggc	caggtccagacgcaggatggc	271
*GAPDH	tgcaccaccaactgcttagc	ggcatggactgtggtcatgag	87
*SDHA	tgggaacaagagggcatctg	ccaccactgcatcaaattcatg	86

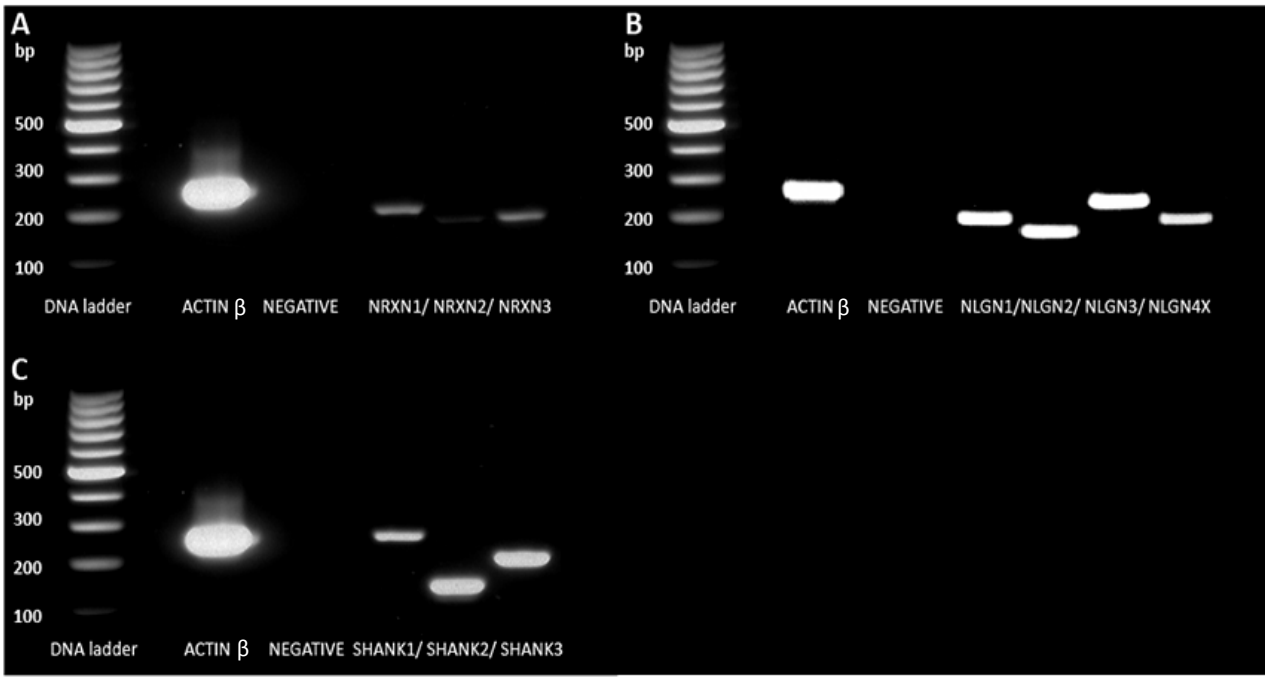


Figure 4.1. Gel electrophoresis confirming the presence of a subset of autism susceptibility genes in the developing human cerebral cortex. The expression of **(A)** NRXNs 1-3, **(B)** NLGNs 1-4X and **(C)** SHANKs 1-3 was detected in the cortex between 8-12 PCW using gene specific primers. mRNA was extracted from tissue samples taken from the anterior, central, posterior and temporal regions of the human cerebral cortex. This RNA was pooled and reverse transcribed for use in non-quantitative PCR reactions. Data shown at 10 PCW.

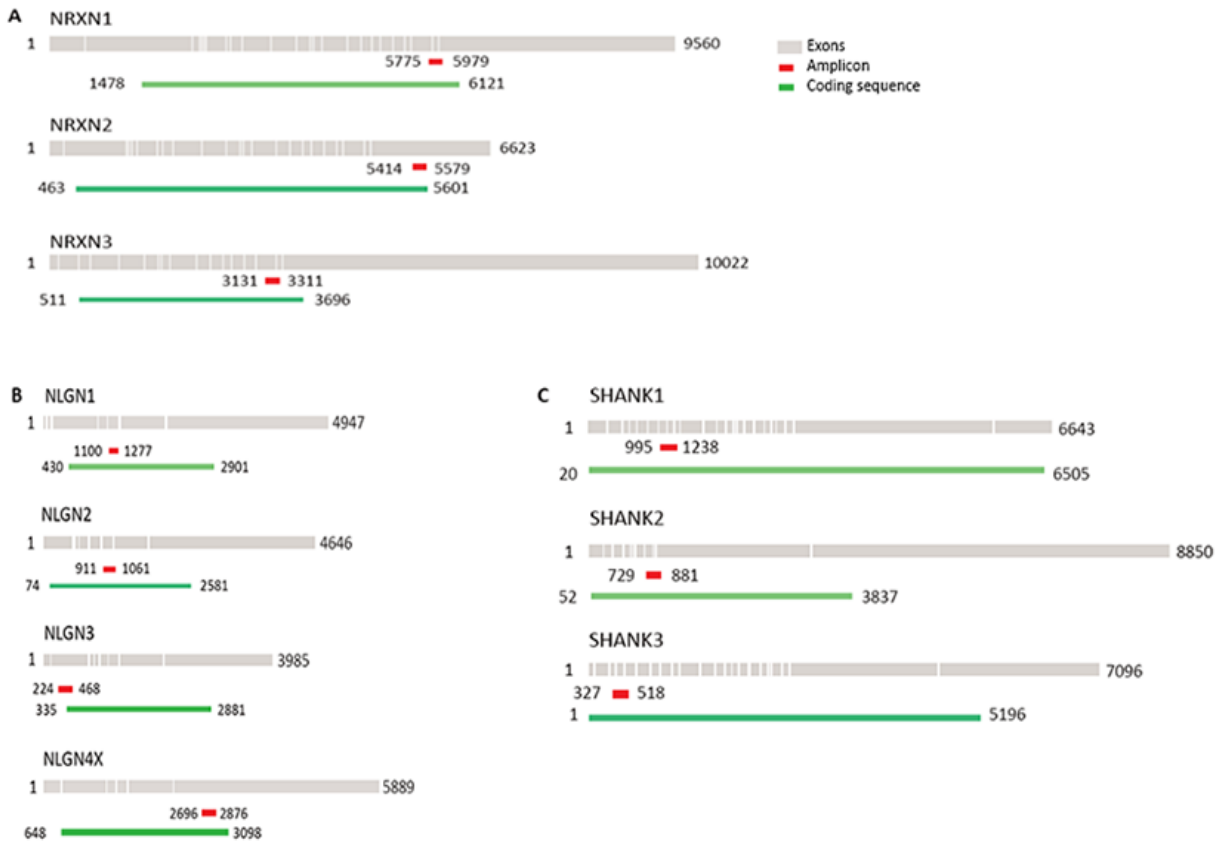


Figure 4.2. Schematic representations of the exons of autism susceptibility genes. (A) NRXNs, **(B)** NLGNs and **(C)** SHANKs. Grey bars represent gene exons and are to scale. Green bars represent the coding sequence of the genes and red bars represent the gene region amplified by the designed PCR primers.

4.2.2 Gene expression values were normalised to three reference genes

For the RNA sequencing data, after normalisation for gene length (2.13), the expression values of different genes within a sample were directly comparable. However, in order to compare gene expression values across samples, the Bioconductor package DeSeq 2 was employed (2.13.2) to account for the variance and the mean of reads per gene across the samples and assumed a negative binomial distribution (Anders and Huber, 2010). Due to the stringent parameters set by such programs, it is difficult to detect small, yet significant differences between samples (Anders & Huber, 2010). Of the *NRXN, NLGN* and *SHANK* genes, only *NRXN3* was identified as differentially expressed between 9 and 12 PCW using this method. For a more accurate comparison with the *NRXN, NLGN* and *SHANK* qPCR data, RPKM values obtained from sequencing were normalised to the expression values of the

same three reference genes, ACTINβ, GAPDH and SDHA used for the qPCR data analysis. These three genes are essential for normal cellular functions and, therefore, are thought to be constant in expression across cell type and across all experimental conditions (Gubern et al., 2009, de Kok et al., 2005).

Quantitative PCR is seen as the 'gold standard' of nucleic acid quantification and was used to validate results obtained from RNA sequencing data and to identify differentially expressed genes between different cortical regions. The comparative CT method assumes that the transcript amplification efficiency values of all primers are 100% and so instead, we used an equation based on the Pfaffl method (Pfaffl, 2001) which takes into account the difference in primer efficiencies. We employed the program data miner (http://www.miner.ewindup.info/) to determine transcript amplification efficiency values for each primer set using the raw fluorescent SYBR readings (chapter 2.3.4). These final expression values were then normalised to the same 3 reference genes as used for the RNA sequencing making the two methods comparable.

For the RNA sequencing data, the $\log_{10}$ of the average RPKM values for the 3 reference genes were compared across conditions (age PCW) and across cortical region. The highest expression values were observed for *ACTINβ* and *GAPDH* whilst *SDHA* had a significantly lower ($p<0.05$) RPKM value in comparison across all ages and cortical regions (figure 4.3). For each sample, the pattern of expression of these three genes remained the same all ages and cortical regions

For the qPCR data, the Ct values (obtained using the data miner program) were compared. These control genes showed the same expression patterns at 8, 10 and 12 PCW as well as in the anterior, central, posterior and temporal regions of the cortex (figure 4.4). Statistical analysis confirmed that *SDHA* had the highest cycle threshold (Ct) value ($p<0.05$) and therefore, the lowest expression level compared to *ACTINβ* and *GAPDH* whose Ct values were not statistically different from each other (figure 4.4). Changing the age of the sample or the cortical region had no effect on the patterns of expression exhibited by the three reference genes making them ideal candidates for normalisation calculations.

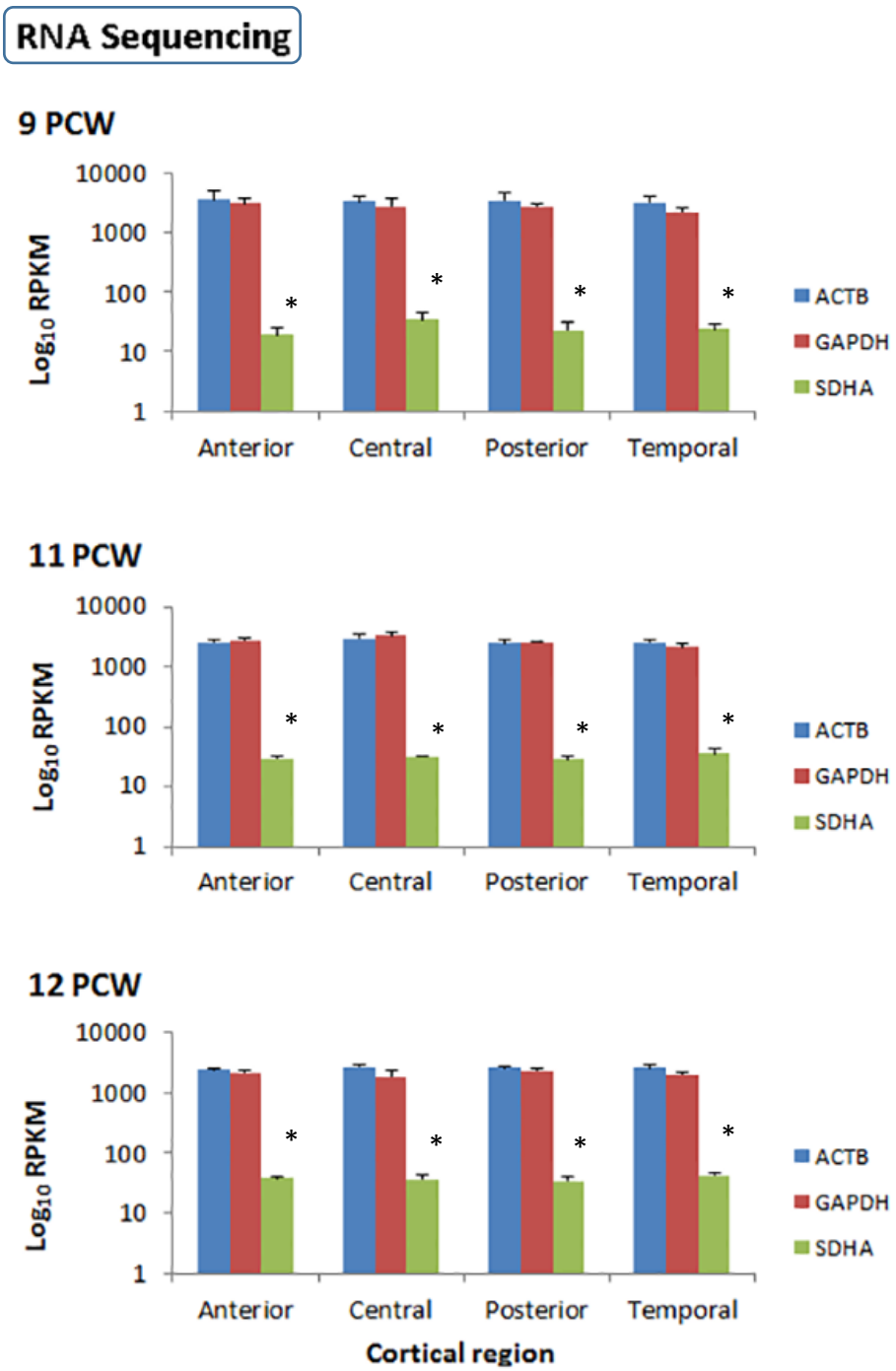


Figure 4.3. Expression (Log_{10} RPKM) of reference genes *βACTIN*, *GAPDH* and *SDHA* obtained from RNA sequencing. The expression values of each of the three reference genes obtained after RNA sequencing and used throughout this study were not significantly different between 9, 11 and 12 PCW or between the anterior, central, posterior and temporal regions of the cortex. *βACTIN* and *GAPDH* were also not significantly different from each other, having RPKM values of more than 1000. The *SDHA* value across samples was less than 100 RPKM and was significantly lower than that of the other two reference genes. *significance $p<0.05$

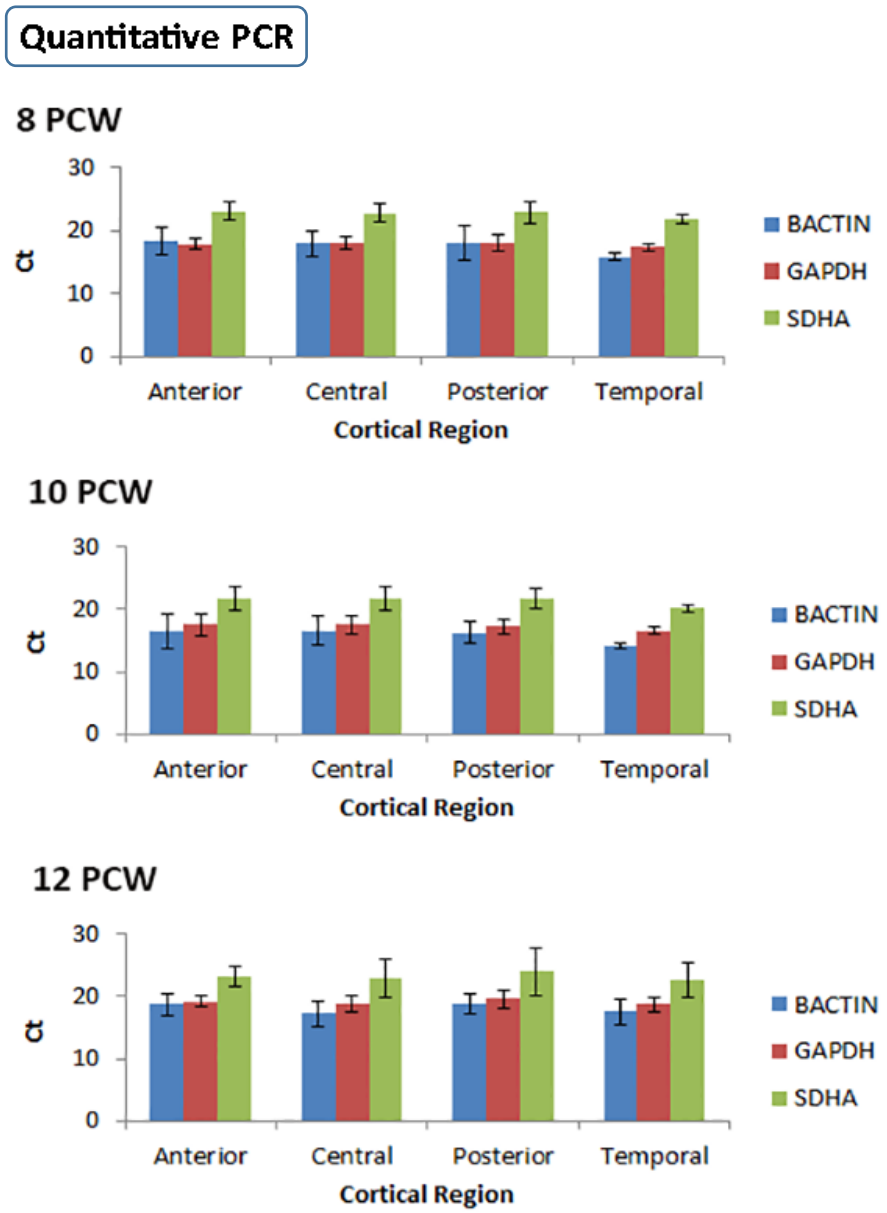


Figure 4.4. Expression (Ct) of reference genes *βACTIN*, *GAPDH*, *SDHA* obtained from quantitative PCR. Lower CT values represent a higher level of expression. The expression values of each of the three reference genes obtained after qPCR and used throughout this study were not significantly different between 9, 11 and 12 PCW or between the anterior, central, posterior and temporal regions of the cortex. The expression of *βACTIN* and *GAPDH* was not significantly different. The *SDHA* expression value was often, but not always, significantly lower than that of the other two reference genes. * significance $p<0.05$

4.2.3 Average expression levels of NRXNs between 8-12 PCW

To obtain average gene expression values for *NRXN*s *1-3*, *NLGN*s *1-4X* and *SHAN*s *1-3* within the cerebral cortex, the values from the anterior, central, posterior and temporal regions of a particular age, sample and hemisphere were combined. The number of brains used for RNA seq and qPCR at each age can be seen in tables 4.1 and 4.2. The expression values were normalised to the expression of the three reference genes. The primer sets used for reference genes and genes of interest in quantitative PCR can be seen in table 4.2.

Due to sample availability, the brains we were able to obtain for RNA sequencing were 9, 11 and 12 PCW of age. The brains used for qPCR were 8, 10 and 12 PCW. The differences in age PCW must be taken into consideration in the comparisons between the two sets of data. At 9PCW in the human cortex, the RNA sequencing data revealed that the expression levels of *NRXNs 1* and *2* showed similar levels of expression but were 10 fold higher than the expression of *NRXN3* (figure 4.5A). They reached a relative expression level of 0.1 which indicates that the level of expression is 10% that of the mean reference gene expression. At 11 and 12 PCW, this pattern of *NRXN* expression remained with *NRXNs 1* and *2* being significantly more highly expressed than *NRXN3* ($P<0.05$; figure 4.5A). However, by 12 PCW, *NRXN1* and *2* expression was each more than 15% of the reference gene expression. For both *NRXNs 1* and *2*, linear correlation co-efficients showed that age and expression were significantly positively correlated ($p<0.05$; figure 4.5A). *NRXN1* showed the greatest change in expression from 9-12 PCW, increasing by almost 2 fold whilst the change in *NRXN2* expression was less than 1.5 fold. *NRXN3* did not significantly increase in relative expression with age.

The qPCR data indicates that at 8 PCW, *NRXN2* expression was significantly higher than *NRXNs 1* and *3* with a relative expression value reaching 0.1 ($p<0.001$; figure 4.5B). At this earlier stage of development, although *NRXN1* seemed comparable to *NRXN3* in its level of expression, the line of best fit in the regression analysis would suggest that its relative expression increased to a level closer to that of *NRXN2* by 9 PCW (figure 4.5B). By 10 PCW, the relative expression values of *NRXNs 1* and *2* were similar and were significantly higher in expression than that of *NRXN3* ($p<0.05$; figure 4.5B). The expression of *NRXN1* had dramatically increased over 5 fold to become the predominantly expressed *NRXN* (figure 4.5B). At 12 PCW, *NRXN1* remained the predominantly expressed *NRXN* with a relative expression value reaching almost 30% of the value of the reference gene expression (figure

4.5B). This is 0.5 fold higher than the expression value obtained from the RNA seq data. However, the error bars show that the data obtained by qPCR was more variable than that obtained by RNA seq. *NRXN2* relative expression at 12 PCW was just less than 0.2 which is similar to the expression value obtained from the RNA seq data. Both *NRXN1* and *NRXN2* were significantly more highly expressed than *NRXN3* at this age ($p<0.01$).

From 8-12 PCW, all three *NRXN* genes significantly increase in expression ($p<0.05$; figure 4.5B). *NRXN1* shows the greatest change in expression from 8-12 PCW, increasing by over 10 fold, whilst the change in *NRXN3* expression with age is less obvious (< 2 fold). *NRXN2* showed a uniform increase in expression during this developmental period, increasing just over 2 fold from 8-12 PCW.

4.2.4 Average expression levels of NLGNs between 8-12 PCW

In the human cortex, the RNA sequencing data indicated that *NLGNs 1* and *3* significantly increase in expression between 9 and 12 PCW ($p<0.01$; figure 4.6A). *NLGN2*, however, significantly decreased in expression between 9-12 PCW ($p<0.01$). NLGN4X relative expression remained constant.

NLGN2 was more highly expressed than *NLGNs 1*, *3* or *4X*. Its expression equalled more than half of that of the reference genes at 9 PCW (figure 4.6A). The next most highly expressed *NLGN* gene at this age, *NLGN3*, had a relative expression 5 fold less than *NLGN2*. The *NLGN* genes with the lowest relative expression levels at 9 PCW were *NLGN1* and *NLGN4X* whose expression levels were 10 fold less than that of *NLGN2*. Although the average relative expression levels of *NLGNS 1, 3* and *4X* were different, these differences were not significant.

At 11 PCW, *NLGN2* was still the predominantly expressed *NLGN* gene and was significantly more highly expressed than *NLGNs 1, 3* and *4X* (figure 4.6A). *NLGN3* was also now significantly more highly expressed than *NLGNs 1* and *4X* at this age. *NLGNs 1* and *4X* showed no significant difference in their relative expression levels compared to 9 PCW and remained the *NLGN* genes with the lowest relative expression values.

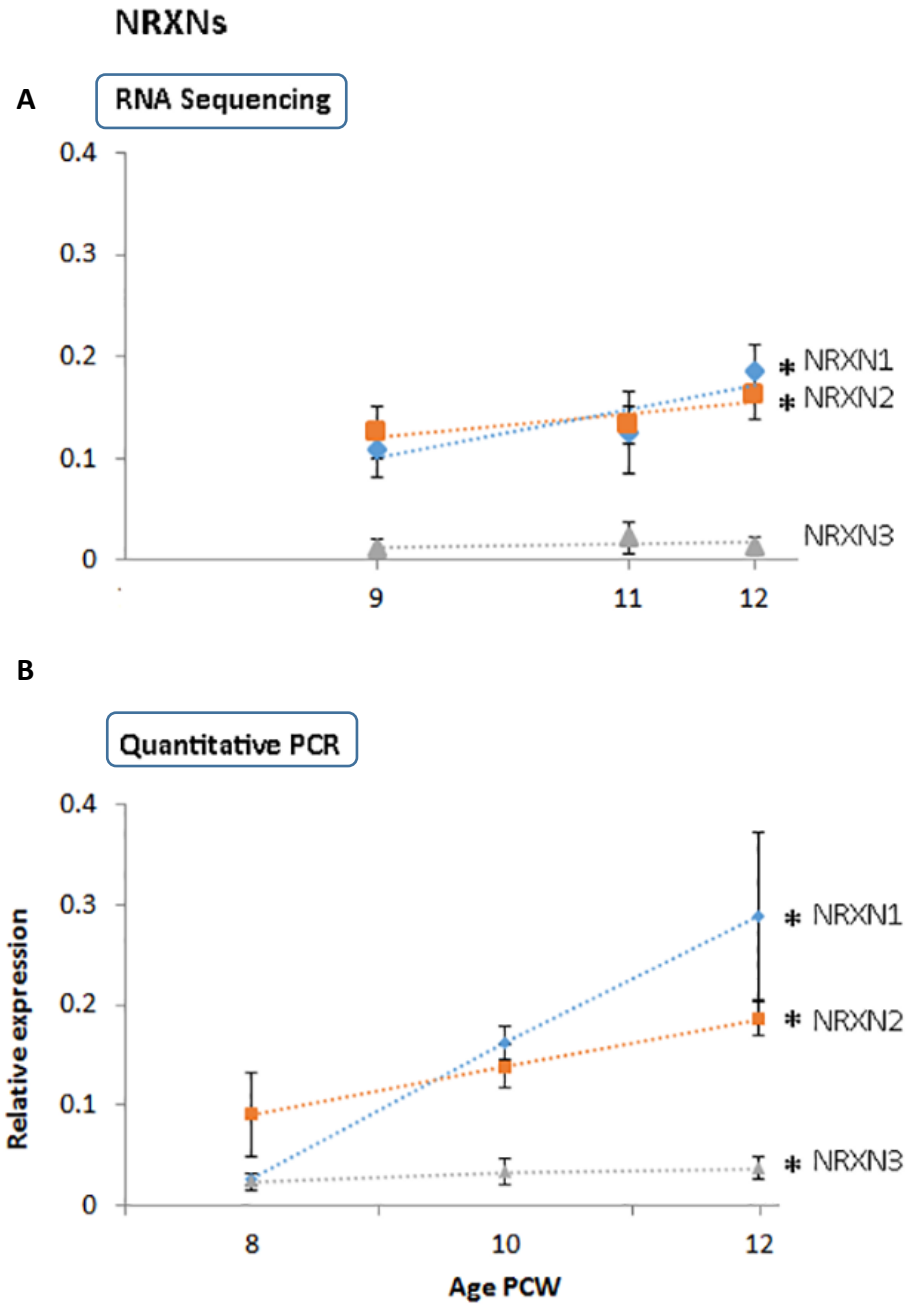


Figure 4.5 Expression of *NRXNs* within the cortex between 8 and 12 PCW. (A) Analysis of RNA sequencing data revealed that *NRXN3* had a significantly lower expression value relative to the three reference genes than *NRXNs 1* and *2*. The expression of *NRXN3* did not change significantly between 9 and 12 PCW. **(B)** Quantitative PCR revealed that the expression of *NRXN3* was significantly lower than that of *NRXNs 2* and *3* at 10 and 12 PCW and that it significantly increased in expression with age. Both *NRXNs 1* and *2* showed a significant increase in expression between 8 and 12 PCW. *significance values $p<0.05$, n=54-55 Error bars represent standard error of the mean

The expression patterns exhibited by *NLGNs 1-4X* at 12 PCW remained more or less the same as at 11 PCW. However, *NLGN1* and *NLGN4X* expression became significantly different from each other (figure 4.6A). At 12 PCW, *NLGN1* expression was 2 fold higher than NLGN4X expression, reaching almost 10% of the reference gene expression.

The *NLGN* qPCR data conflicted with the RNA sequencing data, showing that all *NLGNs* appeared to increase in expression from 8-12 PCW (figure 4.6B). However, only *NLGN*s *2* and *4X* showed a significant increase with age ($p<0.05$) according to the correlation co-efficient between age and expression values. At 8 and 10 PCW, *NLGN1,* rather than *NLGN2*, was the predominantly expressed *NLGN* and was significantly higher in expression than *NLGNs 2, 3* and *4X* ($p<0.05$; figure 4.6B). *NLGNs 2* and *3* had the lowest expression values of the *NLGN* genes from 8-12 PCW. *NLGN3* had a higher relative expression value than *NLGN2* at 8 and 12 PCW, however, this difference was not significant. *NLGNs 2, 3* and *4X* show more than a 2 fold increase in relative expression from 8-12 PCW. *NLGN1*, despite being the most highly expressed *NLGN* shown by the qPCR data, increased in expression less than 2 fold from 8-12 PCW (figure 4.6B). By 12 PCW, the relative expression values of *NLGNs 3* and *4X* had increased so that they were no longer significantly lower in expression than *NLGN1*. *NLGN1* was still significantly higher ($p<0.05$) in expression than NLGN2.

The *NLGN* genes showed a similar relative expression range to the *NRXN* genes (between 0.05 and 0.5). Only half of the *NLGN* genes significantly increased with age and the qPCR data did not correspond well with the RNA seq data unlike the *NRXNs* where at least two of the three *NRXN* genes increased in expression with age and the two experimental methods yielded the same overall patterns of expression.

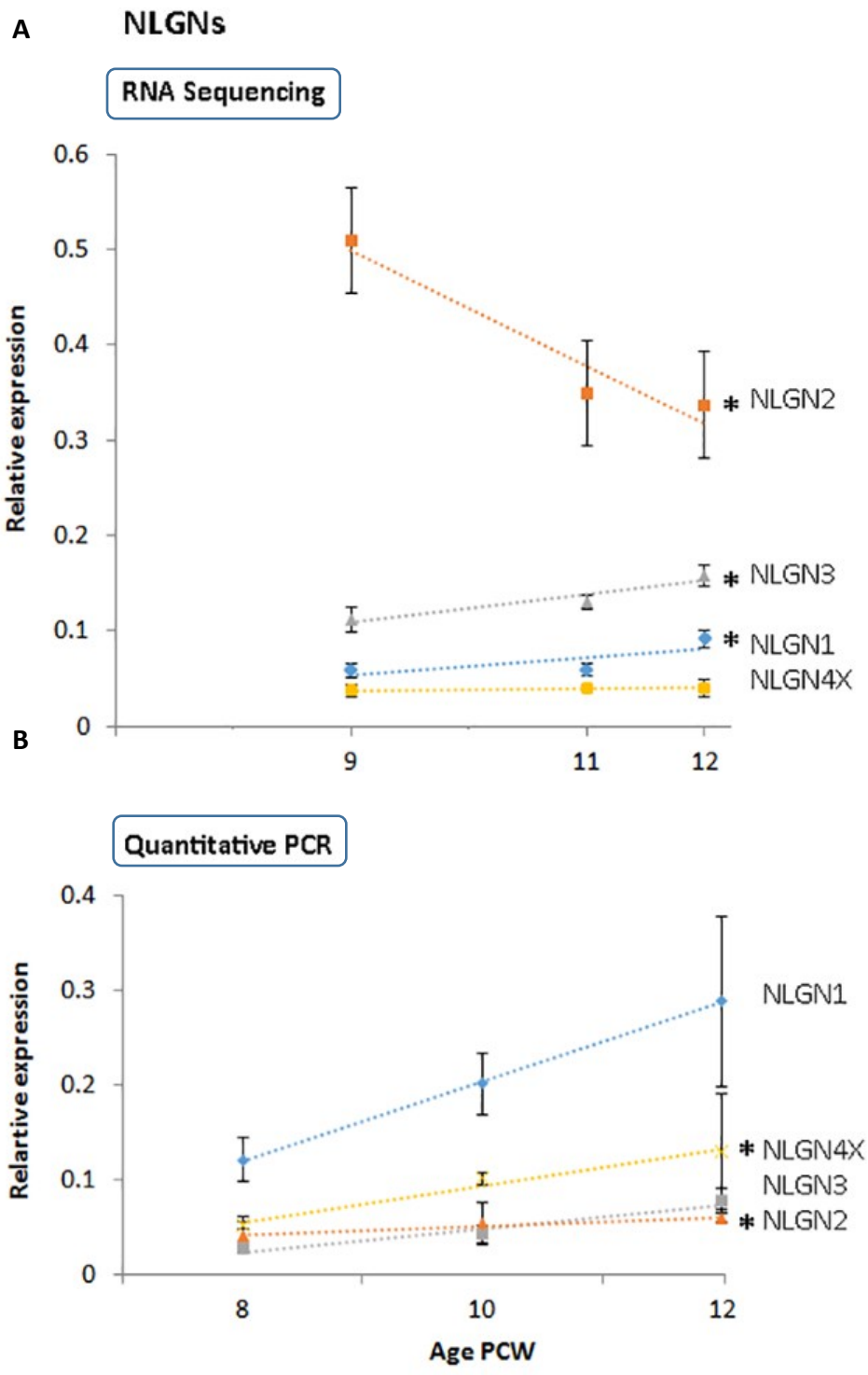


Figure 4.6 Expression of *NLGNs* within the cortex between 8 and 12 PCW. (A) Analysis of RNA sequencing data suggested that *NLGN2* expression was significantly higher than *NLGNs 1, 3* and *4X* expression but decreased significantly between 9 and 12 PCW. The expression of *NLGNs 2* and *4X significantly increased with age.* **(B)** Quantitative PCR revealed that *NLGN1* had a higher relative expression than the other *NLGN* genes. *NLGN2* and *4X* significantly increased in expression with age. *significance values $p<0.05$, RNA Seq n=63, qPCR n=49

4.2.5 Average expression levels of SHANKs between 8-12 PCW

Relative expression of the *SHANKs* was found to be considerably higher when measured by RNA seq than by qPCR. *SHANKs 1, 2* and *3* did not significantly increase in expression from 9-12 PCW (figure 4.7A). The RNA seq data showed that *SHANK1* was significantly ($p<0.05$) more highly expressed than *SHANKs 2* and *3* at 9, 11 and 12 PCW, and reached a relative expression level similar to that of *NLGN2* (figure 4.7A). *SHANK1* was almost 10 fold more highly expressed than SHANK2. At 9 PCW, *SHANKs 2* and *3* were significantly different in expression from each other with *SHANK3* having a twofold higher relative expression level, however this significant difference in expression was lost at 11 and 12 PCW (figure 4.7A).

The qPCR data for *SHANK1* and *3* did not confirm the findings of the RNA seq data. At 8, 10 and 12 PCW, *SHANK2* was significantly more highly expressed than *SHANKs 1* and *3*, with a 5 fold higher relative expression value at 8 and 12 PCW ($p<0.05$; figure 4.7B). The relative expression values for *SHANK2* are less than 10 % of the expression of the reference genes. This level of expression was comparable with that of *NRXN3* and *NLGN4X*. *SHANKs 1* and *3* relative expression values, given by qPCR experiments, were significantly lower than those of *SHANK*2 ($p<0.05$). These genes did not significantly differ in expression from each other at 8, 10 and 12 PCW. *SHANKs 1, 2* and *3* did not show a significant change in expression with age (figure 4A & B).

4.2.6 Identification of gene expression gradients across the cerebral cortex from 8-12 PCW

Expression levels, relative to the three reference genes, of *NRXN*s *1-3* were examined across the cortex between 8 and 12 PCW. Both RNA sequencing and qPCR data were considered. The RNA seq data suggested that at 9 PCW, NRXN2 was significantly more highly expressed in the posterior cortex compared to the anterior cortex (figure 4.8A). However, this was not confirmed by the qPCR data. At 8 PCW, there were no significant differences in *NRXN*s *1, 2* and *3* expression between the anterior, central, posterior and temporal regions of the cortex (figure 4.8B).

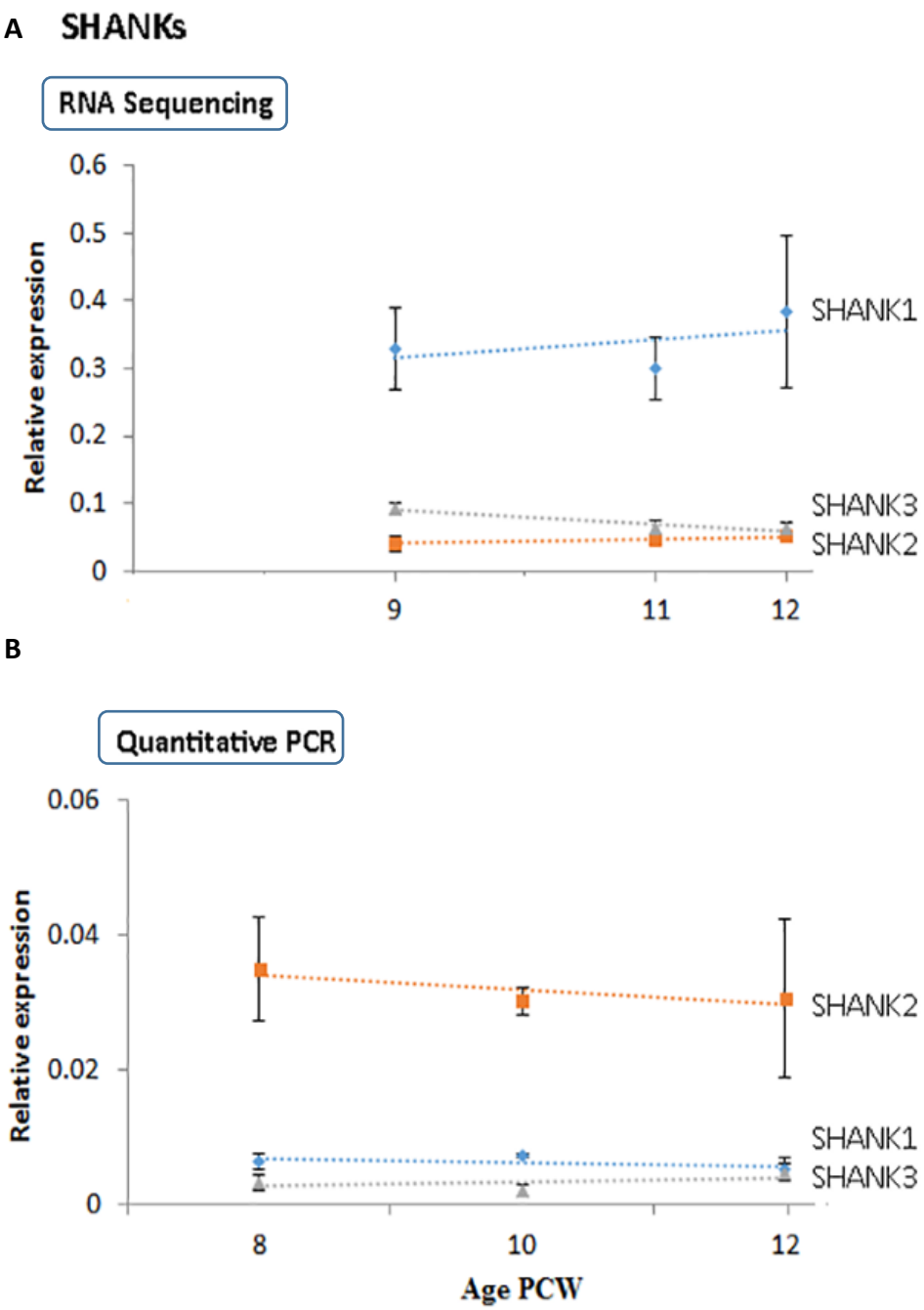


Figure 4.7 Expression of *SHANKs* within the cortex between 8 and 12 PCW. (A) RNA sequencing data suggests that SHANK1 was significantly more highly expressed than *SHANKs 2* and 3. There was no increase in expression of the SHANK genes with age. **(B)** Quantitative PCR data suggested that *SHANK2* had a significantly higher expression than *SHANKs 1* and *3.* There were no significant differences identified in *SHANK* gene expression between 8 and 12 PCW. *significance values $p<0.05$, RNA Seq n=63, qPCR n=30-55

At 11 PCW, the expression of *NRXN1* in the temporal lobe was higher than the other cortical regions but this was not statistically significant (figure 4.8C). The qPCR data at 10 PCW, however, showed that *NRXN1* expression in the temporal lobe was significantly higher than in the posterior cortex (figure 4.8D). The RNA seq data also suggested that *NRXN2* expression was higher in the posterior and temporal regions compared to the central cortex but again this could not be confirmed by qPCR.

At 12 PCW, there were conflicting results regarding the expression of *NRXN1*. The RNA seq data indicated that the expression of *NRXN1* was significantly ($p<0.05$) higher in the temporal lobe compared to the anterior cortex ($p<0.05$; figure 4.8E). Conversely, the qPCR data shows that *NRXN1* expression was significantly higher in the anterior cortex compared to the posterior cortical region ($p<0.05$; figure 4.8E). The expression of *NRXN*s *2* and *3* did not differ significantly between these cortical regions at 12 PCW in either data set.

At 9 and 11 PCW, *NLGN3* expression was significantly ($p<0.05$) higher in the temporal lobe compared to the central region in the RNA data ($p<0.05$; figure 4.9A & C). The relative expression value of *NLGN3* is higher in the temporal region compared to the central region in the qPCR data also, however, this difference is not significant (figure 4.9B). The qPCR identified a difference in expression of *NLGN3* between the anterior and central cortical regions (figure 4.9D). It was significantly more highly expressed in the anterior compared to the central cortex (figure 4.9D). Both sets of data indicate that the central region of the cortex showed a reduced expression of *NLGN3* (figure 4.8C-D) but this difference is not significant in the RNA seq data. By 12 PCW, no expression gradients were identified for *NLGN3* (figure 4.8E & F). *NLGN1* was significantly more highly expressed in the anterior cortex compared to the central, posterior and temporal regions in the RNA seq data but this finding was not validated by the qPCR data ($p<0.05$; figure 4.9E & F). The *NLGN* gene expression data was more variable than the *NRXN* data as shown by the larger error bars.

The RNA sequencing experiments suggested that there was a higher relative expression of SHANK1 in the temporal lobe compared to the other cortical regions (figure 4.10A, C, E). However, statistically, this was only significant at 9 PCW between temporal and central regions ($p<0.05$; figure 4.10A). There were no other statistically significant differences in the expression of SHANKs 1-3 across the cortex (figure 4.10).

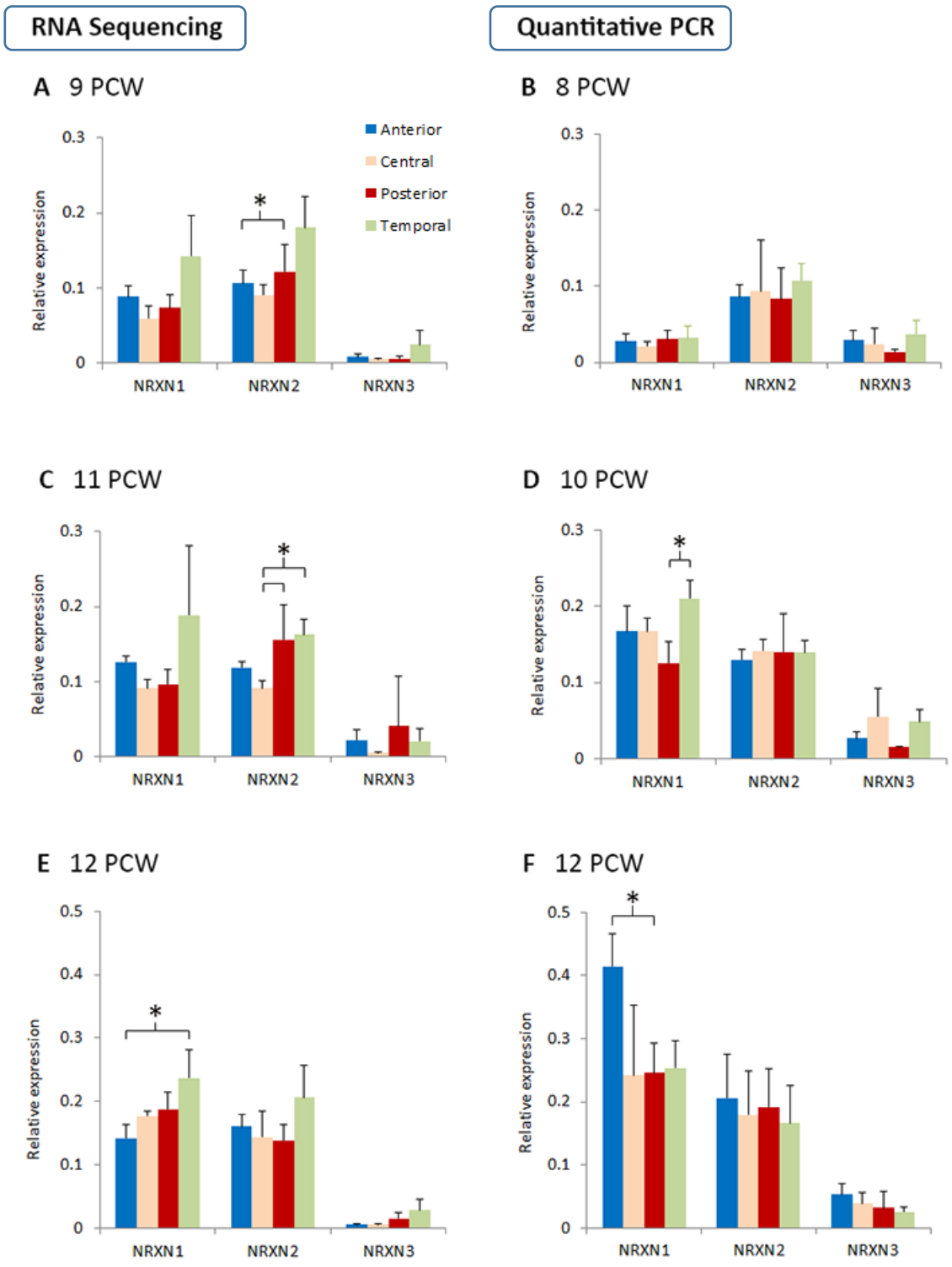


Figure 4.8 Expression of *NRXNs 1-3* relative to three reference genes. (A) At 9PCW, the expression of *NRXN2* in the posterior cortex was significantly higher than that of the anterior cortex. **(B)** Quantitative PCR did not identify any *NRXN* expression gradients at 8 PCW. **(C)** At 11 PCW, RNA seq identified that there was significantly less expression of *NRXN2* in the central cortex compared to the posterior and temporal regions. **(D)** RNA seq identified a

significantly increased expression of *NRXN1* in the temporal cortex. **(E)** At 12 PCW, *NRXN* expression was significantly higher in the temporal cortex compared to the anterior cortex **(F)** qPCR identified a significantly higher expression of *NRXN1* in the anterior cortex at 12 PCW.

*****significance p<0.05, n=3-10 for RNA seq data and 3-5 for qPCR data

Figure 4.9 Expression of *NLGNs 1-4X* relative to three reference genes.

(A) At 9PCW, the expression of *NLGN3* in the temporal cortex was significantly higher than that of the central cortex. **(B)** Quantitative PCR did not identify any *NRXN* expression gradients at 8 PCW. **(C)** At 11 PCW, RNA seq identified that there was significantly higher expression of *NLGN3* in the temporal cortex compared to the central cortex. **(D)** RNA seq identified no significant differences in *NLGN* expression across the cortex at 11 PCW. **(E)** At 12 PCW, *NLGN1* expression was significantly higher in the anterior cortex. **(F)** RNA seq identified no significant differences in *NLGN* expression across the cortex at 12 PCW.

*****significance p<0.05, n=3-10 for RNA seq data and 3-5 for qPCR data

Figure 4.10 Expression of SHANKs 1-3 relative to three reference genes.

(A) At 9PCW, the expression of *SHANK1* in the temporal cortex was significantly higher than that of the central cortex. **(B)** qPCR identified no significant differences in *SHANK* expression across the cortex at 8 PCW. **(C)** RNA Seq analysis identified no significant differences in *SHANK* expression across the cortex at 11 PCW. **(D)** qPCR identified no significant differences in *SHANK* expression across the cortex at 10 PCW. **(E)** RNA Seq analysis identified no significant differences in *SHANK* expression across the cortex at 12 PCW. **(F)** qPCR identified no significant differences in *SHANK* expression across the cortex at 12 PCW.

*****significance p<0.05, n=3-10 for RNA seq data and 3-5 for qPCR data

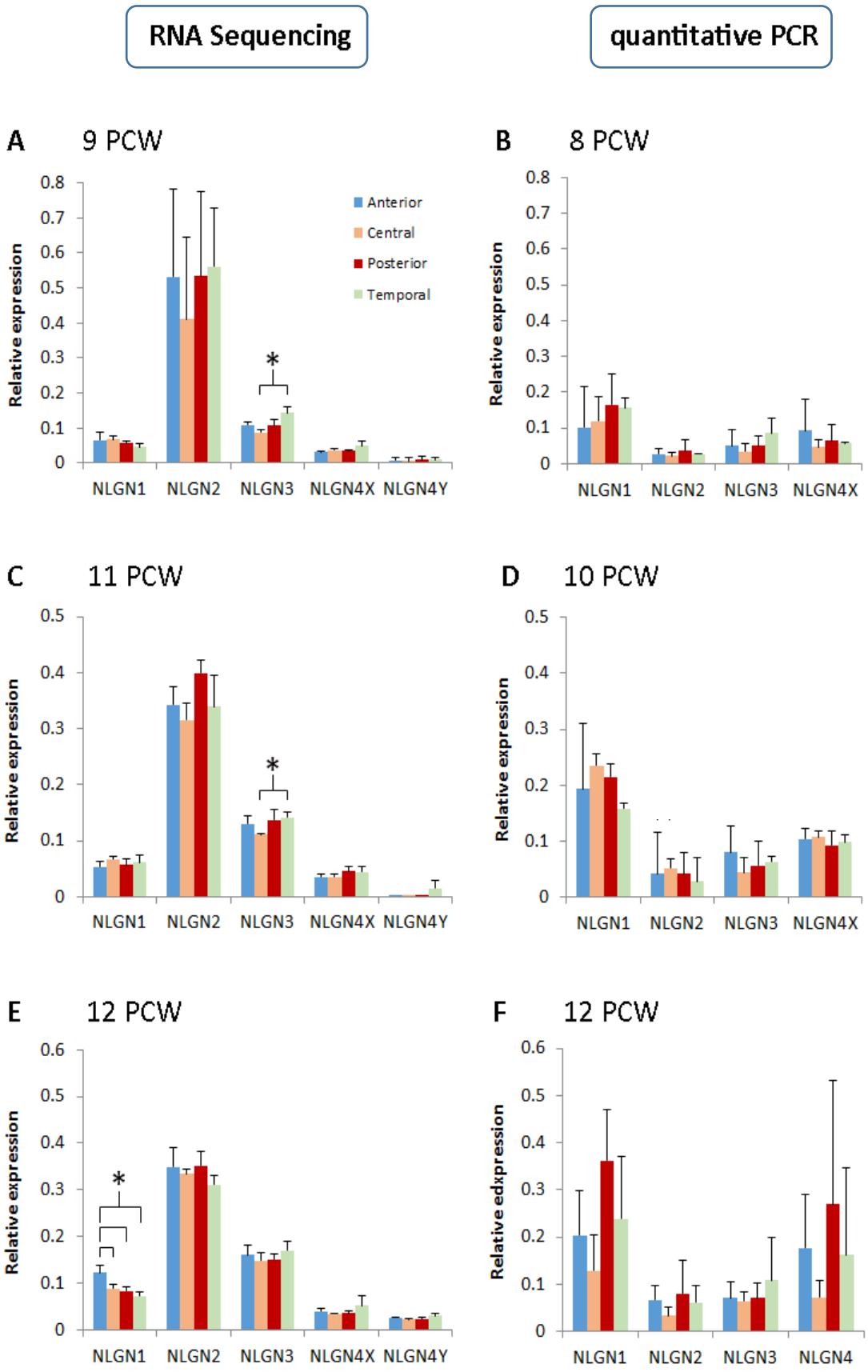


Figure 4.9 Expression of *NLGNs 1-4X* relative to three reference genes.

See page 122 for legend.

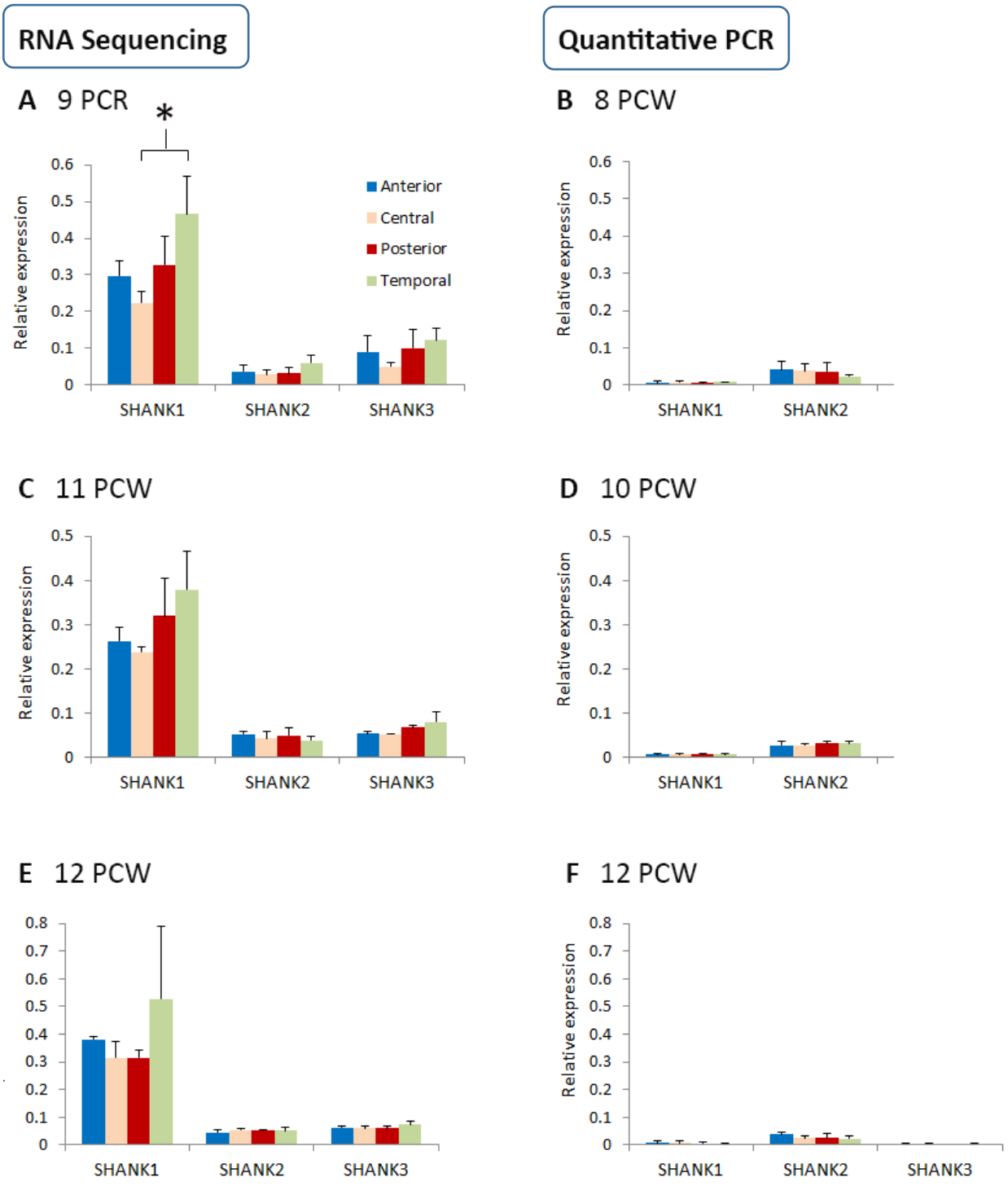


Figure 4.10 Expression of SHANKs 1-3 relative to three reference genes.

See page 122 for figure legend.

4.2.7 Average expression levels of NRXN binding partners between 9-12 PCW

The RNA seq data largely corresponded with that of the qPCR data for *NRXN* expression. The expression of *NLGNs* varied between the two data sets but overall, appeared to show high expression in the cortex as did the *NRXN* genes. In order to get an idea of what other proteins could be interacting with *NRXNs* at this developmental stage, we examined the expression of genes known to code for *NRXN* binding partners relative to the same three reference genes as examined previously. It is important, in order to understand the functions that NRXN proteins are playing at this developmental stage, to have an idea of what proteins they are interacting with. A very low level or an absence of gene expression would suggest that the protein products are not present at this time.

Cerebellin 1 precursor (*CBLN1*) and *cerebellin 2 precursor* (*CBLN2*) genes are both expressed in the cortex between 9 and 12 PCW but have different expression profiles. *CBLN2* expression does not change significantly during this period whereas, *CBLN1* expression significantly decreases ($p<0.05$; figure 4.11A). The expression of CBLN1 is also significantly higher than *CBLN2* at 9, 11 and 12 PCW (supplementary table 4.), reaching relative expression levels comparable those of *NRXNs* in the RNA seq data. *NXPH1*, like *CBLN2,* has a relative expression value less than 0.05 (5% of reference gene expression) and this value does not significantly change between 9 and 12 PCW (figure 4.11A).

Leucine rich repeat transmembrane neuronal (*LRRTM*) genes encode type I transmembrane proteins (Laurén *et al*., 2003) that have similar functions to NLGNs, can bind the PDZ domain of post synaptic density proteins and interact with NRXN proteins across the synaptic cleft (Wright and Washbourne, 2011). All four members of the *LRRTM* family were expressed in the cortex between 9 and 12 PCW and all showed a significant increase in expression during this time period ($p<0.05$; figure 4.11B). *LRRTM2* had the highest relative expression value of the four genes and increased almost 3 fold between 9 and 12 PCW. *LRRTM1* had the lowest relative expression levels of the gene family (<2%), however, it increased over 6 fold between 9 and 12 PCW (figure 4.11B).

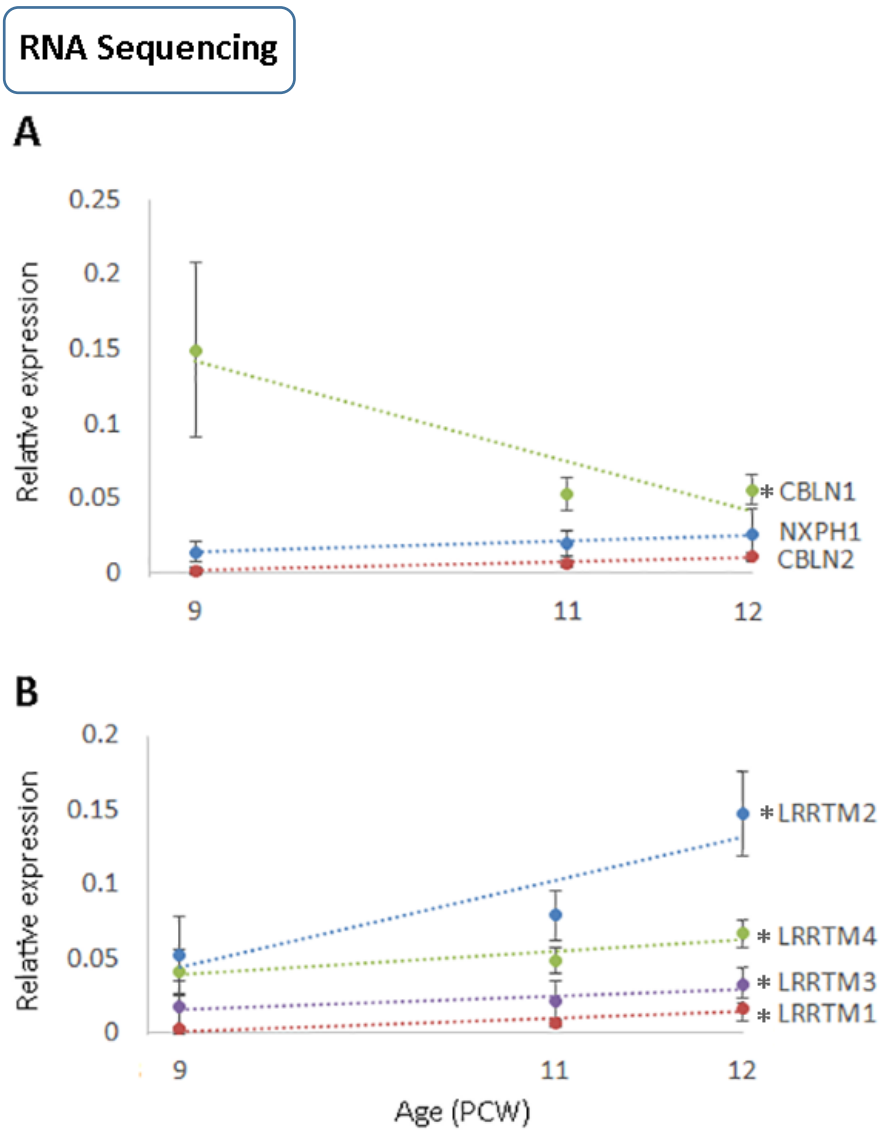


Figure 4.11 Average expression of *NRXN* binding partners relative to three reference genes. (A) *NXPH1* and *CBLN2* do not increase in expression between 9 and 12 PCW. *CBLN* significantly decreases in expression between 9 and 12 PCW. **(B)** All of the *LRRTM* genes significantly increase in expression between 9 and 12 PCW. By 12 PCW, *LRRTM2* was expressed more highly in the cortex than the other *LRRTM* genes.

*significance $p<0.05$, n=63

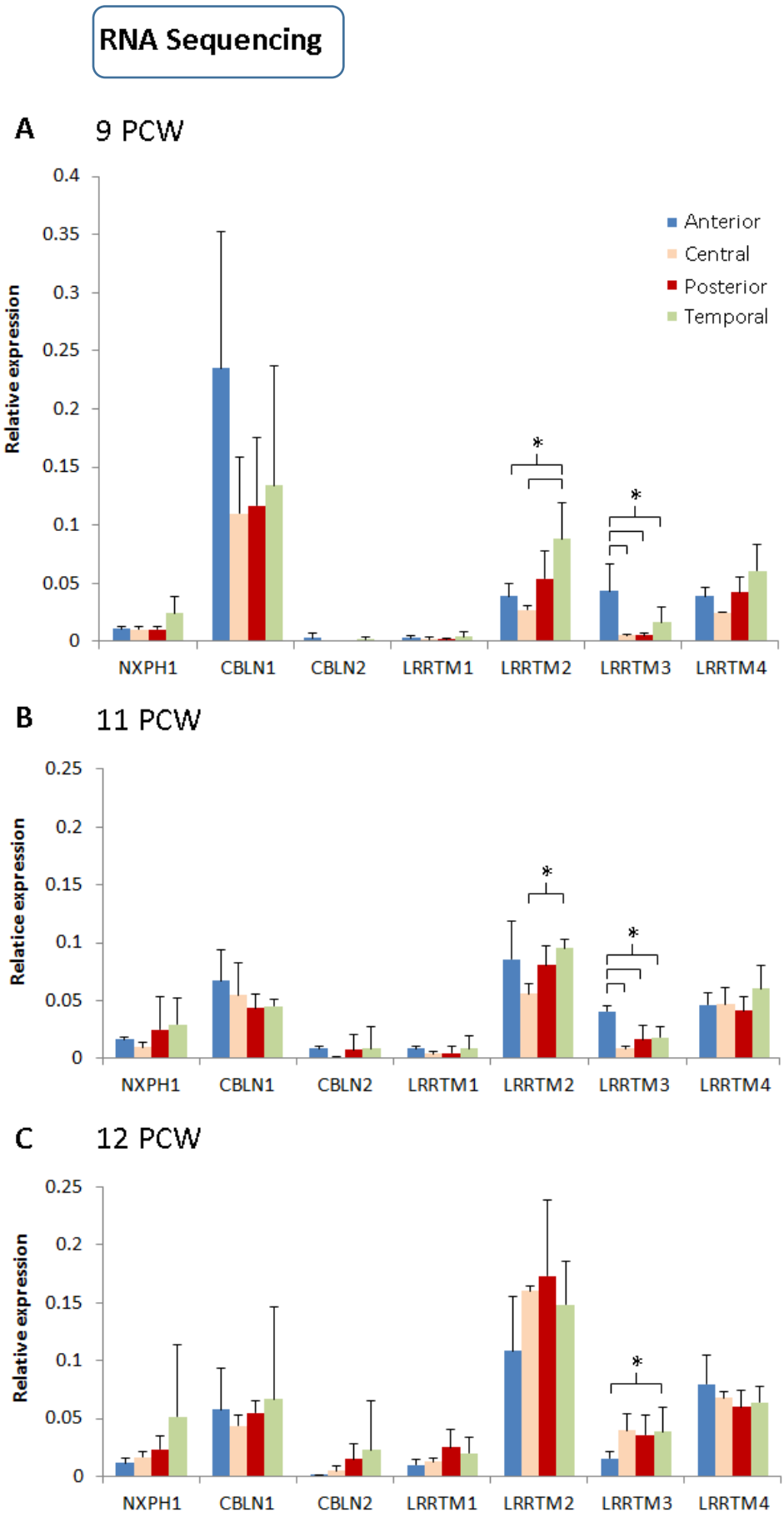


Figure 4.12 Expression of NRXN binding partners across the cortex. See page 128 for figure legend.

Figure 4.12 Expression of NRXN binding partners across the cortex. (A) At 9PCW, the expression of *LRRTM2* in the temporal cortex was significantly higher than that of the anterior and central cortex. The expression of *LRRTM3* was significantly higher in the anterior cortex. **(B)** At 11 PCW, RNA seq identified that there was significantly higher expression of *LRRTM2* in the temporal cortex compared to the central cortex. There was also a significantly higher expression of *LRRTM3* in the anterior cortex. **(C) At 12 PCW,** RNA seq a significantly higher expression of *LRRTM3* in the temporal cortex compared to the anterior cortex. No expression gradients were identified for *NXPH1, CBLN1, CBLN2, LRRTM1* or *LRRTM4* in the cortex between 9 and 12 PCW.

*significance $p<0.05$, n=3-10

No expression differences were identified between the anterior, central, posterior and temporal regions of the cortex for the *CBLN* genes or for *NXPH1* (figure 4.12). There were, however, significant regional expression differences identified in the *LRRTM* family. *LRRTM2* expression in the temporal cortex was significantly higher than expression in both the anterior and central cortex at 8 PCW, and the central cortex at 11 PCW ($p<0.05$; figure 4.12A & B). By 12 PCW, these differences in expression were no longer significant. *LRRTM3* expression in the anterior cortex is significantly higher than the expression in the central, posterior and temporal regions at 9 and 11 PCW ($p<0.05$; figure 4.12A & B). However, by 12 PCW, the expression in the anterior cortex appears to have decreased whilst the expression in the temporal cortex has increased to a significantly higher level than that of the anterior ($p<0.05$; figure 4.12C).

4.3 Discussion

Mapping the expression patterns of autism susceptibility genes in the early fetal brain gives us an insight into what developmental processes their protein products may be involved in. Information showing how these genes change temporally and spatially across the cortex can be related back to the knowledge that we have about these developmental periods. Distinguishing between normal and abnormal gene expression patterns in human brains is essential in order to be able to predict the development of these complex conditions.

We present here, for the first time, a detailed analysis of the expression levels of a subset of autism susceptibility genes in the normally developed human cortex from 8-12 PCW. By employing PCR, RNA sequencing and quantitative PCR, this study has confirmed that these genes are expressed in the developing cerebral cortex during this important developmental period, which sees the establishment and expansion of the transient sub plate and the cortical plate (Bayatti *et al*., 2008a, Bystron *et al*., 2008). Thalamocortical fibres have not yet innervated the cortex (Bystron *et al*., 2008) meaning that neuronal differentiation is under intrinsic cortical control.

4.3.1 The expression of many NRXNs and NLGNs increases between 8 and 12 PCW

Genes that significantly increased in expression with age according to both the RNA seq and qPCR data included *NRXNs 1* and *2* and depending on the data set, *NRXN3* and all four *NLGNs*. The gene expression patterns of NRXNs and NLGNs have not previously been examined in humans at these developmental stages. *NRXN1* and *NLGN3* are known to increase in expression in the cortex during postnatal development before decreasing in expression during aging in mice (Kumar and Thakur, 2015). This decrease in expression with age correlates with a decrease in synaptic plasticity (Sibille, 2013, Bloss *et al*., 2011, Dumitriu *et al*., 2010). Other studies using cell models have shown that the expression of some *NRXNs* and *NLGNs* increases during differentiation. Studies of differentiated human neural progenitor cells (NPCs) found that after 4 days in culture, there was a significant increase in NRXN1, NLGN3 and NLGN4X (Konopka *et al*., 2012) and mouse NPCs, taken directly from the cortical SVZ and cultured in differentiation media for 7 days, showed an increase in *NRXN2* expression (Gurok *et al*., 2004).

This study confirmed that these genes, in particular *NRXNs 1* and *2* and *NLGNs 1* and *2*, are expressed at very high levels within the cortex between 8 and 12 PCW. During this developmental period, the formation of synapses is only just beginning and they are confined to the sub-plate and marginal zone which makes up a relatively small proportion of the cortex (Bystron *et al*., 2008, Konig and Marty, 1981, Konig *et al*., 1975, Molliver *et al*., 1973). It has been proven that a large number of synaptic genes are expressed in cultured subplate neurons from rats before they are plated on a feeder layer and induced to form synaptic connections. However, less than 10% of these are differentially regulated during the early stages of synaptogenesis and it is only at later time periods that an increased number of known synaptic markers change in expression (McKellar and Shatz, 2009). *NRXN3* and *NLGN2* were identified as being upregulated at the later periods of synaptic differentiation (up to 96 hours after feeder layer exposure) rather than the initial periods of synaptogenesis (up to 24 hours).

SHANK genes do not increase in expression with age, although they are expressed in the cortex during this developmental time period. SHANK proteins contribute to the post synaptic density of the post synaptic membrane in mature synapses, linking this structure to the actin cytoskeleton (Qualmann *et al*., 2004). Post synaptic density genes, in addition to presynaptic release genes do not appear to be upregulated during the early stages of synaptogenesis and are likely to act as markers of later synaptic development (McKellar & Shatz, 2009). SHANK2 expression is highest of the three genes in the qPCR data. It has been shown previously, in rats, to be the first of the three genes to be expressed at the PSD (Grabrucker *et al*., 2011). SHANK2 is associated with neuronal differentiation and neurite outgrowth in the retina via ERK1/2 activation (Kim *et al*., 2009). It is co-localised with neurofilament but is present in all retinal layers. The Kim (2009) study showed that SHANK2 increased as the retina differentiates and blocking ERK signalling decreased SHANK2 expression. SHANK2 is unlikely to be involved in the differentiation of cortical neurons via the ERK1/2 signalling at this developmental period as there is no increase in expression with age and RNA sequencing did not detect ERK1/ 2 gene expression (supplementary link). However, the fact that it is being transcribed, possibly at a higher level than the other SHANK genes, suggests that it may be required for neuronal differentiation and neurite outgrowth at later, but imminent, developmental stages.

Therefore, NRXNs and NLGNs appear to be part of a group of synaptic genes that are separate both transcriptionally and functionally to the group that includes SHANKs. Indeed, the expression pattern of the presynaptic vesicle marker synaptophysin is not identical to that of NRXN1 and NLGN3 in the postnatal cortex. In rodents, SYP began to decrease in expression from 10 post-natal weeks whereas a decrease in NRXN and NLGN protein was not observed until after 30 weeks (Kumar and Thakur, 2015).

Previous studies have shown that the expression of 'synaptic' genes occurs before the formation of synapses and this suggests that they may be involved in other functions of neuronal cells. The increase in expression of NRXNs and NLGNs between 8 and 12 PCW correlates with an increase in multiple developmental processes in addition to synaptogenesis including neuronal differentiation, neurite outgrowth and synaptic plasticity (Bystron *et al.,* 2008) and an increase in neuronal migration. Neurexin and neuroligin proteins, and those that they interact with, could be contributing to any of these processes. The level of expression of these genes, in comparison to other synaptic proteins such as the SHANK family of genes, suggests that they have functions other than synaptogenesis and synaptic stabilisation at this developmental period.

4.3.2 Possible **NRXN** *interactions during early development*

Neurexins have a number of binding partners including Neuroligins (NLGNs), LRRTMs, Cerebellins (Cblns), Neurexophilins (NXPHs) and dystroglycans (DEGs; Reissner *et al*., 2014; Matsuda and Yuzaki, 2011; Siddiqui *et al*., 2010; Sugita *et al*., 2001b; Missler *et al*., 1998; Ichtchenko *et al.,* 1996; Ichtchenko *et al.,* 1995). At the presynaptic membrane NRXNs 1 and 2 are increasing in expression between 8-12 PCW. NRXN binding partners that are also increasing in expression are *LRRTM* family proteins, *DLG1* and some of the NLGN genes. As we have shown here with *NRXNs*, *LRRTM* genes show differential expression throughout development in mice. *LRRTM1* and *3* are expressed in the mouse forebrain at E9 (Haines & Rigby, 2007) but *LRRTM2* and *4* are not. *LRRTM2* is expressed later in development than the other *LRRTM* genes at 10 days post coitum (dpc). All *LRRTM* proteins can induce pre-synaptic differentiation in contacting axons in culture as well as recruiting post-synaptic proteins to neuronal sites where they are over-expressed (Linhoff *et al.,* 2009). However, *LRRTM2* has been shown to be the most effective member of the family for inducing synapse formation (Linhoff *et al*., 2009). The corresponding patterns of gene expression for *NRXNs*

and *LRRTMs* between 9 and 12 PCW, suggests that their proteins are not only present at this stage but that they are interacting to induce the recruitment of synaptic machinery. The knockdown of all *NRXN* genes in culture reduces the synaptogenic potential of the LRRTM proteins (Um *et al.,* 2016). Only *LRRTM4* and *3* mutations have been associated with autism, although *LRRTM1* and *2* mutations have been associated with Schizophrenia and Bipolar disorder respectively (de Wit & Ghosh, 2014). The differential splicing of *LRRTM3* and *4* can affect the binding properties of the proteins (Um *et al.,* 2016).

It has been suggested that the function of NRXN proteins depend on the protein that they bind on the post-synaptic membrane (Clarke & Eapen., 2014). Depending on which NLGN is bound; the interaction can induce the recruitment of post synaptic density proteins to the membrane and evoke either inhibitory or excitatory synapse development. *NLGN1* is found primarily at glutamatergic synapses whereas, *NLGN2* is found primarily at GABAergic synapses (Graf *et al.,* 2004). The increase in NLGN1 and the decrease in NLGN2 identified from the RNA seq data would suggest that there is an increase in the formation of excitatory synapse development and a decrease in the formation of GABAergic synapse development. The first mature synapses are expected to be GABAergic (Hennou *et al.,* 2002), although this does not necessarily mean all synaptic outputs are inhibitory. During development, GABA can also be depolarising as expression of the potassium chloride transporter member 5 (KCC2) is initially low during development. In mature neurons it is the action of KCC2 in maintaining high intracellular chloride concentrations that permits the opening of chloride channels via GABAergic receptors to have a hyperpolarising effect (Rivera *et al.,* 1999).

CBLNs bind NRXNs enabling them both to form complexes with certain post-synaptic proteins. For example, in the mouse cerebellum, Nrxn1 interacts with GluRδ2 via Cbln1 (Uemura *et al.,* 2010). Also, CBLN1 acts to form synaptic connections between Purkinje cells and parallel fibres in the cerebellum by mediating the interaction between NRXNs and GRID2 (Mishina *et al.,* 2012). In this study *CBLN1*, like *NLGN2*, decreased in expression between 9 and 12 PCW suggesting that it is not, at this stage, the main NRXN binding protein. *CBLN1* appears to decrease postnatally, when synapse production has decreased, in the human brain but remains high in the cerebellar cortex (brainspan.org). Contrastingly, *CBLN2* expression is higher postnatally than prenatally and is not enriched in the cerebellar cortex (brainspan.org). *CBLN2* in this study does not change in expression between 9 and 12 PCW, although it is expressed. According to the RNAseq data, *CBLN4* is not expressed between 9

and 12 PCW. CBLN4 has a lower binding affinity to NRXNs than CBLNs 1 and 2. An increased affinity for NRXNs is seen when binding complexes contain both CBLN1 and CBLN4 (Wei at al., 2012), however CBLN4 is hypothesised to have other binding partners. *CBLN1* and *2* are able to induce inhibitory pre-synaptic differentiation in cortical cells but the extracellular region of NRXN1β is able to prevent this pre-synaptic differentiation. CBLN4 has a lower binding affinity for the NRXNs than CBLN1 and 2, but all three require the presence of SS4 for binding. CBLNs 1 and 2 have a higher binding affinity for NRXN1 variants in comparison to NRXN2 (Joo *et al.,* 2011) and Cbln1 and 2 bind Nrxns 1α and 2α in vitro but have a lower affinity for Nrxn3α (Wei *et al.,* 2012). Despite their proven affinity for NRXN binding and their presence in the cortex between 9 and 2 PCW, they don't appear to be major candidates for NRXN binding at this developmental stage. Although there may be interactions between NRXNs and CBLNs 1 and 2, the increase in NRXN expression suggests an increase in protein abundance that is not matched by *CBLN1* and *2* expression.

The final NRXN binding partners to be examined were DAG1 and NXPH1. These two proteins have been shown to compete for binding of NRXN1α (Reissner *et al.,* 2014). Nxph1 is found only at inhibitory synapses in mice (Batista-Brito *et al.,* 2008) and dystroglycan is also associated with inhibitory synapses in the mouse hippocampus (Levi *et al*., 2002; Pribiag *et al*., 2014). The DAG1 gene codes for both α and βDAG which are extracellular and transmembrane proteins respectively. In addition to competing with NXPH for binding sites, αDAG is able to bind Nrxn1α at LNS2 to prevents the binding of Nlgn at LNS6. This study has identified DAG1 as increasing in expression between 9 and 12 PCW although neither DAG1 or NXPH1 are differentially expressed between cortical regions as the NRXNs are. It is also not known whether the increased DAG1 expression with age is giving rise to α or β protein forms. Although these genes are expressed in the cortex and DAG1 increases in expression with age, there is limited evidence to suggest that they play a pivotal role in cortical development through their interactions with NRXN proteins. Rather, this study suggests that it is the *NLGN* and *LRRTM* genes that mimic the expression profiles of *NRXNs* and may be, therefore, likely to be the main proteins influencing NRXN function from 9 to 12 PCW.

All of these NRXN ligands mentioned bind to either LNS2 or LNS6 which is present in βNRXNs (Siddiqui *et al.,* 2010; Sujita *et al.,* 2001; Missler *et al.,* 1998). NXPH can bind NRXNs with or without SS4 but the other binding partners are more specific. NLGNs, LRRTMs, and DAGs require the removal of SS4 whereas CBLNs need SS4 present in order to bind NRXNs. It

would be interesting to examine the absence or presence of this splice site in the cortical samples and to examine whether its inclusion/exclusion differs between ages and/ or cortical regions. This would provide valuable and additional insight into what ligands are potentially binding NRXNs at this developmental stage.

4.3.3 NRXN, NLGN and LRRTM gene expression increases in the anterior and temporal cortex between 8 and 12 PCW

The qPCR data for *NRXN1* and *NLGN3* as well as the RNA seq data for *NLGN1*, *LRRTM2* and *LRRTM3* suggests that there is significantly higher expression of these genes in the anterior and/ or temporal cortex between 9 and 12 PCW. The dysfunction of both the anterior and temporal cortex is associated with multiple neurodevelopmental and psychiatric conditions (reviewed in Schwarzbold *et al.,* 2008; de Oliveira *et al.,* 2010; Trimble, 2002) including ASD, depression, dis-inhibition, impaired social judgement and obsessive compulsive disorder. Individuals with tuberous sclerosis have benign tumour growth on the temporal lobe from birth as a result of mutations in either TSC1 or TSC2. These gene mutations and/ or tumours can cause mental retardation, a lack of adaptive social behaviours, a lack of recognition of the significance of people, objects or events and a hypersensitivity to visual stimuli (Hetzler & Griffin, 1981), resulting in additional ASD diagnoses (Benvenuto *et al.,* 2009).

If higher expression of these genes between 9 and 12 PCW is important for the normal development of the brain then ablation of these gradients in the anterior and temporal cortex could be a risk factor for autism. These gradients of expression may be necessary to establish the correct circuitry within and between different cortical regions. Welchew *et al.* (2005) observed abnormal functional connectivity in the brains of Asperger's patients compared to controls. Fronto-striatal circuits are affected in people with Fragile X syndrome, many of which are also diagnosed with ASD (Hoeft *et al*, 2007). In ASD, there are an increased number of connections in the frontal lobe (Courchesne & Pierce, 2005). Abnormalities in fronto-temporal circuitry is also a risk factor for schizophrenia (Winterer *et al.,* 2003; Meyer-Lindenburg *et al.,* 2001; Jennings *et al.,* 1998; Frith *et al.,* 1995). ASD and schizophrenia share many of the same susceptibility genes, including *NRXN1* (Kirov *et al.,* 2009), some of which are transcriptionally co-regulated (Ellis *et al.,* 2016).

A reduction in ASG expression, may contribute to the loss of cell-cell contacts if they have a role as cell adhesion molecules during development. This in turn would disrupt vital connections being established, particularly those required for long range connections with other cortical regions and sub-cortical structures. The same effect would be observed if a decrease in *NRXN* or *NLGN* expression prevented axonal outgrowth or prevented the recognition of these axonal outgrowths by post synaptic counterparts leading to failure to establish these long range connections.

Despite the interesting possibilities if there were higher expression of NRXNs and their binding partners in anterior and/ or temporal cortical regions, this study did not definitively show that expression gradients are present for specific genes. The RNA seq and the qPCR data suggest that gradients may be present, with expression particularly higher in the anterior or temporal lobes, however, the two sets of data are inconsistent. This study has been an interesting insight into the gene expression patterns of ASGs in the developing cortex and has no doubt provided inferences as to where the expression of these genes may be increased.

4.3.4 Discrepancies between the data sets and methods of analysis

Although increases in expression were detected for the NLGN genes in both data sets, the significant differences across time identified in the RNA sequencing data were not validated by the qPCR data. The RNA sequencing data showed a significant increase in expression between 9 and 12 PCW for *NLGNs 1* and *3* but not *NLGN4X.* Conversely, qPCR identified this increase as significant in *NLGN4X* but not *NLGNs 1* and *3.* An increased sample size could possibly resolve the discrepancies between the two methods. It was expected that, due to the significant increase in expression of their binding partners, NRXNs, the expression of NLGNs would also increase.

The expression of NLGN2 appeared to significantly decrease with age in the RNA seq data but significantly increased with age in the qPCR data. NLGN2 is thought to function at inhibitory GABAergic synapses (Babaev *et al.,* 2016; Hoon *et al.,* 2009) interacting with NRXN1. NLGN2 expression is decreased following chronic stress in the hippocampus of adult rats (van der Kooij *et al.,* 2014) and roles in neural circuit remodelling and the synaptic inhibitory/ excitatory balance have been proposed due to changes in aggressive behaviour

following increases/ decreases in its expression (ref). It is important to note that due to sample availability, sample sizes were small (n=3-10) and the brain samples used for RNA sequencing were not the same set of samples as those used for qPCR. Therefore, it is possible that differences between the two sample groups, investigated using two separate techniques, may be due to factors other than those that we have tested for such as environmental influence and changes in response to stress. Post mortem delay differed between samples as well as the conditions that each embryo/ foetus is subjected to within the womb. In addition to post mortem delay, pH has a large effect on gene expression (Birdsill *et al.,* 2011). The pH of each brain was not recorded during dissection but would be an interesting piece of information for future studies. Due to the small sample sizes there is also a degree of caution required when relating the results to the general population. Sample availability is an obvious limitation to this study; however, human material offers a complex and unique insight into the development of the human brain.

Our analysis, normalising RPKM expression values to three reference genes, failed to identify NRXN3 as differentially expressed between the anterior and posterior cortex. The qPCR data set disagreed with the pattern of expression for NRXN3. Rather than being more highly expressed in the posterior as in the RNA seq data, the qPCR data suggests that it is more highly expressed in the anterior cortex although neither finding is statistically significant. This highlights differences between expression values obtained in the qPCR and the RNA seq data. Also differences between using the software package and manually comparing expression to three reference genes in the ability to detect significant changes in gene expression. In this case the manual method, although it identifies that NRXN3 has a higher expression in the posterior cortex than other brain regions, appears to be more stringent.

4.3.5 Neurexins and Neuroligins are required for more than synapse formation

The discovery of an increasing number of proteins with synaptogenic activity supports the findings that the *NRXN-NLGN* interactions are not essential in the process of synapse formation (Missler *et al.,* 2003). Although synapses develop normally in α*NRXN* knockout mice, the absence of protein does elicit behavioural phenotypes suggesting more subtle roles for these proteins in brain development.

The process of synaptic maturation involves the transcription and translation of proteins required for functional synapses, the recruitment of these proteins to the synaptic membranes (Giagtzoglou *et al,* 2009), organisation of these proteins and their interaction with each other within and across synaptic membranes.

As well as the current established functions of NRXN proteins as presynaptic cell adhesion molecules, they are likely involved in the organisation of the presynaptic release machinery. Triple αNRXN knockdown mice, although they are not viable, have the correct number of synapses at birth but neurotransmitter release is severely inhibited (Missler *et al*, 2003). αNRXNs have observed functions in calcium dependant neurotransmitter release (Missler *et al*., 2003). Although synaptogenesis may be aided by the extra-cellular interactions of these proteins, NRXNs may also have roles beyond this. Indeed it has been shown that in the absence of individual NRXN proteins, rodents have functional synapses (Missler *et al.,* 2003).

Although gene expression patterns are informative and give us an indication as to how much protein there will be relative to other genes, it is more convincing to visualise the proteins directly using immunohistochemistry/ immunofluorescence. In the next chapter these techniques will be discussed as will potential roles of neurexins in the developing cortex.

CHAPTER 5

Investigating the cortical localisation of NRXN proteins in the developing human forebrain

5.1 Aim

The aim of this study was to map the distribution of NRXN proteins within the human forebrain between 8 and 12 PCW.

5.2 Results

5.2.1 Markers of cortical lamination

The transcription factor PAX6 is a marker of proliferative radial glia within the ventricular zone (VZ) and sub-ventricular zone (SVZ) of the cerebral cortex (Gomez-Lopez *et al*., 2011, Bayatti *et al*., 2008b, Kerwin *et al*., 2004). Radial glia serve as a means of migration for post mitotic neurons from the proliferative regions to the CP (see section 1.2; Nadarajah and Parnavelas, 2002). Radial glia also migrate from the VZ to the SVZ and proliferate. The developing primate sub ventricular zone of the cortex contains both an inner and outer region. The inner sub-ventricular zone is that contacting the VZ, while the oSVZ is situated between the VZ and the IZ. The outer SVZ is thought to have enabled the expansion and evolution of the human brain (reviewed in Lui *et al.,* 2011). PAX6 controls the length of the cell cycle and prevents the expression of post mitotic neuronal markers (Estivill-Torrus *et al.,* 2002). The anti-PAX6 primary used in this study has previously correctly shown immunopositivity in the VZ and SVZ of the human cerebral cortex (Bayatti *et al*., 2008b, Manuel and Price, 2005). Antibody details can be found in table 2.4.

T box brain 1 (TBR1), another transcription factor, is a marker of post mitotic glutamatergic neurons that reside in the cortical plate (CP) and subplate (SP). TBR1 protein expression is also seen in a subset of cells of the SVZ and IZ as they transition from TBR2 positive intermediate progenitor cells into post mitotic cells that begin migration to the cortical plate (Englund *et al*., 2005). In particular, it is required for the differentiation of layer VI of the cerebral cortex (Bedogni *et al*., 2010). Validation of the anti-TBR1 primary used in this study has been carried out previously, showing immunopositivity in the CP and some cells of the SVZ and IZ and an absence of immunopositivity in the proliferative cortical regions (Bayatti *et al*., 2008b). Antibody details can be found in table 2.4.

Growth associated protein 43 kDa (GAP43) is present in axonal growth cones (Benowitz and Routtenberg, 1997) and between 8 and 12 PCW, these growing axons are present in the subplate (SP), marginal zone (MZ) and intermediate zone (IZ) of the cerebral cortex. Synaptophysin (SYP) is also expressed in these cortical regions and is a marker of synapse formation (Tarsa and Goda, 2002). SYP is the most abundant synaptic vesicle protein (Takamori *et al*., 2006) and is required for their efficient endocytosis (Kwon and Chapman, 2011). The SYP and GAP43 primary antibodies used in this study have been validated in previous studies with SYP showing punctate synaptic staining (Bayatti *et al*., 2008a, Clowry *et al*., 2005) and GAP43 immunopositivity in growing axons of the MZ, SP and IZ of the human cortex (Ip *et al*., 2011, Bayatti *et al*., 2008a). Antibody details for both SYP and GAP43 can be found in table 2.4.

Neurexin antibodies used in this study have never before been used in prenatal brain tissue, however, all have been used for previous publications. Anti-NRXN1 shows immunopositivity in the cell membranes of mouse cortical cell cultures (Kumar and Thakur, 2015) and also of rat pancreatic cells (Suckow *et al*., 2008). Western blots revealed an 84kDa band (Kim *et al*., 2008). Anti-NRXN2 recognises the alpha transcript and immunopositvity has been observed in the neuropil and pyramidal cells of the adult human cerebral cortex (Borsics *et al*., 2010). Immunoblots have also been used to quantify NRXN2 protein levels in rat SCN cells using this antibody (Shapiro-Reznik *et al*., 2012). The NRXN3 antibody used in this study was used by the Human protein atlas and showed immunopositivity in the human cerebral cortex (Berglund *et al.,* 2008). The region of the protein recognised by each NRXN antibody can be seen in figure 5.1.

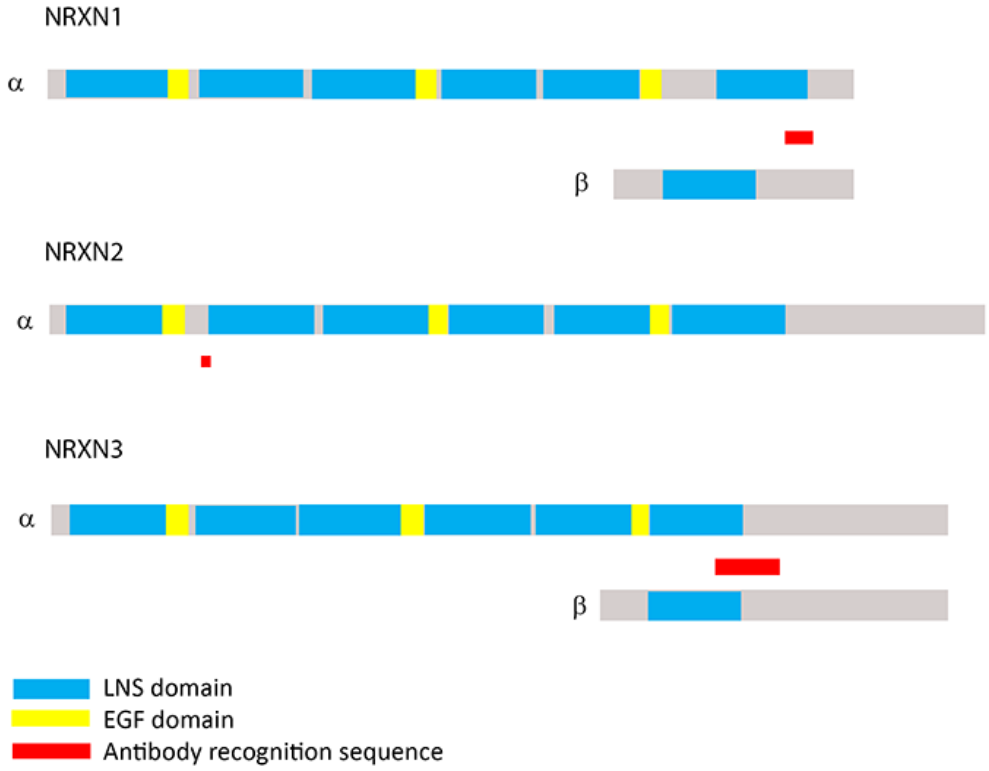


Figure 5.1 Schematic of NRXN protein domains and antibody recognition sequence. The anti-NRXN1 antibody used in this study recognised both the alpha and beta proteins. The recognition sequence fell within the last LNS domain of αNRXN1 but outside of this region in βNRXN1. Anti-NRXN2 only recognised αNRXN2. Anti-NRXN3 recognised both αNRXN3 and βNRXN3. The recognition sequence spanned the end of the last LNS domain of both NRXN3 proteins.

5.2.2 Laminar expression of NRXNs1-3 in the adult cerebral cortex

For further characterisation of the NRXN antibodies, paraffin sections from adult brains were immunostained with anti-NRXN1, 2 and 3. Images were obtained from sections taken from the external pyramidal layer of the prefrontal cortex and the white matter region beneath (figure 5.2). Haematoxylin and eosin (H&E) stained the nucleus and cytoplasm respectively of cells within the adult cerebral cortex (figure 5.2A). Immunohistochemistry revealed that the majority of NRXN immunoreactivity was observed in the grey matter of the cortex, which contains the neuronal cell bodies. SYP showed strong immunopositivity in punctate structures abutting the surface of neuronal cell bodies of the cortical grey matter and was

absent from the white matter (figure 5.2B). This staining pattern was very similar to that of NRXN3 which was seen in punctate structures contacting the neuronal surface but was absent from the inside of cells and from the white matter (figure 5.2E).

NRXN1 and NRXN2 had a slightly different staining pattern to that of NRXN3. NRXN1, and to an extent NRXN2, stained the cytoplasm of pyramidal neurons (figure 5.2C & D), that were also stained by eosin in figure 5.2A. The punctate staining for NRXN2 was more pronounced than that of NRXN1 within the cortical neuropil. There was an absence of nuclear staining in the SYP and NRXN2 and 3 stains (figures 5.2C-E). Punctate staining, in what appears to be the neuropil, could be seen after staining with SYP and NRXNs 1, 2 and 3 (figure 5.2F). This is likely to be presynaptic terminal staining. SYP is found at presynaptic vesicles and is required for their efficient endocytosis (Kwon & Chapman, 2011). NRXNs, in particular the β form, are associated with the presynaptic membrane acting as a linker molecule to the postsynaptic membrane. The α forms of NRXNs have roles in presynaptic neurotransmitter release (Craig & Kang, 2007; Missler *et al,* 2003).

The white matter of the brain, which consists mainly of neuroglia and myelinated axons, showed reduced NRXN 1-3 immunopositivity compared to the grey matter of the prefrontal cortex (figure 5.2). There was, however, some immunopositivity to NRXNs 1 and 2 in the white matter region (figure 5.2C & D), in particular for NRXN2 which stained a number of oligodendrocytes.

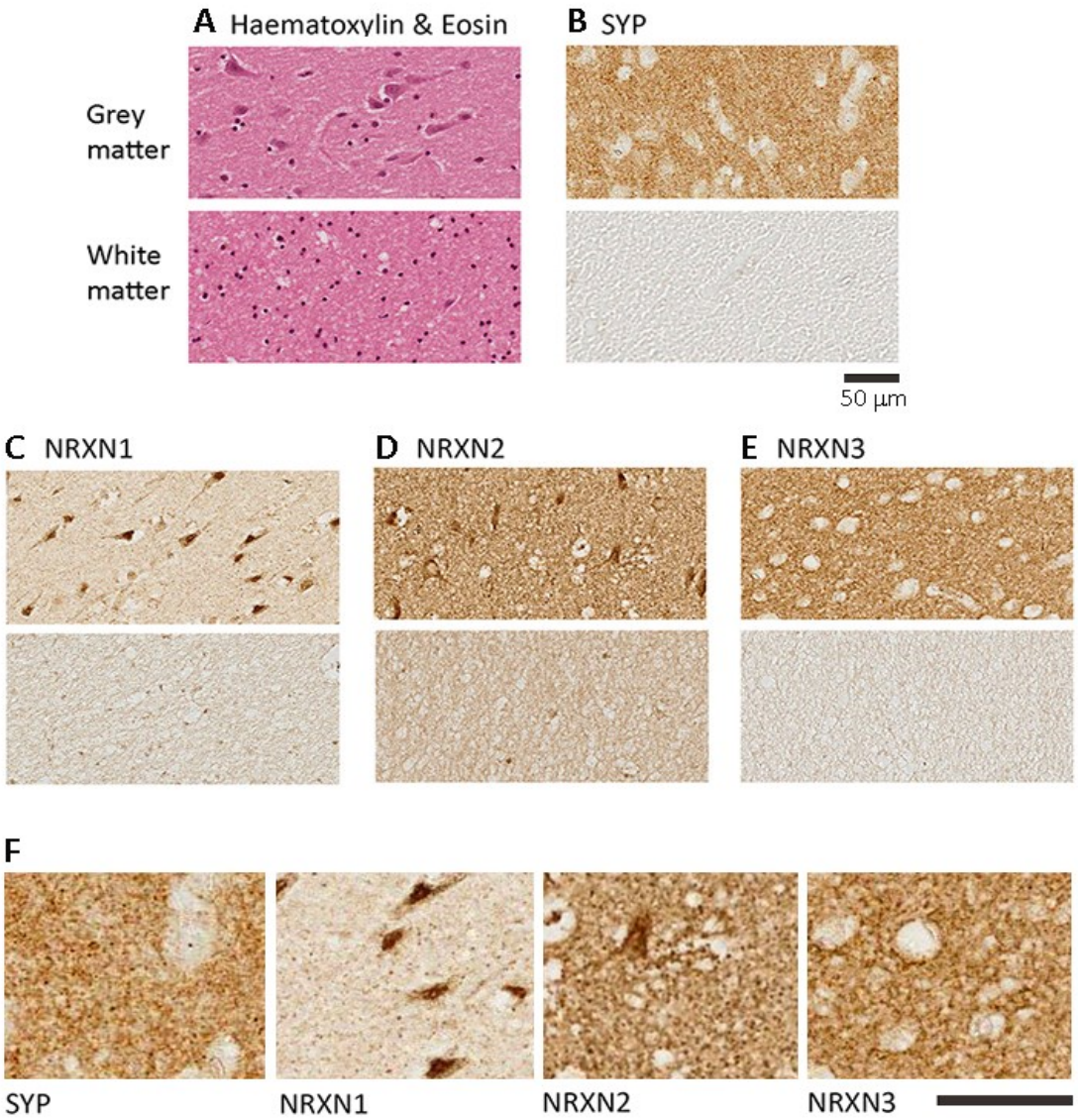


Figure 5.2 Distribution of SYP and NRXN proteins in the adult brain.

(A) Haematoxylin and eosin staining showed the cellular distribution in both the grey and white cortical brain matter. **(B)** Membranous SYP staining was present in only the grey matter of the cortex. **(C)** NRXN1 staining was present in the grey mater including the pyramidal cell bodies and in the white matter to a lesser extent. **(D)** NRXN2 staining was present in the grey and white matter of the cortex including the pyramidal cell bodies. **(E)** NRXN3 staining was similar to that of SYP, being present in the grey cortical matter and mostly absent from the white matter. **(F)** Punctate staining (black arrows) could be seen in SYP and NRXN 1-3 stains. Scale bar represents 50µm.

5.2.3 Laminar expression patterns of NRXN1 between 8-12 PCW

NRXN1 protein was distributed throughout the cortex at 8 PCW, however, there were regions of cells that showed a denser staining pattern (figure 5.3). In particular, the post-mitotic cortical plate (CP) and the apical surface of the proliferative ventricular zone (VZ) showed high levels of NRXN1 immunopositivity. The subplate is a cell sparse region, as was shown by the H&E stain, but cells scattered throughout it expressed NRXN1 quite strongly.

Axons in the SP, at this stage in development, are mostly cortical afferents. SYP immunopositivity was strongest in the SP between 8-12 PCW (figure 5.3). Double labelling of NRXN1 and SYP at 8 PCW showed that they were not co-expressed in cells of the SP, although both appeared to be expressed in the extracellular neuropil (figure 5.4Aii). The cells of the intermediate zone (IZ), sub-ventricular zone (SVZ) and the remaining ventricular zone (VZ) did show NRXN1 expression, however, it was not as apparent as in the CP and apical surface IZ regions. Although expressed in the VZ/ SVZ, double labelling with PAX6 revealed that NRXN1 and PAX6 immunopositivity did not overlap (figure 5.4Ai), possibly due to being present in different cellular locations. PAX6 is a transcription factor and the protein is found in the cell nucleus. There is no evidence that NRXN1 is present in the nucleus in the VZ/ SVZ and therefore, may be confined to the cell cytoplasm/ membrane. Alternatively, NRXN1 immunopositive cells could signify cells at a later stage of differentiation that have progressed from the early progenitor cell state.

At 10 PCW, the protein expression of NRXN1 was more evenly distributed across the cortex (figure 5.3B). The pattern of expression was similar to that of the H&E stain suggesting that at this age, NRXN1 protein can be found in all cortical cells of the CP, IZ, SVZ and VZ. There was, however, a reduction of NRXN1 protein in the SP.

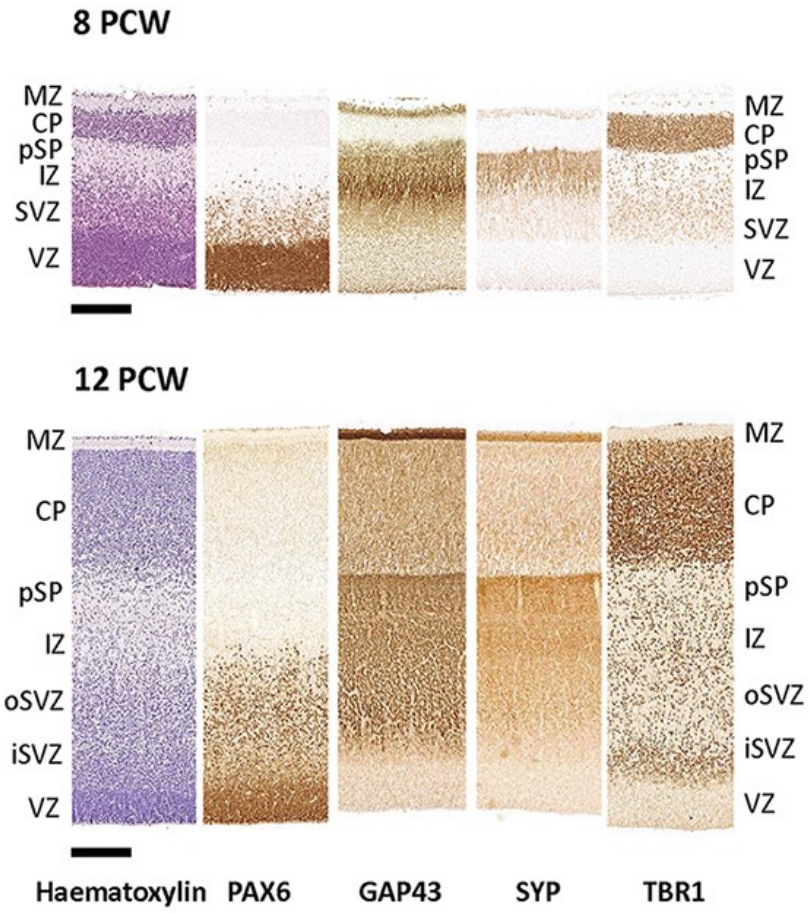


Figure 5.3 NRXN1 protein distribution in the cerebral cortex from 8-12 PCW in comparison to known cell type markers.

(A) At 8 PCW, NRXN1 was expressed in both proliferative and post mitotic cells as defined by PAX6 and TBR1 expression respectively. It was absent from the sub-plate (pSP) as shown by SYP expression. **(B)** NRXN1 was found in most cell types of the cortex at 10 PCW as shown by the haematoxylin stain including those stained by SYP and GAP43, although the staining was less intense. **(C)** At 12 PCW, NRXN1 protein was similarly distributed throughout the cortex, however, it was apparent that the protein is more concentrated in the superficial layers of the post mitotic CP. Scale bar represents 100µm.

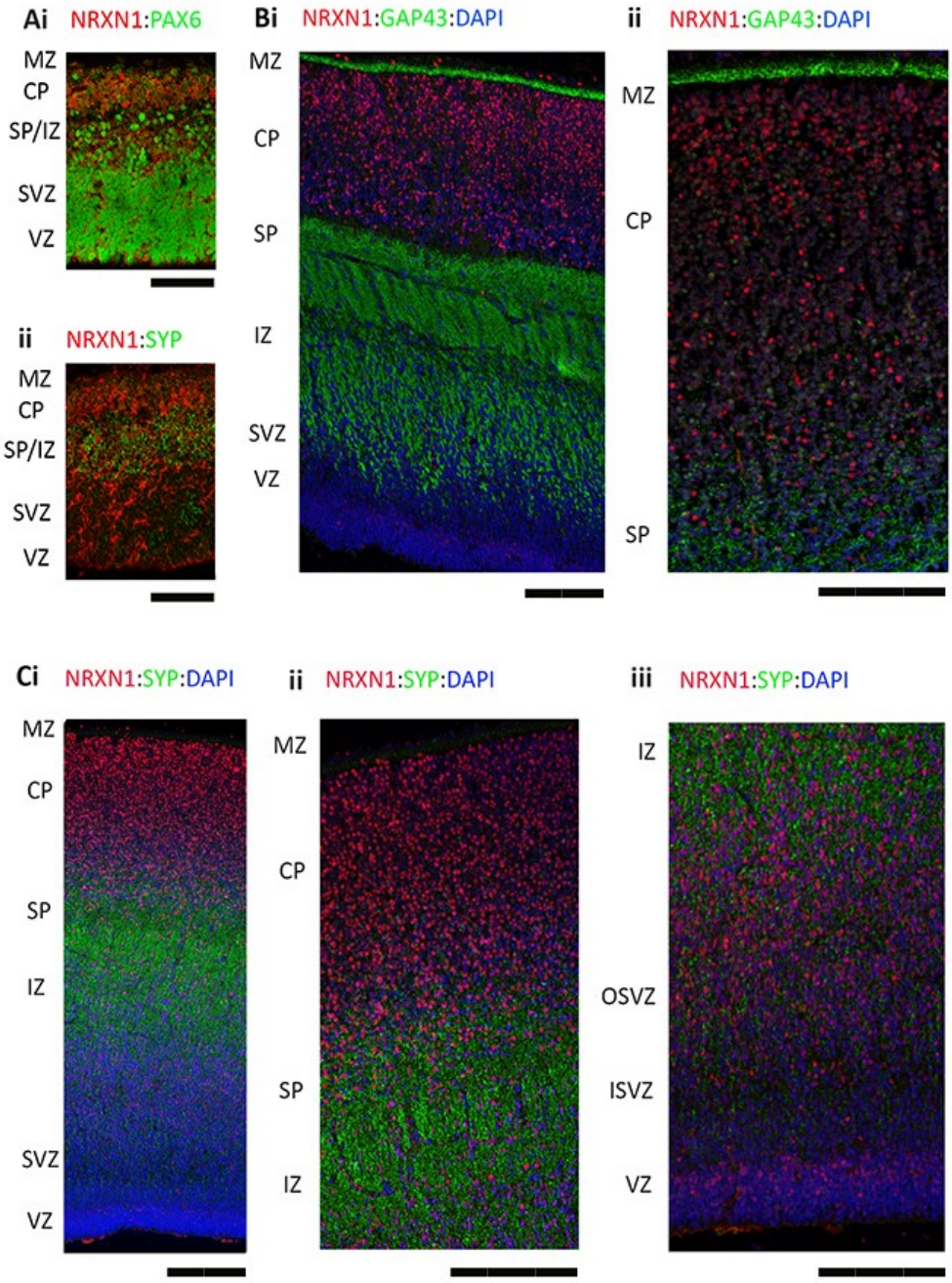


Figure 5.4 NRXN1 was not co-expressed with synaptic or axonal outgrowth markers.

(A) NRXN1 did not overlap in expression with the transcription factor PAX6 **(i)** although it was expressed in regions of cell proliferation or cells that express SYP at 8 PCW **(ii)** at 8 PCW. **(B)** By 12 PCW, NRXN1 protein was concentrated in the superficial layers of the CP and did not overlap in expression with cells expressing GAP43 in the SP/ IZ. **(C)** Cells expressing SYP did not express NRXN1 at 12 PCW **(i, ii)**. NRXN1 expressing cells were present in the proliferative layers VZ and SVZ regions of the cortex but the quantity of protein was not as high as that in the CP **(iii)**. Scale bar represents 100µm

At 12 PCW, NRXN1 protein was again distributed throughout the cortex (figure 5.3C). However, immunostaining was still reduced in the SP region. Double labelling with SYP revealed that, although immunopositivity was observed for both proteins in the SP and IZ, they were not expressed in the same cells or same regions within cells (figure 5.3C). NRXN1 immunostaining was more prominent in the CP at this age, in particular the outermost regions of the CP (figure 5.3). NRXN1 immunopositive cells were present in the intermediate zone although there was no co-expression with the axonal growth cone marker GAP43 (figure 5.3B; 5.4B). There was also strong SYP and GAP43 immunostaining of neurites in the MZ at 12 PCW but they showed no co-localisation with NRXN1 at 12 PCW. NRXN1 was clearly expressed in the CP and to a lesser extent in the SP, IZ and proliferative regions. The lack of co-localisation with neurite markers in the SP/ IZ suggests that the protein was not yet present in the cell membrane or neuropil, but possibly in the cell bodies, which is in contrast to the punctate immunostaining seen in the adult cortex.

5.2.4 Laminar expression patterns of NRXN2α between 8-12 PCW

NRXN2α protein was found at the highest levels in the subplate (SP), marginal zone (MZ) and apical ventricular zone (VZ) at 8 PCW (figure 5.5). This pattern of expression was similar to that of SYP, which was expressed in the MZ and SP regions of the cortex (figure 5.5A). Both SYP and GAP43 immunopositivity was observed in the MZ, SP and the intermediate zone (IZ), however, GAP43 immunopositivity was more apparent in the IZ compared to the SP whereas SYP was more apparent in the SP (figure 5.5A). NRXN2α immunopositivity, like SYP, was more apparent in the SP, however, double labelling of NRXN2α and GAP43 showed that these proteins were co-localised in the neurites of the MZ and the SP/IZ (figure 5.6Aii). There were cells immunopositive for NRXN2α scattered throughout the other regions of the cortex at this age including in the PAX6 immunopositive VZ and SVZ and the TBR1 immunopositive CP (figure 5.5). However, protein expression in these regions was less intense than in the MZ, SP and IZ. Double labelling with the transcription factors PAX6 and TBR1 showed that NRXN2α was located outside of the cell nucleus (figure 5.6Ai & iii) as there was no co-expression observed. Since there are very few cell processes in these regions at this age, NRXN2α protein is likely localised to the cell bodies and/ or the cell membranes.

At 10 PCW, the NRXN2α protein distribution had changed from 8PCW. Immunopositivity was most apparent in the outer layers of the cortical plate (CP) and intermediate zone (IZ) (figure5.5B). The MZ, SP, SVZ and VZ contained NRXN2α protein but the immunopositive staining was less intense than in the CP and IZ regions.

By 12 PCW NRXN2α protein was minimal in the VZ although there was some immunostaining at the apical surface of the VZ and in the SVZ (figure 5.5C). The SP and the IZ were immunopositive for NRXN2α (figure 5.5C). Double labelling revealed that in these regions, cells co-expressed NRXN2α and SYP/ GAP43 in neuronal processes (figure 5.6B & C). The cells in the area of the IZ nearest to the SP showed the greatest NRXN2α/ GAP43 co-expression (figure 5.6C) Double labelling also revealed a decline in co-expression of these two proteins in the regions of the IZ nearest the SVZ (figure 5.6Ci). NRXN2α protein was present in the CP, in particular the intermediate layer of the CP (figure 5.5C; 5.6Bii) most likely in the cell bodies/ cell membrane due to lack of neuropil in this region. At 12 PCW, the GAP43/NRXN2α co-expression in the MZ had disappeared (figure 5.6C).

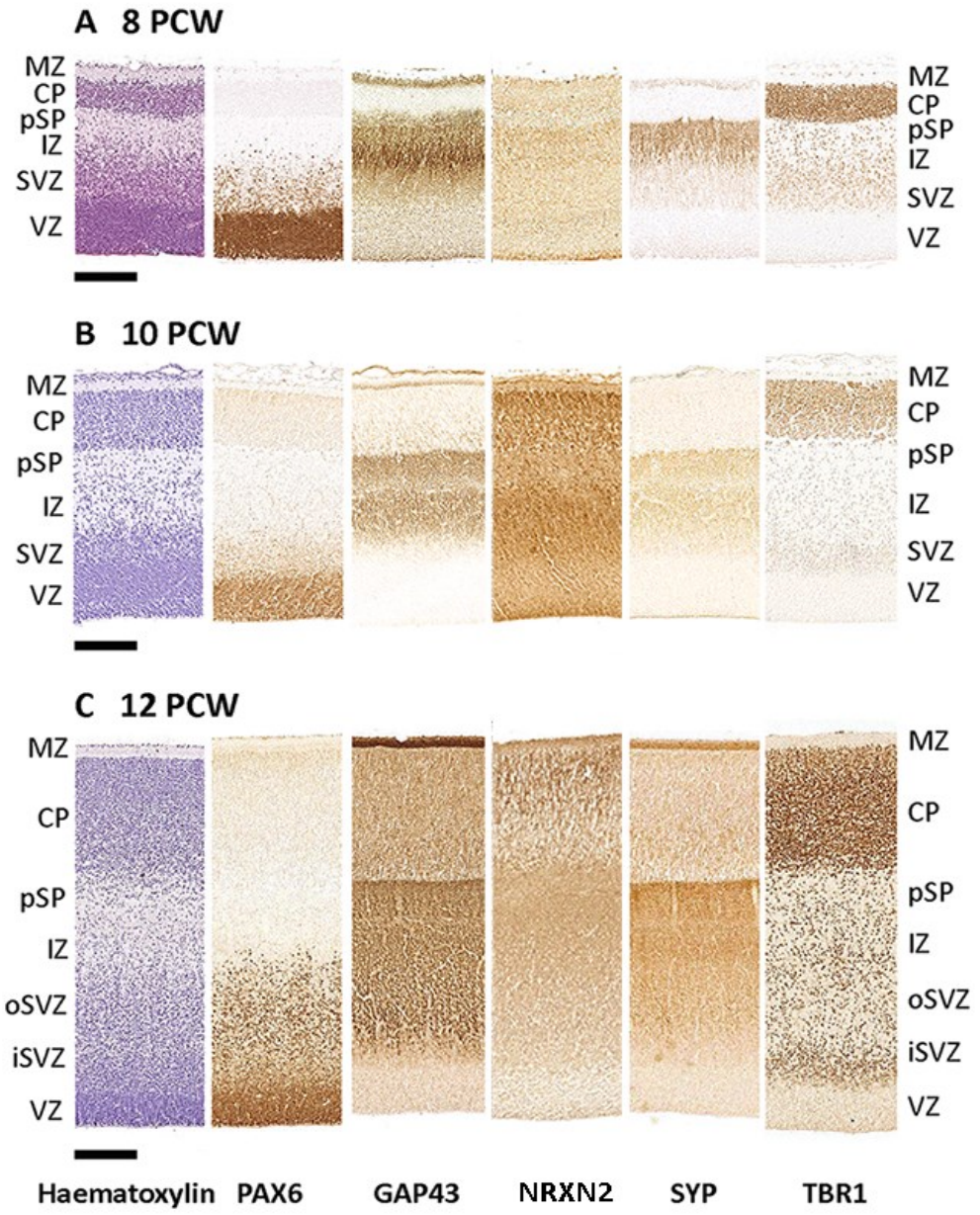


Figure 5.5 NRXN2 protein distribution in the cerebral cortex from 8-12 PCW in comparison to known cell type markers.

(A) At 8 PCW, NRXN2 was expressed in both proliferative and post mitotic cells but was more concentrated in the sub-plate (pSP) as shown by SYP expression. **(B)** At 10 PCW, NRXN2 was concentrated in the outermost region of the CP as well as the SP. **(C)** By 12 PCW, NRXN2 protein was more concentrated in the superficial layers of the post mitotic CP, the SP and IZ and was hardly found in the proliferative VZ. Scale bar represents 100µm.

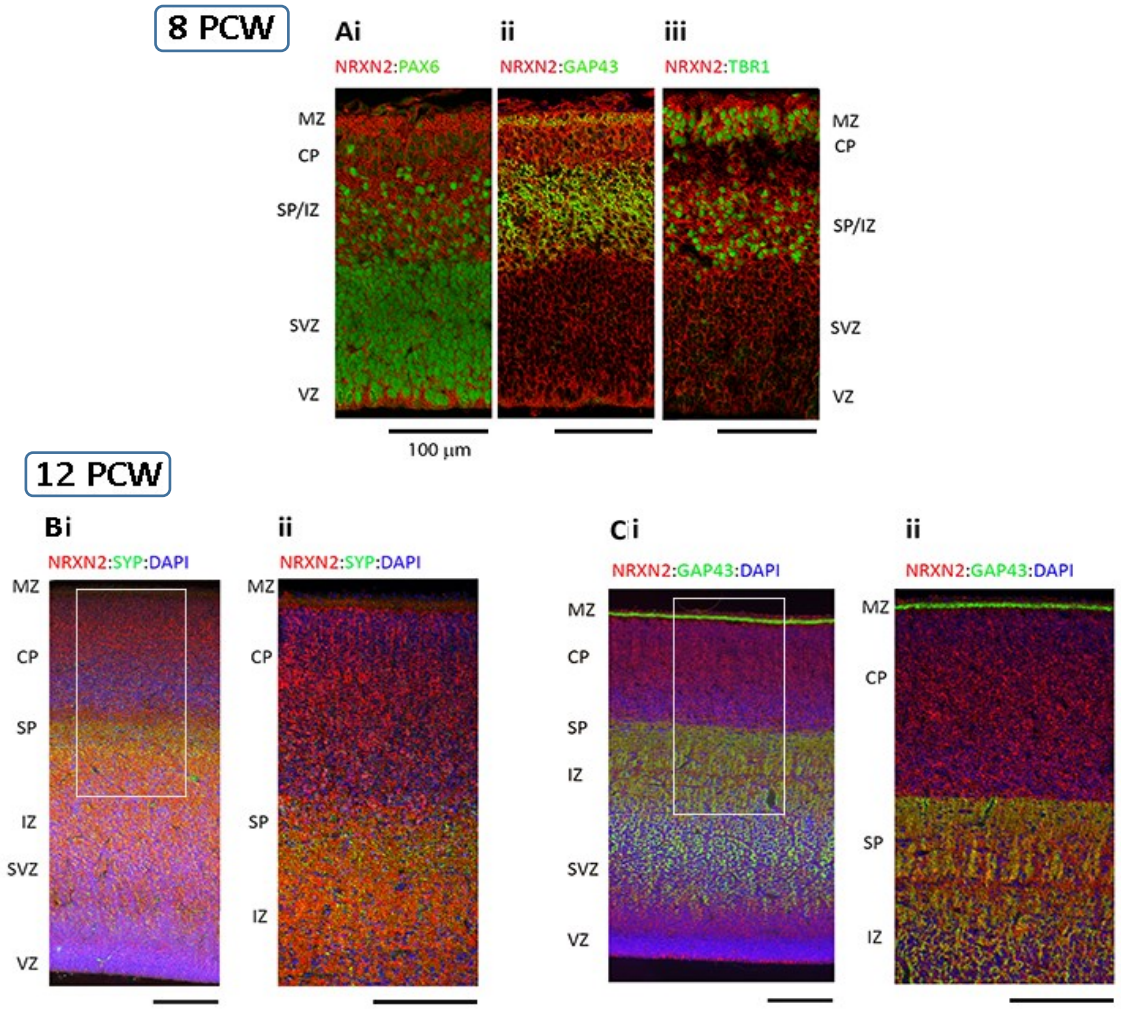


Figure 5.6 NRXN2α co-expression with synaptic markers. (Ai) At 8PCW**,** NRXN2α did not show co-expression with PAX6 in the proliferative cortical regions. (ii) NRXN2α was co-expressed with GAP43 in the MZ and the growing axons of the SP/IZ. (iii) NRXN2α expression was not co-expressed with TBR1 as the proteins appeared to be present in different cellular regions. **(B)** At 12PCW, NRXN2α was co-expressed with SYP in the SP and **(C)** NRXN2α was also co-expressed with GAP43 in the SP and regions of the IZ nearest the SP, but not in the MZ. Scale bar represents 100µm.

5.2.5 Laminar expression patterns of NRXN3 in the cortex between 8-12 PCW

NRXN3 had a very distinct pattern of expression at 8PCW. It was found only in the post mitotic CP that was also immunostained by the transcription factor TBR1 (figure 5.7A). Double labelling revealed that whilst TBR1 was confined to the nucleus and NRXN3 was present in the cell bodies/ cell membranes (figure 5.8B). Proliferative cells in the VZ and SVZ marked by PAX6 and neurites of the SP and IZ shown by SYP immunostaining were not immunopositive for NRXN3 (figure 5.7A). Double labelling with SYP revealed a complete separation of the two proteins within the cortex (figure 5.8A).

At 10 PCW, the protein begun to appear in cells of the SP, IZ, SVZ and VZ regions (figure 5.7B). Nevertheless, the majority of NRXN3 protein was observed in the cortical plate. The pattern of NRXN3 expression at 12 PCW was similar to that of 10 PCW. Although the NRXN3 protein could be seen in the VZ, SVZ, and IZ, the majority of the protein was found in the CP. When compared to SYP immunostaining in the SP (figure 5.7C), there is a lack of NRXN3 immunopositivity in this region. As with NRXN1, NRXN3 appeared to be more intense in the outermost region of the CP compared to the innermost regions (figure 5.7C). Double labelling of TBR1 and NRXN3 suggested there was some co-localisation of the two proteins in this earliest forming layer. However, the many of the cells in the CP did not show co-expression of these two proteins (figure 5.8D) suggesting different cellular localisation. Double labelling with PAX6 also showed some co-expression in the VZ (figure 5.8C) suggesting that NRXN3 was in the nucleus of these cells or that PAX6 was in the cell bodies.

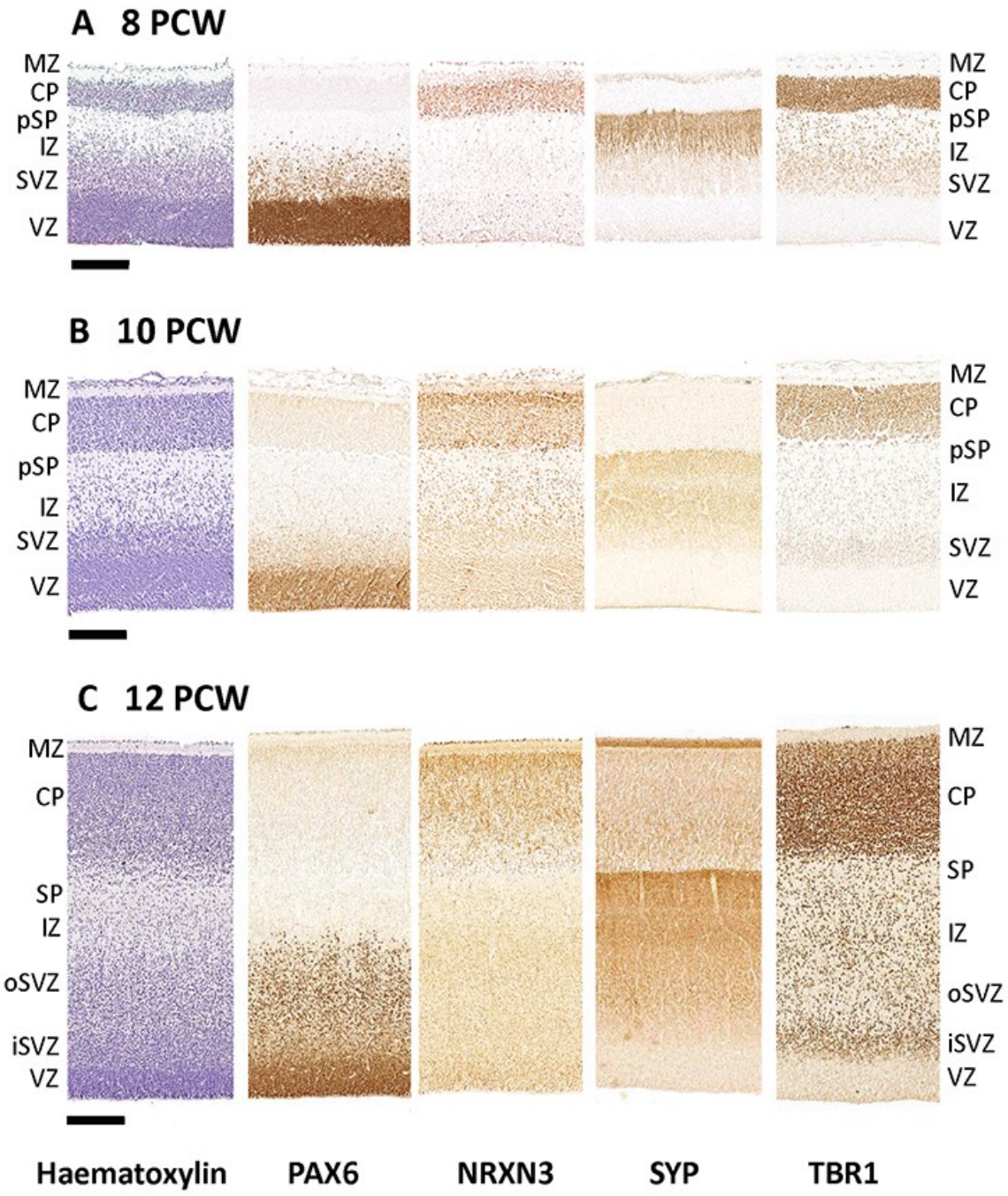


Figure 5.7 NRXN3 protein distribution in the cerebral cortex from 8-12 PCW in comparison to known cell type markers. **(A)** At 8 PCW, NRXN3 immunopositivity was similar to that of TBR1, showing immunopositivity in the post mitotic CP but not in the SVZ. The apical VZ was also immunostained for NRXN3. **(B)** At 10 PCW, NRXN3 immunopositivity was present, to a lesser extent, in the other regions of the cortex, but not at the apical surface of the VZ. The outermost regions of the CP showed the strongest immunopositivity. **(C)** At 12 PCW, NRXN3 immunopositivity was strongest in the outermost CP regions but was absent from the SP. Scale bar represents 100µm.

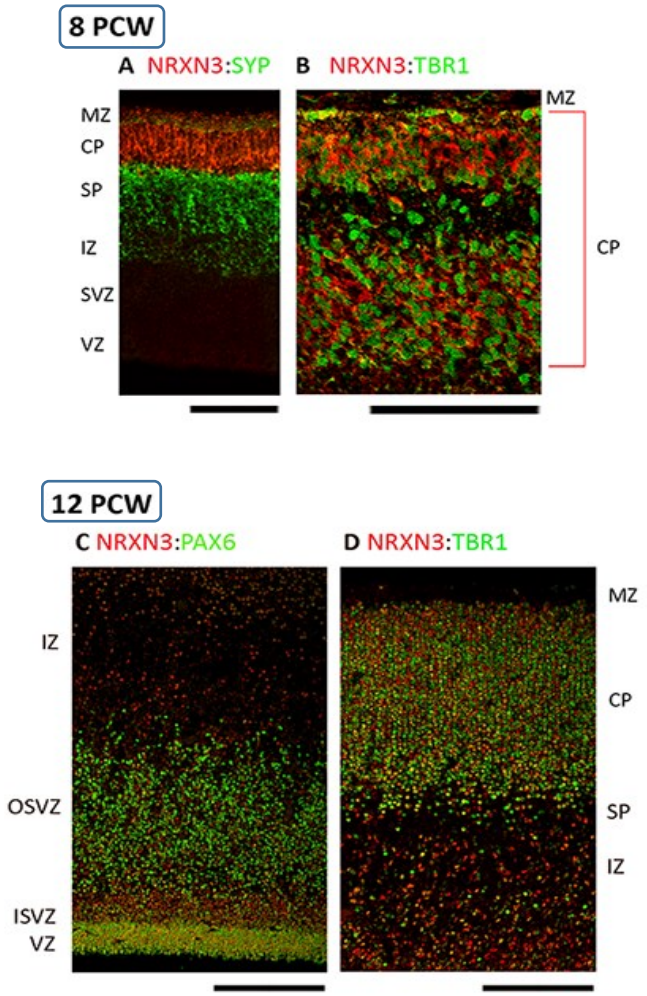


Figure 5.8 NRXN3 double labelling in the cortex at 8 and 12 PCW. (A) Double labelling using NRXN3 and SYP antibodies at 8 PCW showed no overlapping expression. **(B)** Although both expressed in the CP at 8 PCW, NRXN3 and TBR1 were not co-expressed. Immunopositivity of the transcription factor TBR1 was observed in the nucleus whilst NRXN3 immunopositivity was observed outside of this region, most likely in the cell membranes or cell bodies. **(C)** At 12 PCW, NRXN3 showed co-expression with PAX6 in the VZ but not the SVZ. **(D)** At 12 PCW, NRXN3 and TBR1 are both expressed in the CP but co-expression is minimal. Scale bar represents 100µm

5.2.6 Expression of NRXNs 1-3 in the lateral and medial ganglionic eminences and the thalamus at 12 PCW

NRXN1 immunoreactivity was seen throughout both the medial and lateral ganglionic eminences (figure 5.9B). Immunostaining was most dense along the VZ of the GE. NRXN1 protein was also found in the cells of the caudate nucleus. This is a more cell sparse region in comparison to the LGE and MGE and contains post mitotic cells migrating away from the proliferative GE region. The internal capsule was NRXN1 negative. This is a white matter structure in which both corticofugal axons and thalamocortical axons extend through (reviewed in Molnar *et al.,* 2012).

NRXN2 immunopositivity was seen in the proliferative regions of both the lateral and medial GE (figure 5.9C). Like NRXN1, the post mitotic cells of the caudate nucleus were mmunopositive for NRXN2. The axons tracts of the internal capsule were immunopositive for NRXN2 as they were for GAP43 and the synaptic transmembrane protein CASK. GAP43 protein was absent from both the lateral and medial GE. CASK immunopositivity was similar to that of NRXN2 being present in the proliferative regions as well as the post mitotic regions containing a high density of growing axons.

NRXN3 immunopositivity was observed in both the medial and lateral GE (figure 5.9D). Like NRXN1, NRXN3 immunostaining was most dense along the ventricular zone of both the LGE and the MGE. Fewer cells within the caudate and putamen were immunopositive for NRXN3 than they were for NRXNs 1 and 2. The axons of the internal capsule, as with NRXN1, did not express NRXN3.

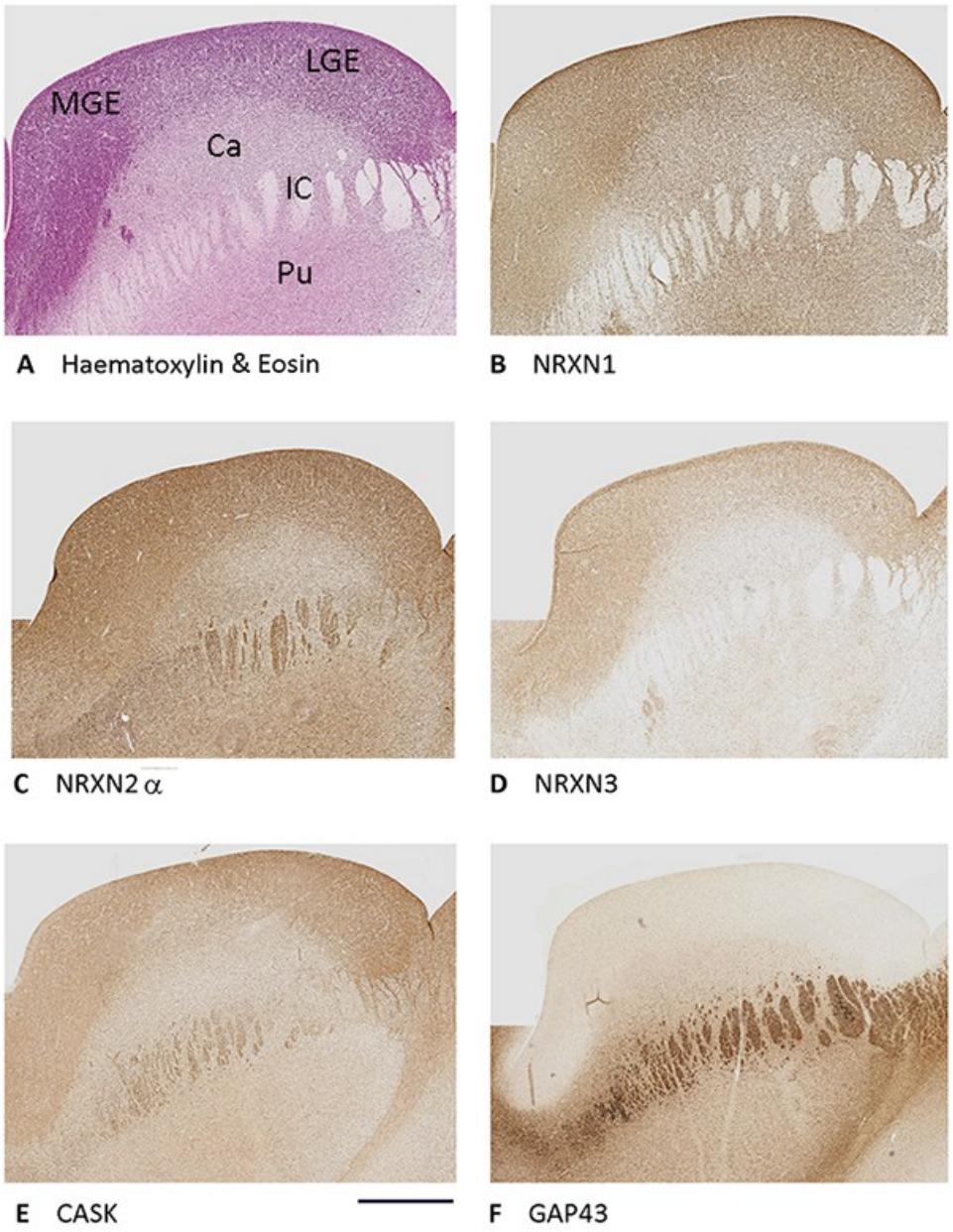


Figure 5.9 Expression of NRXNs in the sub pallium at 12 PCW. (A) H&E stain showing the cell dense staining of the medial ganglionic eminence (MGE) and lateral ganglionic eminence (LGE). The caudate (Ca) and the putamen (Pu) are less cell dense regions. There are no nuclei in the internal capsule (IC). **(B)** NRXN1 immunopositivity was observed throughout the sub-pallium but was absent from the IC. **(C)** NRXN2α immunopositivity was observed throughout the sub pallium including in the axon tracts of the IC. **(D)** NRXN3 immunopositivity was strongest in the MGE and LGE but there was no NRXN3 immunostaining in the IC. **(E)** CASK immunostaining was found throughout the sub-pallium including the IC. **(F)** Growing axons within the post mitotic regions of the Ca and putamen are GAP43 immunopositive, as are the outgrowing axons of the IC. Scale bar represents 1mm.

5.3 Discussion

Neurexins have been identified as presynaptic cell adhesion molecules that are able to link the pre and post synaptic membranes via their interaction with multiple post synaptic proteins (Reissner *et al.,* 2014; Lee *et al.,* 2012; Siddiqui *et al.,* 2010; Zhang *et al.,* 2010; Missler *et al.,* 1998). αNRXNs are also involved in the release of neurotransmitters and aid in the organisation of secretory granules and their secretion in non-neuronal tissues (Mosedale *et al.,* 2012; Dudanova *et al.,* 2006). We have provided, for the first time, evidence that NRXN proteins are present in human neural cells pre-synaptogenesis and that, due to their cortical localisation and cellular protein co-expression, they may have roles in differentiation, migration and axonal/ dendritic outgrowth.

5.3.1 In the adult cerebral cortex, the majority of NRXN protein is present in the grey matter

NRXN protein was present predominantly in the grey matter of the adult cerebral cortex. The grey matter of the cerebral cortex contains the majority of neuronal cell bodies and is the region of the cortex in which the majority of synapses are present as confirmed by the expression pattern of the pre-synaptic marker synaptophysin (figure 5.1). The punctate staining pattern observed for the NRXN proteins is often associated with synapses and is thought to be representative of mature synaptic boutons (Fletcher *et al.,* 1991). In addition to the synaptic like staining, NRXN1 and 2 proteins were also present in the pyramidal cells of the adult cerebral cortex. Ichtchenko *et al.* (1995) showed that NRXNs excluding splice site #4 are enriched in the pyramidal cell layer of the hippocampus.

The white matter of the adult brain consists of the myelinated axons. As there are very few neuronal cell bodies and therefore, very few interactions between pre and post synaptic membranes in this region, the absence of neurexin protein was expected. However, NRXN2 protein was present in the white matter fibres of the cortex and NRXN1 in a number of cell bodies, possibly astrocytes or oligodendrocytes, within the white matter. Indeed, it has been previously shown that NRXNs are not confined to neuronal synaptic terminals and are, in fact, present in non-neuronal perineurial cells which are epithelial cells and at axon-glial interactions (Russel & Carlson, 1997). The knockdown of Nrxn1 expression in iPS cells decreases the expression of the astrocyte marker, GFAP (Zeng *et al.,* 2013) and mouse

models of other neurodevelopmental conditions such as Ret syndrome and schizophrenia have implicated glial cell dysfunction in the pathology of the condition (reviewed in Yamamuro *et al.,* 2015).

Due to the predictable staining pattern of NRXNs in the grey matter of the adult cortex, it was surprising that these proteins were present in the developing cortex in regions that do not contain synapses (see sections 5.3-5.10).

5.3.2 NRXNs are expressed outside of synaptogenic regions in the developing cortex

NRXNs are mainly associated with the development and maintenance of synapses (Graf *et al.,* 2004; Scheiffele *et al.,* 2000), which at this developmental stage, are only present in the SP, MZ and IZ (figure 5.2). In this study, NRXN immunopositivity was observed in the proliferative, migratory and post mitotic regions of the developing cortex between 8 and 12 PCW. Double labelling of NRXN1 and SYP, revealed that, although some cells of the SP and MZ are immunopositive for NRXN1, there is no co-expression of these two markers (figure 5.4). NRXN3 protein is also absent from cells that express SYP (figure 5.8). It is only NRXN2 that was co-expressed with SYP in cells of the cerebral cortex (figure 5.6). NRXN2 knock down in mice causes a decrease in the expression of genes associated with excitatory and inhibitory synaptic transmission as well as reducing the frequency excitatory miniature post-synaptic currents (mEPSCs) (Born *et al.,* 2015; Dachtler *et al.,* 2014). The morphological structure of the brain remains unaffected (Born *et al*., 2015).

In the VZ of the cerebral cortex, radial glial progenitor cells extend processes and shuttle between the basal and apical surface during asymmetric division (Tramontin *et al.,* 2003). NRXN1 immunopositivity appears more pronounced at the apical surface of the VZ at 8 PCW (figure). The cell is usually in the M/G2 phase of cell division at this point of their interkinetic division (Del Bene*.,* 2011). NRXN1 protein expression may peak during this phase of the cell cycle, which allows for protein synthesis and cell growth. It may also symbolise that the NRXNs, despite their presence in proliferative regions, are confined to cells that have longer cell cycles i.e. those that are in the process of differentiation. It has been previously suggested that, due to their large size, there would be no time for transcription of these long genes in rapidly dividing cells (Rowen *et al.,* 2002). As discussed in the previous chapter and

in the literature, all three NRXN genes increase in expression during neuronal differentiation (Konopka *et al.,* 2012; Gurok *et al.,* 2004).

Neural progenitor cells reside in the VZ and SVZ of the cerebral cortex dividing symmetrically (Huttner & Kosodo, 2005) to increase the number of progenitor cells. Progenitor cells can switch to asymmetrical division giving rise to both progenitor cells and differentiating neurons. After surpassing their progenitor like state, these differentiating cells aim to migrate into the cortical plate either by somal translocation or by migrating along these radial glial fibres. At this point, cells need to establish polarity. Previously it was expected that NRXNs played a part in a later stage of neuronal differentiation in which cells had ceased migration, made contact with adjacent cells and had begun to organise synaptic proteins to the cell membrane. We suggest, however, that NRXN1 from 8 PCW and NRXN3 from 12 PCW are important in much earlier stages of neuronal differentiation as the protein is clearly present in the regions of proliferation.

The most intense NRXN1 and NRXN3 immunostaining is observed in the post mitotic cells of the cortical plate (figure 5.2, 5.6), in particular the outermost CP compared to the innermost layers. Due to the cortical plate forming in an 'inside out manner', the outermost layers consist of the most recently migrated neural progenitor cells. The inner and outer regions of the CP can be distinguished by their gene expression patterns, with the outermost cells showing an increase in the expression of genes associated with metabolism whilst the innermost layers show an increase in genes associated with synaptic connections (Miller *et al.,* 2014).

During differentiation of neuronal precursors into mature neurons, cells switch their mode of metabolism from glycolytic to oxidative (reviewed in Kim *et al*., 2014). Stem cells are known to show a decreased amount of oxidative phosphorylation in comparison to their differentiated counterparts (Varum *et al.,* 2011). It has been proposed that this metabolic switch occurs just prior to the switch from proliferation to differentiation (Homem *et al*., 2014). It is possible that NRXNs could induce this switch or, vice versa, the switch in metabolism could induce the expression of NRXNs.

5.3.3 The role of NRXNs in migration

The presence of NRXN1 and 3 proteins in cells of the VZ, SVZ and IZ may suggest that they have functions in cellular migration after cells cease division. Cell adhesion molecules are important in neuronal migration. During neural crest migration, cadherins, integrins and IgCAMs are both up and down-regulated in a tightly controlled process of gene expression (reviewed in McKeown *et al.,* 2013). The expression of the different gene family members differs depending on the migration pathway. Due to the importance of cell adhesion molecules in neuronal migration, it is plausible that neurexins could play a role in the migration of neurons from the VZ to the CP.

Certainly, mutations in *CNTNAP2*, which is similar in structure to *NRXNs 1-3* and classed as an autism susceptibility gene, have been implicated in neuronal migration defects (Strauss *et al.,* 2006). MRI scanning identified focal malformations in the temporal lobe and striatum of three people with *CNTNAP2* mutations. Cortical thickening and abnormal neuronal clustering was observed and ectopic neurons were observed within the sub-cortical white matter. The knock down of Cntnap2 in mice also causes abnormal neuronal migration (Penagarikano *et al.,* 2011). Cells lacking this protein show reduced migration through the deep layers of the mouse cortex and can be found ectopically in the corpus callosum at P14.

5.3.4 The proposed role of NRXN2 in neurite/ axonal outgrowth

NRXN2 and the axonal outgrowth marker GAP43 are co-expressed in cells of the IZ (figure 5.5) as well as both being expressed in the internal capsule of the sub-pallium. CASK, the peripheral plasma membrane protein, is also expressed in the internal capsule. It was found that CASK has the ability to recruit proteins to the plasma membrane to promote neurite outgrowth (Watkins *et al.,* 2013), possibly by regulating the actin cytoskeleton (Vessey and Karra, 2007). Both the rearrangement of the cytoskeleton and the endo/exocytosis of synaptic vesicles that are separate from those containing neurotransmitters are essential for the elongation and stabilisation of neurites and therefore, of axons. Exocytic vesicles require soluble N-ethylmaleimide sensitive fusion attachment protein receptor (SNARE) complexes that include syntaxin proteins for their role in neurite outgrowth (Kunwar *et al.,* 2010; Zylbersztajin, 2011). There is evidence of NRXNs directly interacting with syntaxins and other presynaptic proteins to regulate neurotransmitter release (O'Connor *et al.,* 1993).

Missler *et al.* (2003), using triple knockout mice, discovered that αNRXN was essential for calcium dependant neurotransmitter release. It has also been shown that NRXN1α contributes to secretory granule docking in pancreatic β cells (Mosedale *et al.,* 2012). Using hippocampal cultures from rats, it was shown that NRXN1β can promote neurite outgrowth via its interaction with NLGN1 and subsequent activation of FGFR1 (Gjorlund *et al.,* 2012). The NRXN-NLGN1 interactions are suspected to stabilize filipodia and contribute to the interplay between synaptic stabilisation and dendritic/ axonal outgrowth (Gjorlund *et al.,* 2012; Xuen Chen *et al.,* 2010).

5.4 Summary

The small differences in immunostaining patterns of the three NRXN antibodies suggests that they are likely to have distinct, as well as overlapping, functions in the adult brain and indeed this was shown to be the case in the developing brain also. Between 8 and 12 PCW, although NRXNs 1-3 proteins appeared in many of the same regions, there were also distinct differences in the localisation of these proteins that did not necessarily correspond to their proposed roles in the generation and maintenance of synapses. NRXN2 is most similar in regional specification to the pre-synaptic marker, SYP and is present in cortical and sub-cortical regions of axonal outgrowth. NRXNs 1 and 3 are more likely to be involved in neuronal differentiation before the development of synapses and, at this developmental stage, do not appear to contribute to synaptogenesis in the cortex. The realisation that NRXNs 1 and 3 have novel roles in development will promote research to elucidate these functions. This will allow us to better understand the pathogenesis of neurodevelopmental conditions such as ASD and to develop novel strategies for their treatment.

CHAPTER 6

Investigating the expression patterns, protein localisation and role of proteins associated with NRXN transcription and splicing in the developing forebrain

6.1 Aim of study

The aim of this study was to explore the expression patterns of genes whose protein products regulate *NRXN* transcription (Topoisomerases/ MECP2) and splicing (STAR proteins), within the human cerebral cortex between 8 and 12 PCW. In addition to this, to map the protein localisation of a number of these proteins and analyse the effects of reducing topoisomerase 2B expression in human cortical cell cultures on the expression of *NRXNs*.

6.1.1. Regulation of NRXN transcription and splicing

Neurexins 1-3 are each transcribed from two independent promoters creating the longer alpha *NRXN* and shorter beta *NRXN* transcripts (chapter 1.3.2). Knowledge of proteins that regulate *NRXN* transcription is limited although the knock down of *methyl CpG binding protein 2* (*MECP2*) and *Topoisomerase2* (*TOP2*) was shown to alter the expression levels of these genes (King *et al*., 2013; Runkel *et al*., 2013). The transcriptional regulator MECP2 is primarily involved in the silencing of genes (Fuks *et al*., 2003), although more recently it was also identified as a transcriptional activator (Chahrour *et al*., 2008). Runkel and colleagues discovered that the expression of NRXN is decreased by up to 50% in the brain of *MECP2* knockout mice at P7, suggesting that MECP2 acts as a transcriptional activator of *NRXN* genes in the early postnatal brain. In contrast to this, they discovered that at P20 *MECP2* knockout mice show increased levels of *NRXN* expression (Runkel *et al.,* 2013).

Topoisomerase enzymes govern the topological state of DNA. TOP1 and TOP3B are involved in the breaking of a single strand of the DNA helix to relive topological tension. Topoisomerase 2 genes, *TOP2A* and *TOP2B,* code for two separate proteins that are required for the breaking of both DNA strands to control DNA topology (reviewed in (Nitiss, 2009, Wang, 2002). *Neurexins* are relatively large genes within the human genome spanning more than 1kb (chapter 1.3.2). The inhibition of topoisomerase 1 (TOP1), using the chemotherapeutic agent topotecan, causes a decrease in the expression of *NRXNs 1* and *3*

(King *et al*., 2013) which are the largest of the *NRXN* genes, as well as decreasing the expression of other long genes in cultured cortical mouse neurons differentiated from iPSCs. Inhibiting TOP2B using the inhibitor ICRF-193 also decreases the expression of long genes in these cells. Lentiviral shRNA knockdown of these genes had the same effect suggesting that TOP1 and TOP2 are required for the correct expression of NRXNs 1 and 3 in mouse cortical neurons (King *et al.,* 2013). Interestingly, TOP2A did not have an effect on the expression of NRXN proteins (King *at al.,* 2013). These studies suggest that MECP2 and Topoisomerases are transcriptional regulators of NRXN genes in mice.

In addition to the alpha and beta *NRXN* transcripts generated from two promoters, each NRXN gene contains six splice sites that potentially are able to generate thousands of isoforms (Treutlein *et al*., 2014, Tabuchi and Südhof, 2002). The regulation of *NRXN* splicing is controlled by the STAR family of proteins (consisting of KHDRBS1, 2 and 3) in mice (chapter 1.3.2). To date, no other candidates for the control of NRXN splicing have been identified.

As of yet, no evidence exists to support the role of these proteins in the control of *NRXN* transcription or splicing in the human brain. We have shown that *NRXNs* were expressed during this developmental period (chapter 4 & 5) and it is essential to explore whether these suggested transcriptional regulators are also expressed. Co-expression of these genes with *NRXNs* would provide evidence for the theories developed in mouse and cellular models that TOP2B and STAR proteins also regulate *NRXN* transcription and splicing during human development.

6.2 Results

6.2.1 Genes associated with NRXN transcriptional regulation were expressed in the human cortex from 9 PCW

Analysis of the RNAseq data (carried out as described in chapter 4.2.3) revealed that *topoisomerases* were expressed in the cerebral cortex between 9 and 12 PCW. *TOP1* was expressed at higher levels than *TOP3B*, reaching almost 40% of the combined reference gene expression. *TOP3B* expression reached less than 5% that of the reference gene expression. There was no significant change in expression of either *TOP1* or *TOP3B* between 9 and 12 PCWs (figure 6.1A). The expression patterns of *TOP2A* and *TOP2B* were different between 9 and 12 PCW. *TOP2A* appeared to decrease in expression with age whereas *TOP2B* appeared

to increase with age, although neither of these changes is significant (p>0.05) (figure 6.1A). *TOP2B* had a higher relative expression level than *TOP1 and TOP2A*, being expressed at similar levels to the reference genes. *TOP2A* also showed a high level of expression, reaching half that of the reference genes. Regulator of transcription, *MECP2*, was also expressed in the cortex between 9 and 12 PCW (figure 6.1A). There was no significant change in expression during this period. The expression level of *MECP2* was less than 10% of the control gene expression (figure 6.1A). There were no significant differences in expression identified between the anterior, central, posterior and temporal cortical regions at 9, 11 or 12 PCW (figure 6.1 B, C, D).

6.2.2 Genes identified as regulators of NRXN splicing were expressed in the human cortex from 9 PCW

KHDRBS1, 2 and *3* were expressed between 9 and 12 PCW. *KHDRBS1* showed the highest level of expression compared to *KHDRBS 2* and *3*, reaching more than 80% of reference gene expression (figure 6.2A). The expression of *KHDRBS2* and *3* was less than 20% of the reference gene expression. *KHDRBS2* and *3* expression, but not that of *KHDRBS1*, significantly increased between 9 and 12 PCW (figure 6.2A).

KHDRBS1 expression was significantly higher in the temporal cortex compared to the central cortex at 9 PCW and *KHDRBS1* expression in the temporal and posterior cortex was significantly higher than in the central cortex at 11 PCW ($p<0.05$; figure 6.2B & C). There were no identified expression gradients for KHDRBS1 at 12 PCW (figure 6.2D). There was no significant difference in *KHDRBS2* expression between the anterior, central, posterior and temporal regions of the cortex ($p<0.05$; figure 6.2B, C & D). *KHDBR3* expression in the temporal cortex was significantly increased in comparison to the central and posterior cortex at 9PCW and expression in the temporal cortex was significantly higher than in the anterior, central and posterior cortex at 11 PCW ($p<0.05$; figure 6.2B & C). This expression gradient had disappeared by 12 PCW (figure 6.2D).

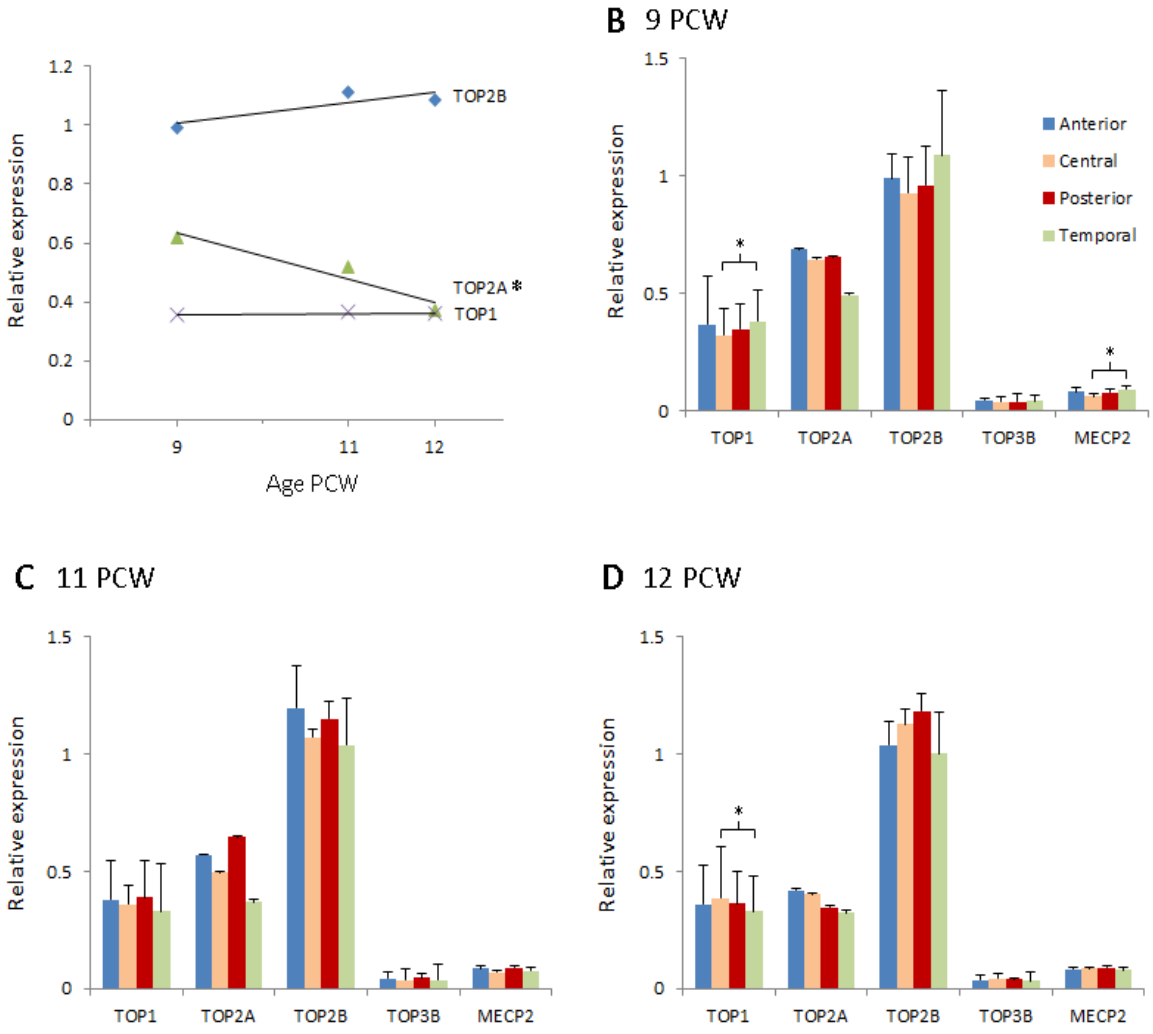


Figure 6.1 Genes associated with NRXN transcriptional regulation were expressed in the human cortex from 9 PCW. (A) *TOP1, TOP2B* and *MECP2* did not increase in expression between 9 and 12 PCW. *TOP2A* significantly decreased in expression during this period whilst *TOP3B* showed a small, but significant increase in expression. **(B)** There was no significant difference in expression of *TOP2A, TOP2B* or *TOP3B* across cortical regions at 9PCW. Expression of both *TOP1* and *MECP2* in the temporal cortex was significantly higher than its expression in the central cortex. **(C)** There were no significant differences in the expression of any of these genes across the cortex at 11PCW. **(D)** There were no significant differences in the expression of *TOP2A, TOP2B, TOP3B* or *MECP2* across the cortex at 12 PCW. The expression of TOP1 was significantly higher in the central cortex compared to the temporal cortex. ***p<0.05; n=between 3 and 10**

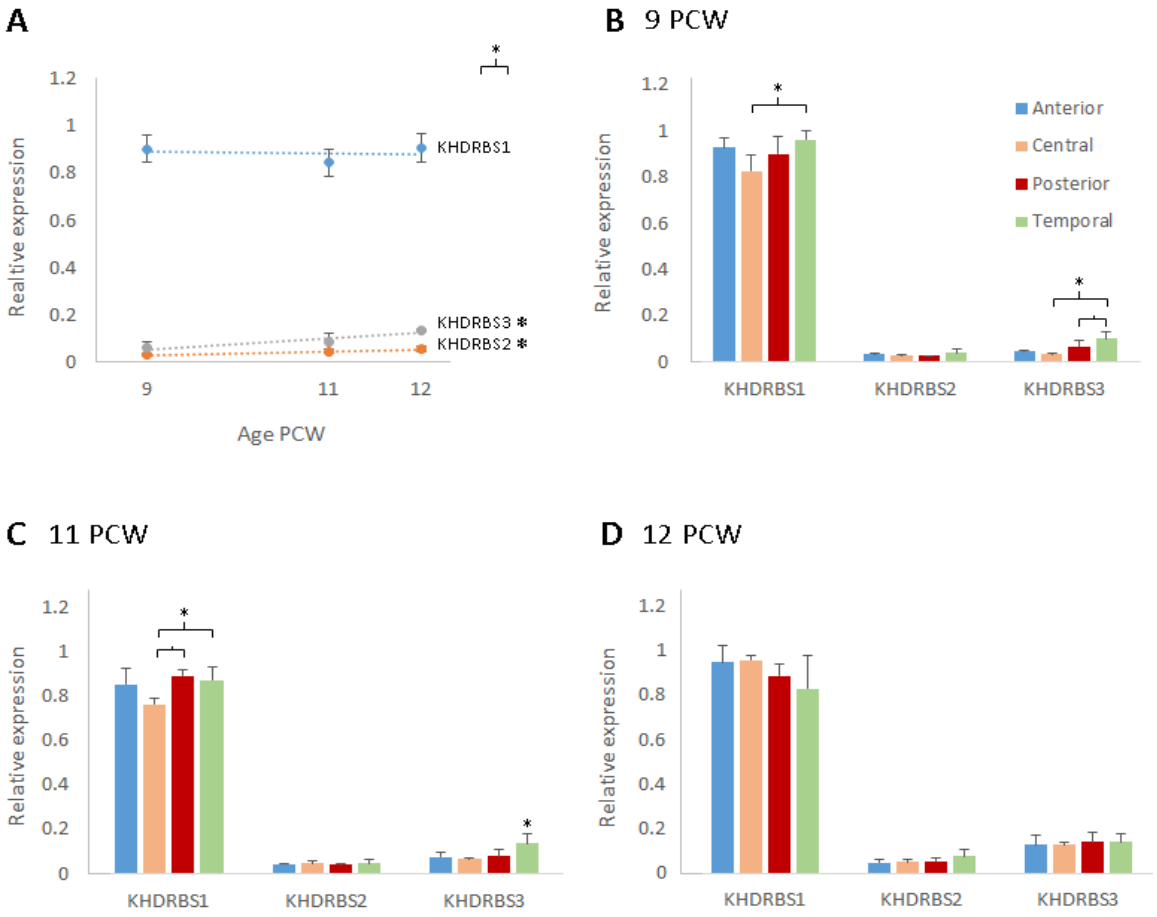


Figure 6.2 Regulators of NRXN splicing, KHDRBS, were expressed in the human cortex from 9 PCW. (A) *KHDRBS1* was significantly higher in expression that *KHDRBS2* and *3* but did not increase in expression with age. *KHDRBS2* and *3* both significantly increased in expression from 9 to 12 PCW. **(B)** There was no significant difference in expression the of *KHDRBS2* across cortical regions. Expression of *KHDRBS1* in the temporal cortex was significantly higher than its expression in the central cortex. The expression of *KHDRBS3* in the temporal cortex was significantly higher than that of the central and posterior regions. **(C)** The expression of *KHDRBS1* in the temporal cortex was significantly higher than that of the central and posterior regions at 11 PCW. There were no significant differences in the expression of *KHDRBS2* across the cortex. *KHDRBS3* expression was significantly higher in the temporal cortex compared to all other cortical regions examined. **(D)** There were no significant differences in the expression of *KHDRBS1, 2* and 3 across the cortex at 12 PCW. ***p<0.05; n=between 3 and 10**

6.2.3 Markers of cell proliferation, MGE and LGE

KI-67 (KI67) is a marker of cell proliferation and is present in all phases of the cell cycle except G_0 (reviewed in Scholzen and Gerdes, 2000). It is essential for the formation of the perichromosomal compartment which is important in the assembly and organisation of the nucleolus (Booth *et al*., 2014). The antibody against the KI67 protein has been used in previous publications (Chan *et al*. 2006; Meyer *et al*. 2002b).

PAX6 and TBR1 antibodies are those described in chapter 5.2.1. In addition to being a marker for proliferating cells of the cortical VZ/ SVZ (Bayatti *et al*., 2008a, Kerwin *et al.,* 2004; Magdalena *et al.,* 1998), PAX6 is also a marker for the lateral ganglionic eminence (LGE).

NKX2.1 is a transcription factor and a marker of the medial ganglionic eminence (MGE) in humans (Butt *et al*., 2008). NKX2.1 is required to generate neurons of the globulus pallidus and ventrally derived interneurons (Flandin *et al.,* 2010).

Antibodies against TOP2A and TOP2B proteins used in this study were donated by Professor Caroline Austin (table 2.). Each antibody recognised the C terminal domain (figure 6.3A). TOP2A recognised residues 1244-1531 and TOP2B recognised residues 1263-1621. The antibodies have been used previously for quantitative immunofluorescence in human cell lines (Lee *et al*., 2016).

Antibodies to KHDRBS2 and 3 were kindly donated by Professor Scheiffele, University of Basle (table 2.4). Immunogen sequences used to generate the antibodies were VNEDAYDSYAPEEWAT (residues 308-324) and PRARGVPPTGYRP (residues 243-256) respectively (figure 6.3B). The antibodies have been used previously for Immunofluorescence studies in mouse brain (Iijima *et al*., 2014).

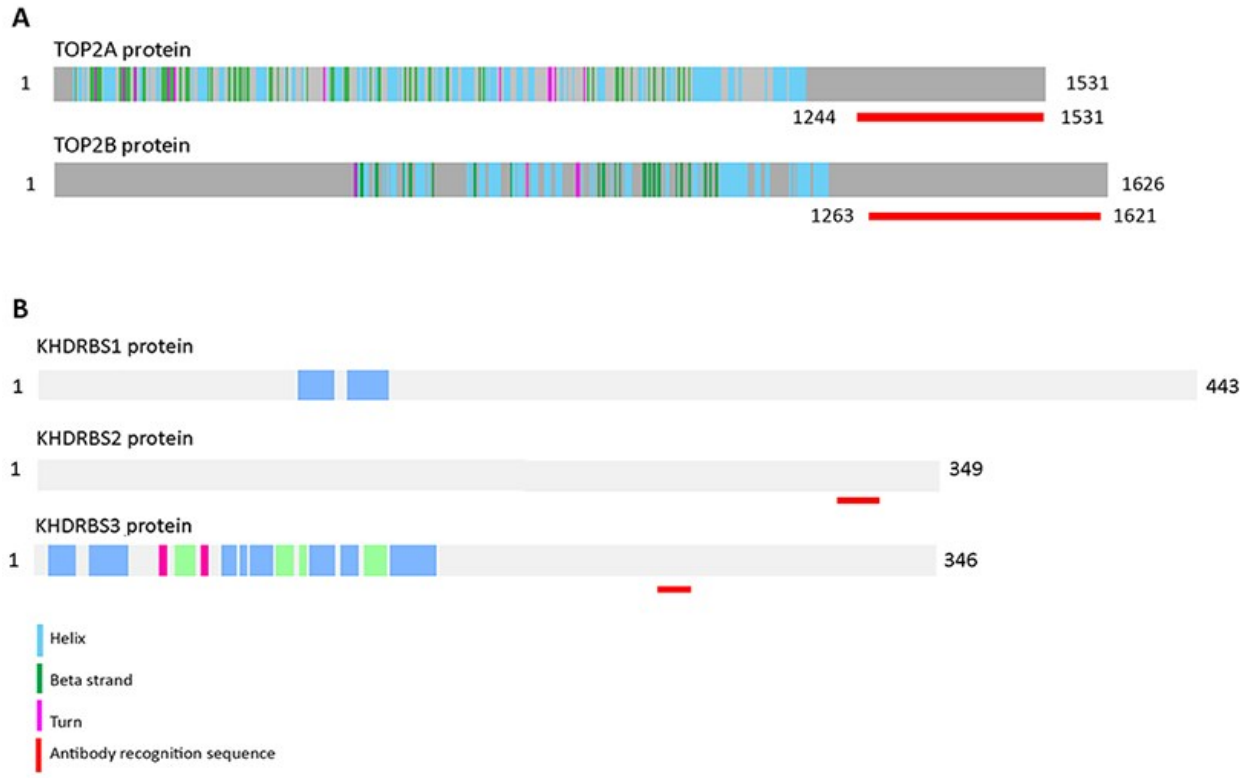


Figure 6.3 Antibody recognition sequences used in this study. (A) TOP2A and TOP2B antibodies were designed to recognise residues 1244-1531 and 1263-1621 of the C-terminal domain respectively. **(B)** Antibodies against KHDRBS2 and KHDRBS3 recognised residues 308-324 and residues 243-256 respectively.

6.2.4 TOP2A was expressed in regions of cell division

Although not previously identified as affecting NRXN expression, TOP2A protein expression in the developing human cortex was examined using an antibody that recognised the C terminal domain (figure 6.3). Due to sample availability, brains were aged between 9 and 12 PCW. During this developmental time period, TOP2A immunoreactivity was identified in the proliferative ventricular and sub-ventricular zones of the cerebral cortex that also stain positive for the radial glial marker PAX6 (figure 6.4). A higher proportion of these cells were PAX6 positive suggesting that not all radial glial cells express TOP2A. TOP2A staining was stronger at the apical surface of the VZ, as was that of the marker of cell division, KI67 (figure 6.4). KI67 protein is most abundant in cells at the G2/M phase of division (Scholzen and Gerdes, 2000). Double labelling with KI67 and TOP2A showed that these two proteins were highly co-localised throughout the VZ and SVZ (figure 6.5A).

As seen with KI67, there was also a dense staining of TOP2A positive cells in the inner SVZ (figure 6.6). These cells are intermediate progenitor cells that also express TBR2 (Lui *et al*., 2011, Bayatti *et al*., 2008b). Double labelling with PAX6 and TOP2A revealed that the majority of cells showing co-localisation were at the apical surface of the VZ and the inner SVZ (figure 6.5C). Double labelling of TOP2A and TOP2B also showed strong co-localisation in these regions (figure 6.5D white arrows). Very few cells of the intermediate zone (IZ) were immunopositive for TOP2A at 9 PCW and even less so by 12 PCW (figure 6.4). TOP2A protein was not detected in the post mitotic cortical plate (CP) between 9 and 12 PCW (figure 6.4; figure 6.5E).

6.2.5 TOP2B is expressed in both proliferative and post- mitotic regions of the cortex

TOP2B immunoreactivity was observed in both the proliferative PAX6 positive regions of the cortex and in the post mitotic neurons that are immunopositive for TBR1 (figure 6.4). TOP2B immunopositivity was weaker in the VZ compared to the CP and SVZ at 9PCW but by 11 PCW, staining was uniform throughout the cortex (figure 6.4). The ventricular surface of the cortex showed strong TOP2B immunopositive staining at 9 PCW (figure 6.4A), however, by 11 and 12 PCW, this distinctive VZ staining had disappeared (figure 6.4B & C).

Double labelling of TOP2B with the marker of proliferation, KI67 revealed a lack of co-localisation suggesting that, as these proteins are both nuclear, they are present in different cells within the proliferative regions (figure 6.5B). Double labelling with TBR1 showed a high level of co-localisation of these two proteins in the CP and in the IZ (figure 6.5Fi, ii & iii). TOP2B immunopositivity was strongest in the outer regions of the cortical plate, which consist of the most recently migrated post mitotic neurons (figure 6.5Fii).

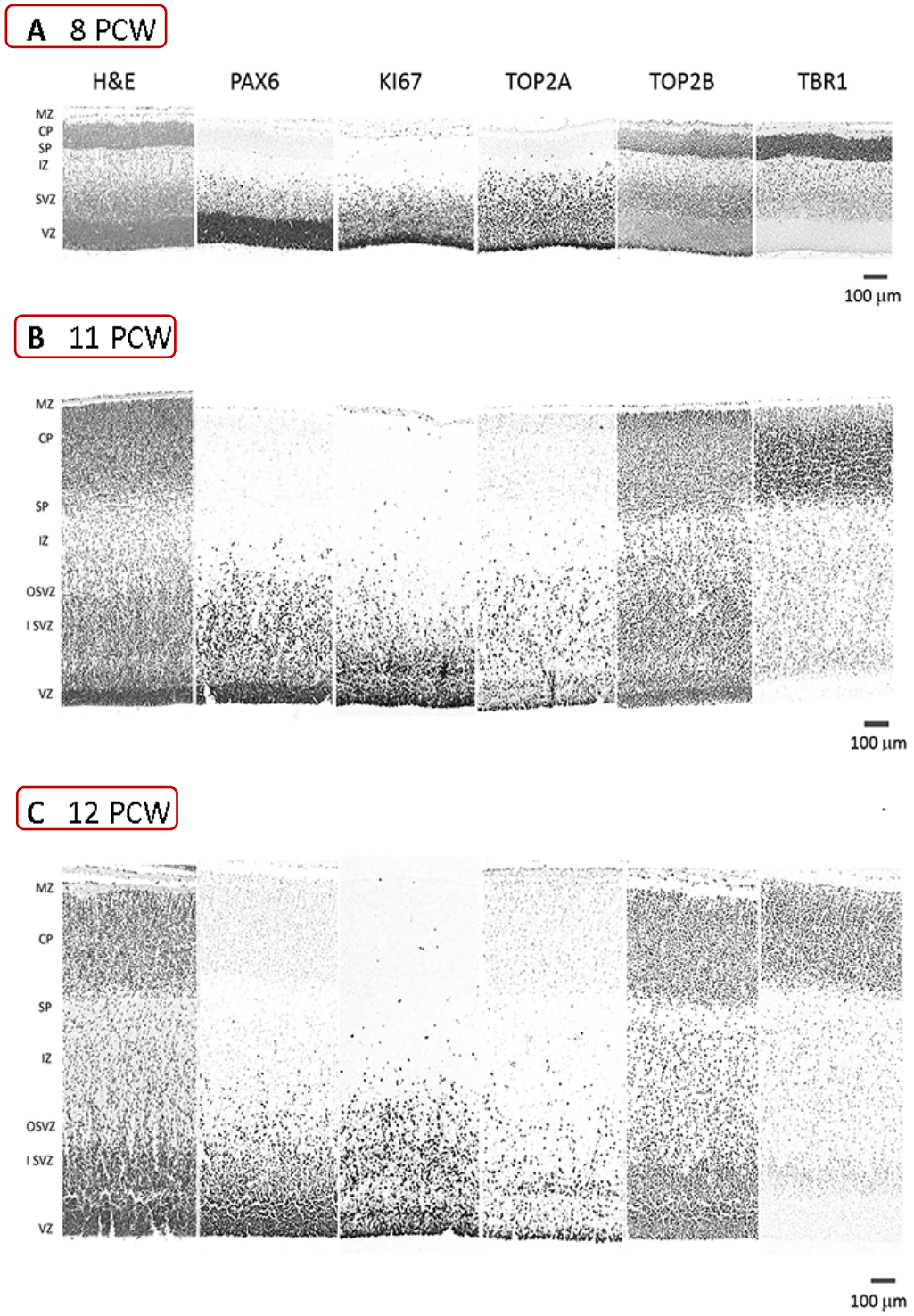


Figure 6.4 TOP2A and TOP2B Immunopositivity in the human cortex between 8 and 12 PCW. TOP2A was expressed in the proliferative VZ and SVZ at **(A)** 8, **(B)** 11 and **(C)** 12 PCW similar to the expression pattern of KI67, but less dense than that of PAX6. TOP2B was expressed throughout the cortex at **(A)** 8, **(B)** 11 and **(C)** 12 PCW in both the proliferative regions and the post mitotic neurons of the cortical plate that were immunopositive for TBR1.

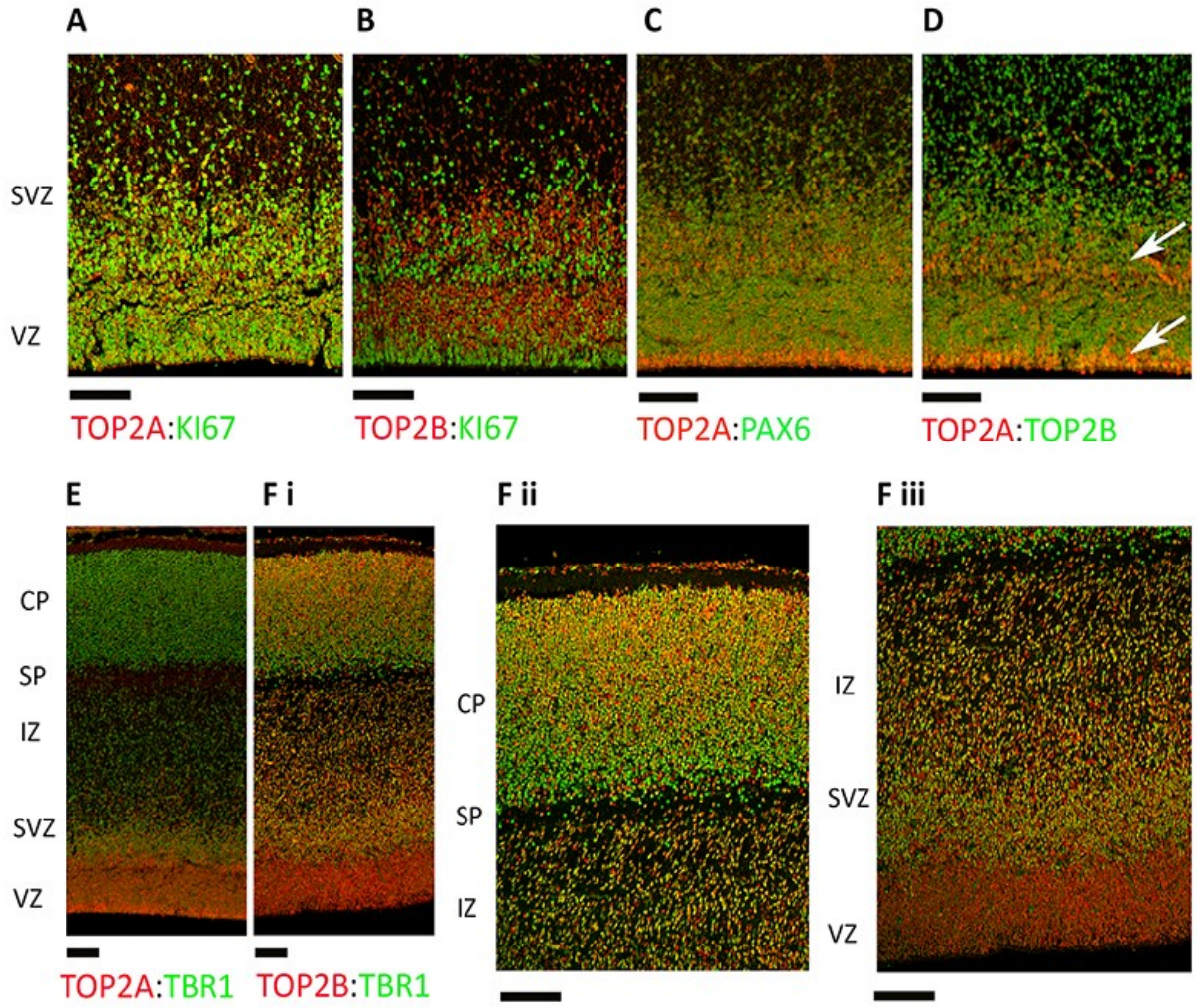


Figure 6.5 Double labelling of TOP2A and TOP2B with cell type specific markers at 12 PCW. **(A)** TOP2A and KI67 were strongly co-localised in the VZ and SVZ of the human cortex at 12 PCW. **(B)** TOP2B and KI67 appeared to be expressed in different cell types in the proliferative regions of the cortex. **(C)** TOP2A and PAX6 showed co-expression in cells at the apical surface of the VZ and the inner SVZ. **(D)** TOP2A and TOP2B showed co-expression in cells at the apical surface of the VZ and the inner SVZ. **(E)** TOP2A and TBR1 were not expressed in the same cells, being confined to the regions of proliferation and differentiation respectively. **(Fi)** TOP2B and TBR1 were co-expressed in the cells of the CP and IZ. (ii) TOP2B immunopositive cells were more apparent in the outer regions of the CP. (iii) TOP2B immunopositive cells were present in the VZ where TBR1 positive cells were absent. There was strong co-expression of these two markers in the SVZ and the IZ.

6.2.6 TOP2A is more prominent in the Medial Ganglionic Eminence (MGE) than in the Lateral Ganglionic Eminence (LGE).

The proliferative cells of both the MGE and the LGE showed TOP2A and KI67 immunopositivity, although this was less dense for both proteins at the LGE:MGE boundary (figure 6.6). TOP2A immunopositivity was stronger in the MGE compared to the LGE (figure 6.6). The LGE and MGE were distinguished by their expression of NKX2.1 and PAX6 respectively (figure 6.6).

TOP2B immunopositivity was observed throughout the MGE and LGE but, in contrast to TOP2A, appeared to be stronger in the LGE (figure 6.6). The ganglionic eminence, although considered a region of cell proliferation, does contain post mitotic cells that migrate out of this region.

6.2.7 KHDRBS 2 and 3 expression in the pallium and sub-pallium

Both KHDRBS 2 and 3 immunopositivity was most apparent in the cortical plate of the cortex at 8, 11 and 12 PCW (figure 6.7A, B and C) that was also immunopositive for the transcription factor, TBR1. There was some KHDRBS2 immunopositivity in the proliferative regions of the cortex between 8 and 12 PCW (figure 6.7). KHDRBS2 showed very little immunopositivity in the proliferative regions of the cortex, that were immunopositive for the radial glial marker PAX6.

A haematoxylin and eosin (H&E) stain was used to show the cell density of regions within the subpallium. The GE contained the most densely packed cells whilst the caudate was comparatively cell sparse (figure 6.8A). Both KHDRBS2 and 3 immunopositivity was observed at the ventricular surface of the ganglionic eminence (figure 6Bi & Ci). As with the cortex, the proliferative regions of the subpallium showed some immunopositivity to KHDRBS2 (figure 6.8Bi). There was an absence of KHDRBS3 staining in the proliferative GE (figure 6.8Ci). Both proteins were observed in the post mitotic caudate region as well as in the putamen (figures 6.8ii & iii). KHDRBS2 stained cells in the caudate appeared larger than haematoxylin stained nuclei (figure 6.8Bii). This could signal the presence of the protein outside of the nucleus or a more open chromatin state.

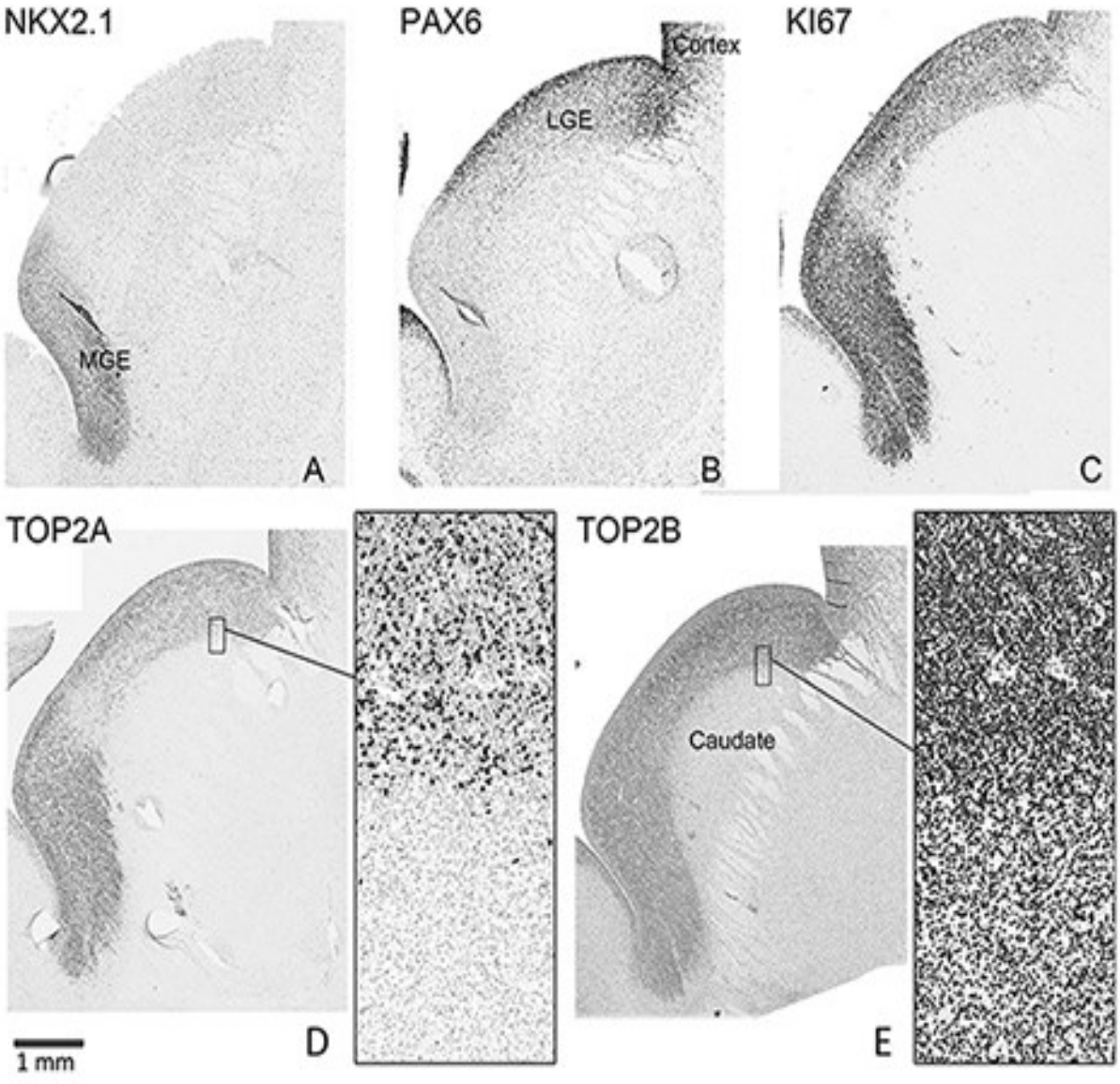


Figure 6.6 TOP2A was more prominent in the Medial Ganglionic Eminence (MGE) than in the Lateral Ganglionic Eminence (LGE). (A) NKX2.1 showed strong immunopositivity in the medial ganglionic eminence (MGE) whereas, **(B)** PAX6 immunopositive cells were found in the lateral ganglionic eminence (LGE). **(C)** Both the proliferative MGE and LGE were immunopositive for KI67 with less intense staining at the MGE:LGE boundary. **(D)** TOP2A immunopositivity, like KI67, was shown in both the MGE and LGE but is absent from the caudate. **(E)** TOP2B immunopositive cells were present in the proliferative MGE and LGE as well as the post mitotic caudate.

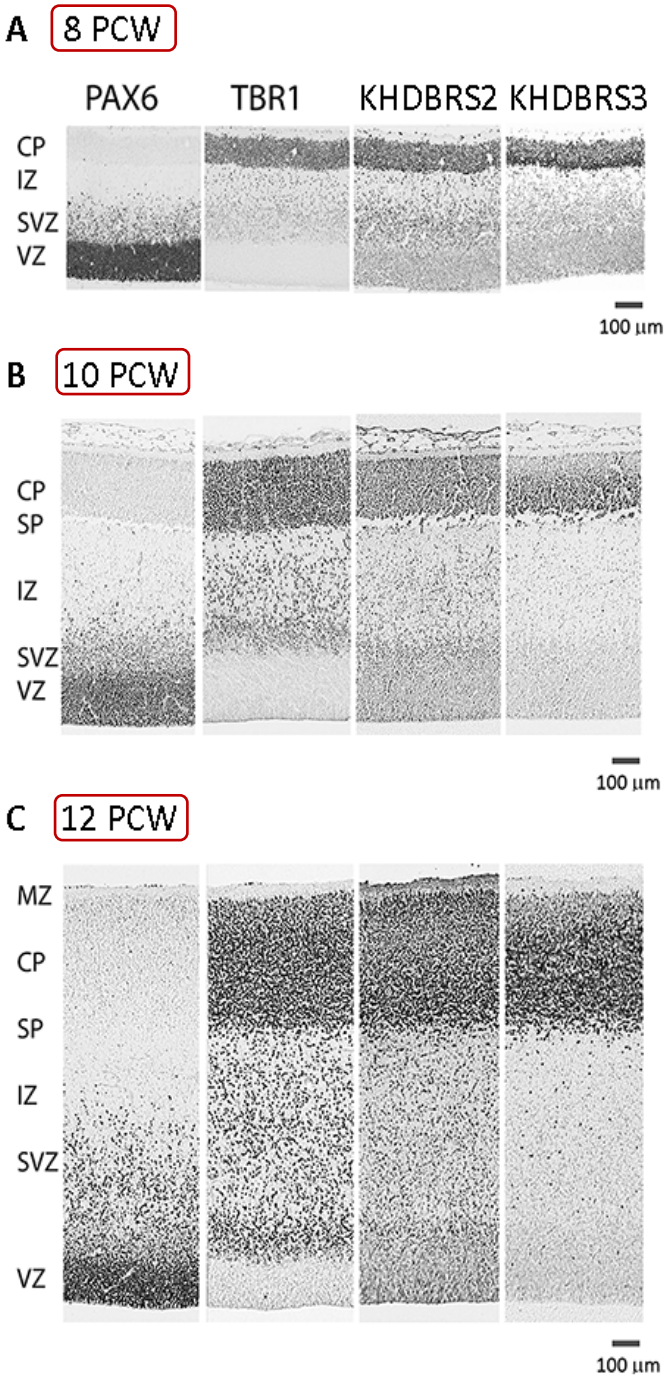


Figure 6.7 SLM1 and 2 were expressed most strongly in the post mitotic regions of the cortex. KHDRBS2 showed the most intense staining in the cortical plate as did the marker of post mitotic neurons, TBR1 at **(A)** 8, **(B)** 10 and **(C)** 12 PCW. There was also some immunopositivity in the PAX6 positive proliferative VZ and SVZ regions. The CP was also immunopositive for KHDRBS3 at **(A)** 8, **(B)** 10 and **(C)** 12 PCW, however, there was a clear reduction of immunostaining in the regions of proliferation.

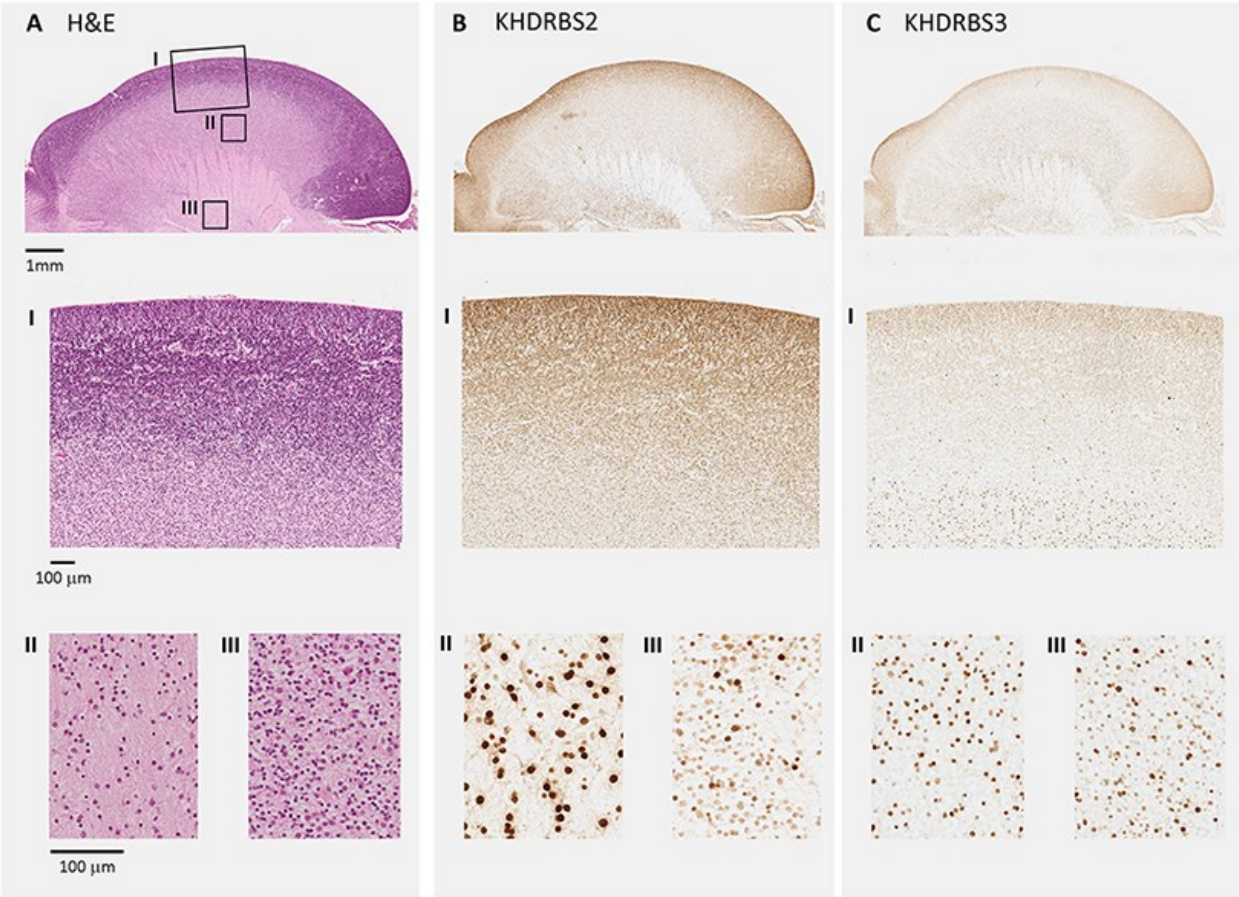
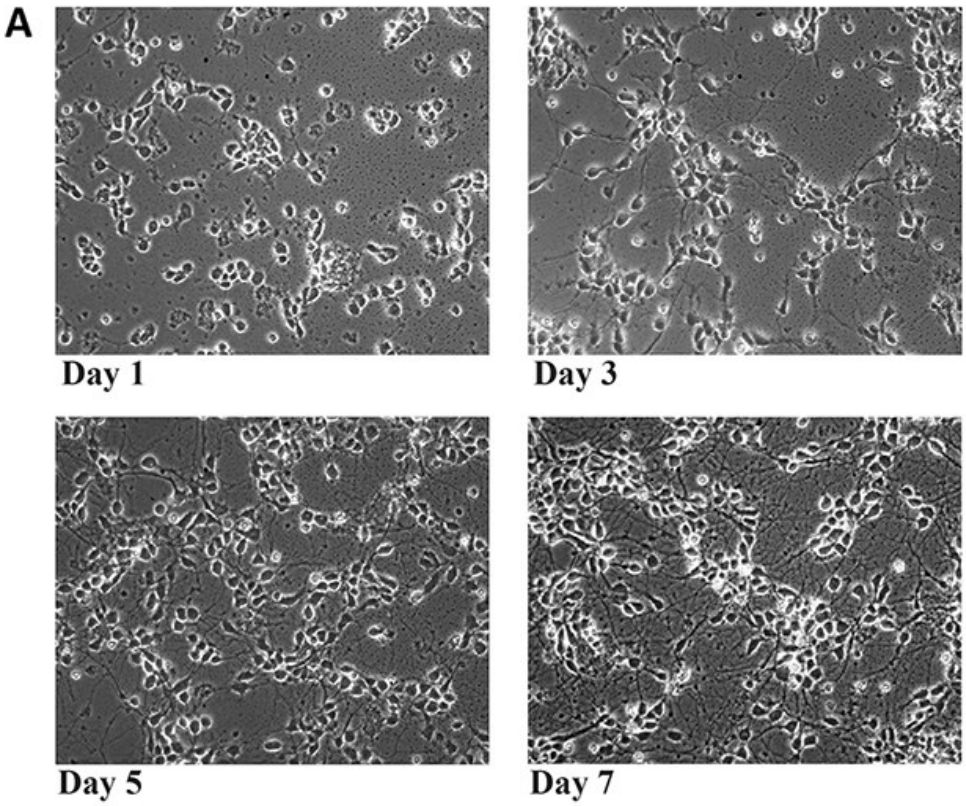


Figure 6.8 KHDBR2 and 3 proteins show different patterns of expression in the GE. (Ai & ii**)** Haematoxylin and eosin (H & E) staining showed a cell dense region in the proliferative MGE/LGE/CGE? and a comparatively reduced cell density of the caudate region. **(**iii**)** The putamen is a cell dense region compared to the caudate, with an increased number of haematoxylin stained nuclei. **(B**i**)** The ventricular surface of the GE was immunopositive for KHDRBS2 and there were immunopositive cells in both the (ii) caudate and the (iii) putamen. Staining in the caudate appeared to be more intense than that of the caudate and the cells appeared larger than the haematoxylin stain. **(C**i**)** There were some KHDRBS3 immunopositive cells at the ventricular surface of the GE, however, the staining was greatly reduced in the proliferative regions of the sub-pallium. (ii) The majority of the cells of the caudate appeared to be immunopositive for KHDRBS3, and (iii) a number of cells in the putamen stained positively for the protein.

6.2.8 Topoisomerase inhibition reduced the expression of NRXNs 1 and 2 in cortical cell cultures

Cells were isolated from the anterior region of the human cortex at 11 and 12 PCW as described in chapter 2.10. After 24 hours in survival media, cells were bathed in neural differentiation media and cultured for 7 days. Between days 1 and 7, the number of cells increased and connections formed between them (figure 6.9A). Markers of proliferation (PAX6), intermediate progenitor cells (TBR2) and post mitotic neurons (TBR1) were used to characterise the cell cultures during the seven day period. Cell counts were carried out as described in chapter 2.13. There was no significant difference during this time period in the expression of PAX6 or TBR1 (figure 6.9B). However, the number of cells expressing TBR2 was significantly higher on day 1 than on any of the remaining days (figure 6.9B).

Cortical cultures were established using cells taken from the anterior cortex of 10-12 PCW human brains (chapter 2.). Three separate cell cultures were used in this study, details of which can be seen in table 6.1. The levels of *NRXN* expression in these cultures were similar to those observed in the 12 PCW brain. *NRXN3* had the lowest relative expression and *NRXN1* had the highest relative expression. The expression level of *NRXN2* appeared to be the most variable between the cell cultures. ICRF-193 inhibition of TOP2B in neural progenitor cells caused a significant decrease in the expression of *NRXN1* ($p<0.05$). *NRXN1* showed a 1.5 fold decrease in expression (figure 6.). *NRXN2* expression was reduced in some cultures but not others. Although the average expression was decreased, this change was not statistically significantly. *NRXN3* expression was unaffected by ICRF-193 inhibition, with no significant difference between the treated cultures and the controls (figure 6.10).



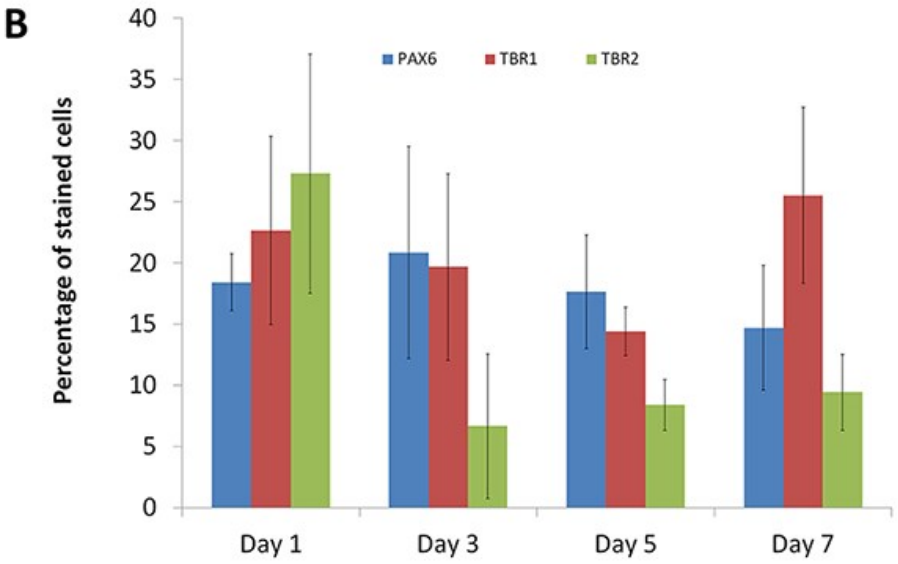


Figure 6.9 Cortical cell cultures. (A) Cortical cell cultures isolated from 12 PCW anterior cortex at days 1-7 after the addition of neural differentiation media. Cells increased in number and the number of cellular connections increased. **(B)** Cells expressed the radial glial marker PAX6, the post mitotic marker, TBR1 and the intermediate progenitor cell marker, TBR2. The expression of PAX6 and TBR1 remained stable throughout this 7 day period, however, TBR2 significantly decreased in expression after the addition of neural differentiation media. *$p<0.05$; n=3

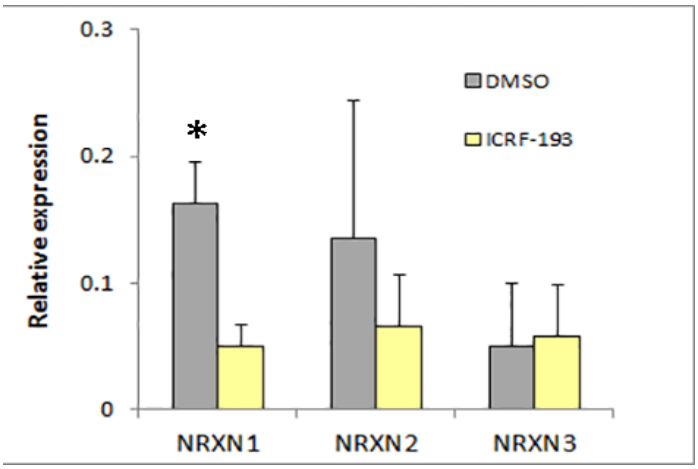


Figure 6.10. Topoisomerase 2 inhibition with ICRF-193 led to a decrease in the expression of NRXN1. There was a significant reduction in *NRXN1* expression in cell cultures established from the prenatal anterior human cortex following treatment with the TOP2 inhibitor ICRF-193 ($p<0.05$). No significant differences in expression were seen after treatment in either *NRXN2* or *NRXN3*. N=3

6.3 Discussion

The presence of these proteins in the human cortex between 8 and 12 PCW has not previously been reported. This study has shown that regulator of *NRXN* transcription, TOP2B and regulators of *NRXN* splicing, KHDRBS2 and 3, were present between 8 and 12 PCW in the human pallium and sub-pallium. We have confirmed the role of TOP2B as a regulator of *NRXN* expression in human cortical cells. Although a direct role for STAR proteins of *NRXN* splicing was not observed here, the expression of these genes during this developmental period and the presence of these proteins in cortical and sub-cortical structures where NRXN is also present, suggests that they may have the same role in human development as has been shown for mice.

6.3.1 Expression of topoisomerase enzymes in the proliferative cortical regions

The majority of cells that expressed TOP2A, also expressed KI67 confirming the role of TOP2A in cell division (Lodish *et al.,* 2000). The proportion of dividing cells in the cortex decreases between 8 and 12 PCW, which explains the decrease in TOP2A expression with age. Whilst PAX6 expression was also observed throughout the proliferative regions of the cortex, it did not overlap in expression with TOP2A protein as KI67 did. TOP2A was predominantly found in cells at the apical surface of the VZ suggesting that it is found in cells in the G2/M phase of cell division. Indeed, it has been shown that TOP2A expression is low but gradually increases during the S phase, before peaking at G2/M phase (Negri *et al*. 1992; Woessner *et al*. 1991; Heck *et al*. 1988). In contrast to this, cells in the S phase cells throughout the ventricular zone of the coretex have been shown to express PAX6 in mice (Englund *et al.,* 2005). Alternatively, TOP2A/ KI67 expressing cells, that do not express PAX6, could have migrated into the cortical proliferative regions from the MGE. Cells of the MGE have been shown to tangentially migrate into the cortex (Lavdas et al., 1999). Using embryonic brain slices, Wichterle et al. (1999) showed that MGE cells migrate towards the cortex and that MGE cells, when transplanted into multiple brain regions, are able to differentiate into neurons. Double labelling of KI67 and PAX6 will be required to discover if indeed there are dividing cells in the proliferative cortical regions that do not express PAX6. Also, using markers such as BrdU and phosphohistone H3 (Englund *et al.,* 2005) to distinguish what phase of the cell cyle TOP2A, KI67 and PAX6 immunopositive cells are in.

TOP2B protein was also observed in the proliferative regions of the cortex and a proportion of these cells express both TOP2A and TOP2B, suggesting that there is an overlap in the expression of these proteins, likely at the period when cells transition from a state of cell division to the beginnings of differentiation. Cells that express TOP2B do not express KI67 adding weight to this theory that TOP2B immunopositive cells are not actively dividing. Multiple post mitotic markers, including TBR1, MAP2 and CTIP2 (I *at al.,* 2011; Bayatti *et al.,* 2008a), that have no known roles in cell division are also expressed in the SVZ. It has been shown previously that in the absence of TOP2A in human cell lines there is an increased rate of non-disjunction, which is the failure of sister, chromatids or homologous chromosome to separate during cell division (Gruel *et al*., 1998). In the absence of Top2b, neurogenesis is normal (Yang *et al.,* 2000). This information, together with our findings, suggests that TOP2A, but not TOP2B, is essential for correct cell division.

6.3.2 Expression of topoisomerases in post mitotic neurons

TOP2B protein was seen throughout the cortex, which correlated with the finding from the RNA seq dataset that it was expressed more highly than the other topoisomerase genes. The results have confirmed the findings from rodents of Capranico *et al.* (1992), Tsutsui *et al.* (1993) and Tiwari *et al.* (2012) that TOP2B is present in post-mitotic neurons. Unlike TOP2A, it was present in the cortical plate of the cortex as has also been shown in rodents (Thakurela *et al.,* 2013). This is consistent with the suggestion that TOP2B regulates the expression of genes implicated in cell differentiation. Cells that co-express TOP2B and TBR1 were more apparent in the superficial layers of the CP where there was also strong NRXN1 and NRXN3 immunopositivity. This layer is comprised of cells that have most recently migrated here from the proliferative regions of the cortex (Rakic, 2009). TOP2B protein abundance appeared to be reduced in the more mature deep cortical layers and studies in mice have shown that *Top2b* expression in the brain decreases during development (Capranico *et al.,* 1992) signifying that *Top2B* is downregulated in maturing neurons. However, despite an increase in neuronal maturation with age, the expression of *TOP2B* did not change significantly between 9 and 12 PCW. This is likely due to the counter effect of an increasing population of early post mitotic cells with high levels of *TOP2B* expression.

In the deep layers of the human cortical plate, NRXN proteins were also reduced. Although postnatally NRXN expression remains constant, TOP2B may be an important transcriptional

regulator prenatally. Reducing *Top2b* expression in rodent cell lines reduces the expression of NRXNs 1 and 3, in addition to a number of other long genes >67kb, (Li *et al*., 2014; King *et al.,* 2013) which is unsurprising given their overlapping expression patterns. In this study, by reducing the expression of *TOP2B* in human cortical cell cultures, we were able to show that the expression of NRXN1 was significantly reduced. NRXN3 was not identified as being reduced in expression, despite using the same concentrations of ICRF-193 as was used for the King *et al.* (2013) study. In part, this could be due to the difference in species and cell type. King *et al.* (2013) used rodent cortical cells and differentiated human iPS cells whereas the cells used in this study were taken directly from 11 and 12 PCW human fetal brains. Using both mRNA and protein data, we have provided evidence that TOP2B and NRXN show similar patterns of expression and therefore have the potential to interact between 8 and 12 PCW. Knockdown studies in mice have implied that Tob2b has an indirect role in axon pathfinding, target finding (Nevin *et al.,* 2011; Yang *et al.,* 2000) and neurite growth (Nur-E-Kamal *et al.,* 2007) and it is possible that this role may be fulfilled through its regulation of *Nrxn1* expression. Similarly, in humans, TOP2B could potentially have indirect roles in these processes via the transcriptional regulation of *NRXN* genes.

6.3.3 Increased expression of NRXN transcriptional regulators in the temporal lobe

TOP1 and *MECP2* showed an increased expression in the temporal lobe compared to the central cortex at 9 PCW. The inhibition of TOP1 by the inhibitor topotecan impairs synaptic transmission by reducing the expression of long genes including *NRXN1* and *NLGN1* (Mabb *et al*., 2014). Therefore, we would expect an increase in TOP1 expression alongside an increase in *NRXN1* expression. However, no significant difference in *NRXN1* expression in the temporal lobe was observed at 9 PCW. The increase in TOP1 expression may have a delayed effect on *NRXN1* expression in the cortex.

MECP2 acts mainly as a transcriptional repressor but has also been shown to act as a transcriptional activator. If MECP2 was to act as a transcriptional repressor of NRXN at this age, this would not correlate with the increased levels of NRXN expression in the region. Instead, this would suggest that MECP2 is not acting as a transcriptional repressor of *NRXNs 1* and *2*. Looking more closely at the data, the increase in the expression of *NRXNs 1 and 2* in the temporal cortex was not present at 9 PCW, whereas, the increase in *MECP2* expression was only present at 9PCW. It is therefore, possibly that a decrease in *MECP2*

expression in the temporal cortex after 9 PCW alleviates the transcriptional repression of *NRXN1* and *2* transcription, leading to an increase in *NRXN* expression in the temporal cortex.

There was an increase in KHDRBS1 expression in the temporal lobe compared to the central cortex at 9 and 11 PCW. A similar increase was observed in the RNAseq dataset for *NRXN2* and in the qPCR data for *NRXN1* at 10 PCW. The fact that the expression of KHDRBS1 mimics that of *NRXNs 1* and *2* suggests that there is not differential splicing of these genes by KHDRBS1 across the cortex but rather that the increase in the KHDRBS1 protein meets the demand of the increased *NRXN* transcripts. However, the fact that the expression of the other two STAR family genes doesn't mimic that of *NRXNs 1 and 2* gives rise to the possibility that there may be different levels of splicing taking place across the cortex. If the amount of KHDRBS2 and 3 proteins remains constant, but NRXN protein increases in the temporal lobe, then there would be less opportunity for alternative spicing. This theory is obviously dependant on the increased levels of KHDRBS expression translating to increased protein levels as well as assuming that the observed level of STAR proteins was insufficient to keep up with the increased amount of NRXN transcripts.

6.3.4 Expression of TOP2 in the subpallium

TOP2A showed the same expression pattern as KI67 in the ganglionic eminences, being present in both the medial and lateral GE, further confirming its role in cell division. Pallial projection neurons, striatal interneurons and cortical inhibitory interneurons are born in the MGE (Wang *et al*. 2014; Hansen *et al*. 2013; Hernandez-Miranda *et al*. 2010; Xu *et al*. 2008; Marin *et al*. 2000). This region is more highly developed in primates, likely due to the larger cerebral cortex and the requirement of a larger number of interneurons (Hansen *et al.,* 2013).

TOP2A was absent from the post mitotic caudate whereas, contrastingly, TOP2B was present in this region. These findings reiterate the staining patterns within the cortex, that whilst TOP2A is concerned with cell division, TOP2B is acting in both the proliferative and post-mitotic regions of the developing human brain. Previously we showed that NRXNs are similarly distributed across these sub-pallial regions and TOP2B could potentially be influencing NRXN transcription in sub cortical structures in addition to within the cortex.

6.3.5 KHDRBS1 has a distinct expression pattern

The comparatively higher expression level of *KHDRBS1* compared to *KHDRBS2* and *3* corresponds to the protein expression in the cerebral cortex. While KHDRBS2 and 3 were predominantly found in the post mitotic regions of the cortex, KHDRBS1 was distributed equally across the post-mitotic and proliferative regions. Distinct patterns of protein expression are also present in postnatal rodent brains with KHDRBS1 being more widely distributed throughout the brain compared to the other two proteins (Iijima *et al.,* 2014). The significant increase in KHDRBS2 and 3 expression with age is likely due to the expanding cortical plate, which is where the majority of KHDRBS2 and 3 immuno-positive cells reside.

6.3.6 KHDRBS2 and 3 are predominantly expressed in the post mitotic cells

The mRNA and protein expression patterns of KHDRBS2 and 3, being more similar to each other than to that of KHDRBS1, suggests overlapping functions. In rodents, KHDRBS3 knockout causes an increase in KHDRBS2 expression (Traunmuller *et al*., 2014) and in yeast it has been suggested that these two proteins serve redundant functions in actin cytoskeleton organisation (Fadri *et al*., 2005). The functions of the STAR family of proteins appear to be more complex in neurons than they are in yeast and include the alternative splicing of NRXNs. KHDRBS 2 and 3, unlike KHDRBS 1, do not require depolarising neuronal activity in order to exert their function in NRXN splicing (Iijima *et al*. 2014; Traunmuller *et al*. 2014; Ehrmann *et al*. 2013; Iijima *et al*., 2011). Reduced expression of KHDRBS2 and 3 leads to an increase in the inclusion of AS4 in mouse neurons (Traunmuller *et al.,* 2014; Erhmann *et al.,* 2013; Iijima *et al.,* 2011). The removal of this splice site is essential for the binding of NRXNs to NLGNs. The majority of KHDRBS2 and 3 protein was found in the post-mitotic regions suggesting that the majority of NRXN-NLGN interactions are occurring in this region also.

The KHDRBS2/3 complex has been shown to affect mTOR signalling, presumably via binding to mTORC2 subunits, which can influence the growth of non-proliferating cells including neurons (Fadri *et al*., 2005; Jakinto & Hall, 2003). Abnormal mTOR signalling, and presumably the effect this has on the development of the brain, has been identified as a risk factor for autism (chapter 1.).

Our data suggests that, in the developing human brain, KHDRBS2 and 3 were present in the same regions of the cortex, although double labelling has not been carried out to ascertain

whether they are expressed in the same cells. This would corroborate the findings of Iijima *et al.* (2014) that they are suited to regulate splicing in a cell type specific manner, and indeed we have seen that KHDRBS2 and 3 immunopositivity at 12 PCW differs in the outer regions of the human CP. There are also differences in the protein localisation within the proliferative cortical regions. Although KHDRBS2 staining is predominantly found in the CP it is also present in some cells of the proliferative regions. KHDRBS2 is able to form complexes with KHDRBS1 and KHDRBS1/2 double knock out mice, but not single knockouts, had reduced brain sizes and disorganised Purkinje cells in the cerebellum (Iijima *et al*., 2014).

The expression of these two proteins in the subpallial regions was different. There was KHDRB2 and KHDRB3 protein expression in the caudate, which correlates with their post mitotic CP localisation, but KHDRBS3 protein was more obviously located in the post mitotic regions of the sub pallium. This corresponded with its expression in the cortex, where it was predominantly expressed in the CP. Although found in overlapping regions of the cerebral cortex, the functions of the STAR family of proteins, particularly in the subpallium, may differ due to their interactions with each other and with other proteins.

Chapter 7 Summary and Future work

This thesis aimed to identify autism susceptibility genes that are expressed during 8-12 PCW in the human cortex. We hypothesised that the synaptic adhesion molecules *NRXNs* and *NLGNs* would be expressed at this developmental time period and also that their protein expression may not be limited to synaptogenic regions of the cortex. It was also hypothesised that proteins that regulate *NRXN* expression would be present in the developing brain and that these proteins may be able to be used to manipulate the expression of *NRXNs* in culture. The findings of this study were expected to be informative as to the pathogenesis of autism.

7.1 Main findings of the study

RNA sequencing of a collection of brains aged between 9 and 12 PCW was able to generate a vast amount of data of which only a portion has been analysed for this thesis. PCA analysis demonstrated that different anatomical brain structures have clearly defined gene expression patterns even at this early stage in development. In addition to the gross anatomical differences, we have identified regional and temporal gene expression changes within the cortex although these appear to be less obvious transcriptionally, as shown in the PCA and cluster analysis. A larger number of genes change temporally compared to spatially suggesting that although the cortex is becoming regionalised between 9 and 12 PCW, the structure as a whole is trying to establish itself as separate from the subcortical regions of the CNS.A number of autism susceptibility genes were differentially expressed both spatially and temporally and the gene sets that are changing with age and across the cortex are different. The expression of these genes during early brain development suggests that although the symptoms of ASD do not become apparent until approximately 2 years of age, the incorrect expression and/ or function of these molecules between 8 and 12 PCW could potentially lead to abnormal brain development and contribute to the autistic phenotype.

The RNA seq and qPCR NRXN expression data correlated very well showing that *NRXN1* and *NRXN2* increase in expression with age and have high expression levels relative to the reference genes whereas *NRXN3* has a comparatively lower expression. The cortical region that was most often significantly different from the others was the temporal lobe. The temporal lobe is directly connected to the sub-pallium and is more likely to be the brain region that is exposed to the signalling of sub-pallial structures. Alternatively, the dissections

may have taken some sub-pallial material that is changing the results. Indeed, the expression of the temporal lobe sample datasets was the most variable of all the cortical regions but it was also the largest section of the cortex that was dissected.

NRXN protein was expressed in multiple cell types across the telencephalon including those in proliferative regions and those in post mitotic regions. The expression patterns of the NRXNs, although often overlapping, were also distinct for each protein. NRXN1 showed the broadest expression being present in almost all cells of the forebrain but is absent from the neuropil and fibre tracts of the internal capsule. NRXN2 co-localises with GAP43 and SYP in the neuropil of the intermediate zone and subplate as well as being present in the GAP43 and CASK positive fibre tracts of the internal capsule. NRXN3 is present in the neuropil/ cell bodies of the post mitotic cells of the cortical plate at 8 PCW but by 12 PCW was also present in the proliferative regions. NRXN3 immunostaining was less apparent in the proliferative regions of the sub-pallium and, like NRXN1, was absent from the internal capsule. NRXNs 1 and 2 immunopositivity was always more pronounced in the newly forming outer layer of the cortical plate. Our results suggest that NRXNs have functions outside of their role in synaptogenesis at this stage of development. Cells that reside in the areas in which the NRXNs were expressed have roles in proliferation, migration and neuronal differentiation. NRXN2 is the strongest candidate for a role in synaptogenesis and axon outgrowth or guidance as it overlaps in expression with genes of similar functions.

We have also shown that the regulators of NRXN transcription, MECP2 and Topoisomerase 2B, were expressed in the cortex between 9 and 12 PCW. TOP2B immunopositivity was similar to that of NRXN1 being present in both proliferative and post mitotic cells, whereas the closely related TOP2A is confined to dividing cells. We provided evidence that TOP2B regulates the transcription of NRXN1 in cortical cell cultures as its inhibition using ICRF-193 resulted in a significant reduction in NRXN1 expression. In addition to regulators of NRXN transcription, the STAR family genes KHDRBS2 and KHDRBS3 were also expressed in the cortex between 9 and 12 PCW. Like NRXNs 1 and 2, the strongest immunopositivity was observed in the cortical plate. KHDRBS2 was also present in the VZ and SVZ but KHDRBS3 was greatly reduced in regions of proliferation in both the cortex and the sub-pallium. This suggests that splicing by KHDRBS3 does not take part in proliferative regions between 9 and 12 PCW. This may reduce the exclusion of SS#4 in the NRXN transcripts in those regions and consequently reduce the ability of NRXNs to bind NLGNs.

In summary ASGs are present in the developing cortex between 9 and 12 PCW and their functions at these ages are largely unexplored. The functions of many of the proteins encoded by these genes differ between anatomical structures, brain regions and cell types. Although studies that delineate functions for these molecules in unrelated brain or other anatomical regions are extremely insightful and often transferable, it is possible that these proteins have completely novel functions in the neural progenitor cells of the cerebral cortex. The expression patters of the NRXN1 and 3 genes suggest that they are required for additional functions that extend beyond synaptogenesis and synaptic stabilisation. The main aim moving forward would be determine what functions the *NRXN, NLGN* and *SHANK* proteins have between 8 and 12 PCW and how this could relate to the development of conditions such as ASD. We have learnt a lot about the potential functions of these proteins from mice however the functions of these proteins in humans and in particular their functions during development, are largely unexplored. In particular roles in cell differentiation, cell migration, axonal outgrowth and neuronal proliferation should be further examined. Our cortical cell cultures provide an excellent model system for this work and bypass the problems posed by iPSCs such as methylation marks and incomplete differentiation.

7.2 Limitations of the study

One of the main limitations of the study is the small number of samples at each age. This reduces the statistical power making it more difficult to detect differential expression. Small, but significant, changes in expression between groups are unlikely to be identified. Although the dataset is extremely interesting, caution must be taken in the interpretation of the results. Multiple factors affect the quality and the accuracy of the data including but not limited to, the sample preparation, the read length, the sequencing depth and the programs used for quality control and mapping of the data. In addition to problems that can arise from the sequencing process, it is important to consider the genetic variation between human samples and the effects of termination procedures, substances that the embryo/ fetus is exposed to within the womb and the post mortem delay and changes in brain pH that can all affect gene expression. These prove problematic for the qPCR data also and since different brains were used for the RNA seq and qPCR, this may explain some of the variation seen in the analysis of the *NLGN* and *SHANK* genes.

In theory, because autism is a condition that, at present, cannot be diagnosed prenatally, some of the samples used may harbour mutations that could have led to the development of ASD or another neurodevelopmental/ neuropsychiatric condition. Due to data protection and ethical standards, it is not possible to obtain a medical history or any information regarding the origins of the sample. Therefore, it is important to note that it is possible that the samples used in this study may have mutations that lead to the observed gene expression patterns.

A limitation when culturing primary human fetal cells is the low proliferation rate. This makes them unsuitable for many gene editing techniques such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR; Xiong *et al.,* 2016). Since they do not recover well from freezing, the cultures are again subject to tissue being available.

7.3 Implications of the findings and recommendations for future research

It will be important for future studies to genotype all samples used and screen for DNA abnormalities as well as chromosomal abnormalities to ensure that all results reported correspond to the normal human genotype. Genotyping samples would also enable researchers to gather a collection of samples that had genetic mutations in ASD susceptibility genes. This could bypass the ethical implications of obtaining information about the women opting for the termination.

This thesis has presented evidence for alternative functions of *NRXN* proteins during development compared to in the adult brain. It is likely that many other susceptibility genes that are expressed at these ages have alternative or additional functions to those seen in the adult brain. The findings of this thesis suggest that neurons should be seen as more than synapses, particularly during the early stages of brain development. There are many cellular processes that neurons exhibit that are independent of synaptogenesis or are required in order to differentiate into a mature neuronal cell. Discovering novel developmental functions of ASD susceptibility genes could change the associations observed in the current gene module models and elucidate novel signalling pathways or functional hubs that, when perturbed, cause the condition. It also has the potential to provide new targets for the development of treatments.

Cell models provide an important platform for studying the functions of ASD genes during early development. Primary fetal brain cultures likely have gene expression profiles and characteristics that, compared to neural stem cell models, are more similar to those seen in neural progenitor cells in vitro. Although manipulation of the genetic material of these cells is possible, it is not possible to select for those cells that have been manipulated and so functional studies are limited. It will be useful, in future, to direct these cells towards a proliferative state by changing the components of the culture media and possibly the cell culture vessel. Creating proliferative stocks of cells obtained from primary cultures will also diminish the requirement for fresh tissue. Human neural stem cell models could also provide a platform from which to test the early functions of these ASD susceptibility genes as they are undifferentiated, well characterised and more easily manipulated, though not without their own limitations (Jakel *et al.,* 2004).

These findings could help towards developing a molecular prodrome. If we are able to map out gene expression patterns during normal development than we can use deviations in expression from these normal ranges as risk factors for the condition. However, we need to consider the ethical implications of presenting this information to families during pregnancy. The American Academy of Paediatrics (2016) states that genetic testing should be driven by the best interest of the child. It would be irresponsible for scientists and medical professionals to provide information about these genetic mutations until we have a more thorough understanding of their effects. Due to the heterogeneity of the condition, it may not be possible to give a definitive diagnosis. The likelihood of the child developing autism would have to be quantified in order for clinicians and families to weigh up the risks of treatment and/ or testing versus autism severity and to make informed prenatal treatment decisions. It may be more efficient to search for common upstream targets of these genes so that a more efficient and cost effective method of prenatal/ early diagnosis can be obtained. Molecular screening tests are being developed and Schmunk *et al.,* (2017) recently developed a high-throughput assay to detect defects in calcium signalling that are predictive of autism.

If individuals are diagnosed with mutations in ASD susceptibility genes, this will likely affect their family planning and may also encourage terminations of the pregnancy. At present, unless there is a family history of ASD and a research interest, there are no genetic screens for the condition. There is also a risk of genetic discrimination with families opting to abort a

fetus with an autistic prodrome or to select against genetic traits that could lead to autism. In vitro fertilisation techniques aim to use fertilised eggs that are free from genetic mutations. There is a movement of people, referred to as the neurodiversity movement, who see ASD not as a disability but rather as a natural difference (Jaarsma & Welin, 2012). These individuals do not welcome a 'cure' for the condition but instead suggest changing how it is viewed and adapting accordingly (Kapp *et al.,* 2013).

Because of the limited supply of human embryonic and fetal tissue it will be important to pool together data from multiple studies. Improved study design will enable a better use of the resources and will ensure that RNA/ DNA is available from the same samples in order to validate results. The published findings that the MMR vaccine was a risk factor for autism sparked major public concern about the safety of all vaccinations (Gross *et al.,* 2009). This has subsequently led to a rise in a number of disease cases that were once rare and are preventable thanks to vaccination programs. It is, therefore, vital that the applications of research such as this are sensitive to personal opinions and ethical guidelines.

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Appendix

Supplementary Table 3.1. Top 200 protein coding genes that are differentially expressed between 9PCW and 12 PCW using fold change (p adjusted <0.05) **(blue – upregulated, red – downregulated)**

Gene Name	Log 2 fold	P adjusted
HSPA6	4.789757	3.02686E-26
PTPRQ	4.295245	1.47248E-20
BCAS1	4.216468	1.61969E-31
KRT31	4.120775	1.52382E-16
CIDEA	4.035745	2.63098E-28
KRT34	4.016802	2.53216E-15
RASL10A	3.855068	1.07419E-30
HTR1A	3.810911	3.02686E-26
OTOR	3.637189	8.38909E-17
KRT33B	3.500152	7.06345E-12
ZFP57	3.47717	1.09182E-10
GPX3	3.330594	2.98985E-25
AQP4	3.253096	1.2034E-10
CRYM	3.163538	1.81394E-20
KRT33A	3.152875	1.37788E-08
SPINK5	3.144915	9.09149E-15
CHRM5	3.135105	5.69478E-13
HSPA1A	3.059664	2.62294E-21
INHBA	3.050151	1.21654E-18
SLCO1C1	3.03587	6.57456E-26
ACY3	2.991134	1.0051E-24
DEFB112	2.978308	3.61808E-09
TMOD1	2.9035	9.06073E-28
HEPACAM	2.879263	1.23245E-11
LIN28B	2.865438	9.06073E-28
GPR50	2.855074	2.53589E-13
HTR2B	2.82659	1.82462E-15
KRT19	2.815798	2.39691E-10
ITGB5	2.797474	9.36529E-20
CCL3L3	2.77031	1.30666E-12

MAG	2.761447	2.88455E-07
FAP	2.747006	2.42919E-12
CD2	2.731503	1.17217E-06
DAZ2	2.726465	1.52308E-06
TTLL2	2.705767	1.66424E-18
ATP2C2	2.685292	1.82576E-17
HSPA1B	2.634536	7.48666E-17
C3orf80	2.609697	1.11569E-08
DAZ1	2.59296	5.8732E-06
TAAR9	2.582372	3.55933E-07
CCL3	2.567333	6.32138E-13
SV2C	2.546575	8.46655E-16
OSM	2.504266	1.56731E-10
TMC3	2.501266	4.65308E-07
DAZ3	2.492306	1.46396E-05
ADRB1	2.487501	3.30174E-12
LPL	2.473995	4.36254E-21
CRYAB	2.46982	2.1389E-06
EBF2	2.463912	3.89506E-10
GADD45B	2.461292	1.15003E-21
ADIRF	2.455737	1.6506E-05
NDST4	2.446381	3.46265E-19
GPR17	2.437764	1.41841E-08
DIRAS3	2.436985	4.14963E-17
HMGA2	2.428826	1.01582E-13
RAMP3	2.42796	8.46829E-08
RGS6	2.422529	1.03774E-19
NXPH3	2.421848	8.37792E-13
OSTN	2.416467	6.48328E-10
HTR3A	2.411307	6.04459E-07
FA2H	2.377963	1.4195E-06
VSX1	2.340006	2.51674E-09
IL12RB2	2.329874	1.83695E-06
SLC35F2	2.327911	3.67506E-19
ERICH5	2.323898	1.49827E-12
SERPINE1	2.320634	1.08846E-23

EYA2	2.3083	1.86441E-15
AGT	2.304714	3.83779E-05
SULT1E1	2.303907	1.09634E-08
PAPLN	2.292464	1.24496E-10
KRT13	2.287289	1.39552E-06
SYT10	2.253876	3.0857E-14
KCNK9	2.24358	5.12897E-12
FGF10	2.241131	9.39778E-06
IQCJ-SCHIP1	2.221233	2.63526E-07
CH25H	2.220792	1.27725E-08
UNCX	2.20735	5.48003E-07
MYL4	2.189925	8.77987E-12
TNC	2.184324	4.13656E-09
MOG	2.183881	1.40862E-06
SULT1C2	2.181035	1.22875E-05
TBX21	2.180791	0.000171936
FAM163A	2.174863	6.14639E-07
CDC42EP1	2.173741	8.70251E-11
LCE5A	2.163304	8.22105E-05
MT1M	2.157847	1.39312E-05
SLN	2.148791	5.89218E-09
NKX2-2	2.144897	2.62651E-11
FRMPD4	2.139676	9.7448E-10
ARHGAP36	2.137153	6.10509E-07
PTGDR2	2.128119	1.10302E-12
SCNN1G	2.12441	2.83834E-13
TMEM102	2.111661	3.40225E-09
SIX2	2.110486	0.000181156
BBOX1	2.108356	4.573E-05
ADAMTS5	2.108111	3.18695E-16
KRT32	2.108022	0.000174529
PCDH11Y	2.104755	1.4912E-11
LHX9	2.101447	1.04035E-11
SPRR2D	2.095641	0.000265192
TLR2	2.095366	1.79073E-08
CRYGC	2.093131	6.54999E-05

GABRP	2.086788	1.04123E-13
SIX1	2.086667	2.12289E-05
FOSL2	2.080639	8.8706E-11
GLRA2	2.079189	3.01112E-10
HAPLN4	2.07628	3.43901E-05
SATB2	2.070723	3.06611E-08
CLDN23	2.068993	3.1945E-06
BNC2	2.064444	5.30264E-06
HS3ST4	2.048937	1.21654E-18
F11	2.045579	0.000317099
HLA-C	2.045282	2.45145E-12
HRH2	2.04192	2.27641E-10
LIX1	2.03841	3.78511E-10
FFAR3	2.037553	0.000357933
FRMD7	2.035523	0.000139582
CDH7	2.0326	1.15468E-07
DCDC2C	2.0304	5.23935E-05
KIF26B	2.026417	8.33892E-13
DMRTB1	2.024068	0.000209034
ZFP42	2.02342	0.000313891
DEFA1B	2.023142	0.000528869
C10orf82	2.021508	6.38576E-09
GPAT2	2.018198	2.67161E-07
NDRG1	2.002483	5.39501E-23
PSMB9	2.00132	7.43381E-06
IFI44L	2.000429	1.97527E-12
TUBA3D	1.996973	0.000448678
AC011155.1	1.990526	4.30033E-06
LMX1B	1.990156	0.000515944
DLK1	1.983053	6.65286E-05
WFDC1	1.975597	3.87858E-06
CCND1	1.97263	2.25718E-13
RPRM	1.964752	6.10758E-10
SOWAHB	1.960087	2.51845E-15
NR4A2	1.959099	8.01416E-14
HSPA12A	1.95812	3.17242E-08

TAP1	1.955397	1.69388E-12
GSTM1	1.951048	1.79921E-09
L1TD1	1.943111	1.6178E-11
OTOL1	1.941835	0.001051675
LGALS3	1.94036	6.29471E-12
DYNC1I1	1.937347	5.30468E-38
GADL1	1.936371	7.54856E-05
RHCG	1.932235	9.02773E-12
SH2D2A	1.931472	2.51845E-15
CFH	1.926696	6.22016E-06
SEMA7A	1.925203	5.50184E-17
OLAH	1.923195	1.90353E-05
LCN2	1.922863	0.00033607
HKDC1	1.921033	1.50604E-08
CSF3	1.920389	0.000116954
C1orf95	1.919961	1.35004E-17
C6orf141	1.911174	1.68013E-05
MPZL2	1.9057	0.000800295
RXRG	1.90109	0.000385838
NAPRT	1.892101	1.10835E-06
C2CD4B	1.891664	3.65459E-10
MYH4	1.889875	0.000391397
SLC44A4	1.889602	1.45388E-05
CYR61	1.886435	3.3257E-21
FOS	1.88246	2.51179E-12
SHISA9	1.881594	2.96285E-12
NEFM	1.880194	5.7098E-08
CRABP1	1.880164	5.95828E-06
GFY	1.872993	1.67664E-05
LIMCH1	1.872801	1.41115E-23
TSHZ3	1.868857	5.627E-12
EVA1C	1.867855	2.3722E-11
G0S2	1.855041	0.00051163
PRPS1L1	1.852949	0.000309747
LRRTM1	1.851177	6.78139E-08
NHS	1.846459	1.10352E-19

OR5M8	1.842173	0.001093831
OGN	1.841665	0.00186276
LEF1	1.839851	1.12736E-09
DEFB110	1.83813	0.001796698
VHLL	1.83707	9.52753E-06
COL6A3	1.833995	0.000999464
HGF	1.829095	1.08438E-08
SCRG1	1.826111	3.58193E-06
FRMPD2	1.821707	6.44925E-05
C8orf34	1.81811	7.07945E-10
ENOSF1	1.812963	2.11301E-14
TSHZ2	1.808606	1.73225E-05
LUM	1.805274	6.894E-05
TAS2R10	1.80082	2.14117E-09
GPR64	1.796264	2.81778E-05
HSPB1	1.795849	1.36556E-05
PLAG1	1.79409	1.89089E-12
SALL4	1.788668	5.72588E-10
DNAJB1	1.784406	3.05484E-10
C7	1.780551	0.001501459
PLD4	1.780056	2.82919E-10
CPNE8	1.779081	3.56988E-05
DEFA3	1.776043	0.002779504
CPLX1	1.775468	8.6088E-08
TRPC6	1.769692	7.25162E-08
FPR1	1.769488	2.37489E-12

Supplementary Table 3.2. Top 200 protein coding genes that are differentially expressed between 9PCW and 12 PCW using P value (fold change >2)

Gene Name	Log 2 fold	P adjusted
HDHD1	1.036406213	2.81543E-39
DYNC1I1	-1.937347079	5.30468E-38
BCAS1	-4.21646826	1.61969E-31
RASL10A	-3.855068264	1.07419E-30
CIDEA	-4.035745122	2.63098E-28
LIN28B	2.865437872	9.06073E-28
PSTPIP2	1.247049312	9.06073E-28
TMOD1	-2.903499997	9.06073E-28
SCD5	-1.419099014	1.07917E-27
MAFF	-1.654376105	1.51164E-27
HTR1A	-3.810910701	3.02686E-26
HSPA6	-4.789756785	3.02686E-26
SLCO1C1	-3.035869614	6.57456E-26
GPX3	-3.330593594	2.98985E-25
ZNF658	1.03244712	5.36152E-25
ACY3	-2.991134193	1.0051E-24
LARGE	-1.23692116	1.0459E-24
TMEM74	-1.359305117	2.67493E-24
STARD5	-1.687079232	4.96278E-24
HIF3A	1.596976634	1.05742E-23
SERPINE1	-2.320634042	1.08846E-23
LIMCH1	-1.872800611	1.41115E-23
NDRG1	-2.002482897	5.39501E-23
NUDT13	1.244054419	2.65742E-22
GADD45B	-2.461291738	1.15003E-21
WASF1	-1.504638348	1.22331E-21
HSPA1A	-3.059663682	2.62294E-21
CYR61	-1.886434695	3.3257E-21
LPL	-2.473995173	4.36254E-21
PTPRQ	4.295245357	1.47248E-20
CRYM	-3.163538495	1.81394E-20
DUSP1	-1.129791759	4.16332E-20

KCTD12	-1.68006269	9.14605E-20
ITGB5	-2.797474446	9.36529E-20
SRSF6	1.0743457	9.57695E-20
RGS6	-2.422529019	1.03774E-19
NHS	1.846459295	1.10352E-19
NDST4	2.446380774	3.46265E-19
SLC35F2	-2.327910539	3.67506E-19
EDN1	-1.383276716	4.45242E-19
TMEM108	-1.628263585	4.87966E-19
HS3ST4	-2.048937259	1.21654E-18
INHBA	-3.050150611	1.21654E-18
TTLL2	-2.705766923	1.66424E-18
TMEM159	-1.438084747	4.95828E-18
C1orf95	-1.919960968	1.35004E-17
GAB2	-1.377893767	1.59839E-17
APIP	1.141853264	1.82576E-17
ATP2C2	-2.685291763	1.82576E-17
SYTL4	1.118449158	1.9742E-17
FAM110B	-1.029880659	1.99311E-17
DIRAS3	2.436985305	4.14963E-17
SEMA7A	-1.925202754	5.50184E-17
HSPA1B	-2.63453646	7.48666E-17
FAM149A	-1.098434915	7.64179E-17
OTOR	-3.637188584	8.38909E-17
KRT31	-4.120775404	1.52382E-16
DDIT4	-1.599122088	2.31166E-16
FLRT1	-1.381080465	2.35519E-16
NCK2	-1.084683862	2.37454E-16
SCN11A	1.181506407	3.04383E-16
ADAMTS5	2.108111086	3.18695E-16
CCNJ	1.059928847	4.59961E-16
ADM	-1.716435374	5.52721E-16
SV2C	2.546574702	8.46655E-16
FAM227A	1.09627181	9.39765E-16
SLC26A2	1.146537114	9.80205E-16
TBC1D8B	1.637220293	1.04264E-15

AGAP1	-1.422589131	1.04264E-15
CRYGS	1.282597393	1.54015E-15
HTR2B	-2.826589998	1.82462E-15
EYA2	2.308299509	1.86441E-15
TPH1	1.646422971	2.40592E-15
SIK1	-1.333134736	2.51247E-15
SH2D2A	-1.931472479	2.51845E-15
SOWAHB	-1.960087246	2.51845E-15
KRT34	-4.016801799	2.53216E-15
LDB2	-1.390108841	4.37779E-15
ACRC	1.342084915	5.55959E-15
SPINK5	3.144914762	9.09149E-15
C3	-1.58772598	1.0706E-14
PHLDB2	1.625391163	1.16833E-14
AHNAK2	-1.357824211	1.24489E-14
CCDC173	1.480312709	1.63209E-14
ADAMTSL1	-1.768522459	1.63209E-14
JUN	-1.651705159	1.81004E-14
SLC9C2	1.337764398	1.92627E-14
ENOSF1	1.812963208	2.11301E-14
IGSF3	-1.171048211	2.34902E-14
B3GALT2	-1.337461195	2.46475E-14
MAT2A	1.07080628	2.61853E-14
SYT10	2.253876006	3.0857E-14
TDRD7	-1.166108991	3.11517E-14
GRM3	-1.733214717	3.81266E-14
SOBP	-1.624788977	3.99874E-14
PRSS23	-1.15678247	5.01199E-14
NR4A2	-1.959099345	8.01416E-14
CLCN5	1.531298943	8.06955E-14
APLN	-1.353922846	9.11376E-14
PLCH1	1.243028579	9.35657E-14
DDX26B	1.187741516	1.00719E-13
HMGA2	2.42882644	1.01582E-13
GABRP	2.086788024	1.04123E-13
IL27RA	-1.582028049	1.15579E-13

PDZD7	-1.646697369	1.45895E-13
ME3	-1.058124764	1.77206E-13
LYPD5	-1.054422089	1.81243E-13
FAM101B	-1.518205101	1.95695E-13
CCND1	1.97263006	2.25718E-13
ANKHD1-EIF4EBP3	1.327599442	2.30173E-13
GPR50	2.855073641	2.53589E-13
RTN4RL1	-1.75920198	2.60826E-13
MAPT	-1.721959135	2.68703E-13
SCNN1G	-2.124410007	2.83834E-13
TUBB2B	-1.085178153	3.6874E-13
SMOC1	-1.739225078	5.07846E-13
MMRN1	1.594108018	5.50153E-13
CHRM5	-3.135105025	5.69478E-13
CCL3	-2.567332649	6.32138E-13
GABRE	1.62782374	6.34506E-13
ATP1A1	-1.014127683	7.83391E-13
KIF26B	-2.026416883	8.33892E-13
NXPH3	-2.421847619	8.37792E-13
PPFIBP2	-1.125915165	8.58532E-13
LRP5L	-1.336537205	9.74886E-13
PTGDR2	-2.128118729	1.10302E-12
TLR3	-1.586177556	1.12974E-12
CASP1	-1.684565373	1.13644E-12
MTUS2	-1.385670095	1.2368E-12
ZNF730	1.537212974	1.30208E-12
CCL3L3	-2.770310379	1.30666E-12
SH3KBP1	-1.073934725	1.39878E-12
ASTN2	-1.752090772	1.48054E-12
ERICH5	-2.323898407	1.49827E-12
RND1	-1.248056037	1.51521E-12
TRIM67	-1.509887841	1.56406E-12
TAP1	-1.955396556	1.69388E-12
MAP3K15	-1.399503664	1.83792E-12
PLAG1	1.794090145	1.89089E-12
IFI44L	2.000428647	1.97527E-12

NODAL	-1.414278922	2.12005E-12
FPR1	-1.769487583	2.37489E-12
FAP	2.747006322	2.42919E-12
TMEM178A	-1.246773516	2.45145E-12
HLA-C	-2.045282181	2.45145E-12
FOS	-1.882460041	2.51179E-12
NELL2	-1.116779016	2.59991E-12
SLA	-1.675145546	2.70948E-12
STMN4	-1.557584041	2.80599E-12
KIAA1217	1.645829508	2.92586E-12
SHISA9	-1.881593626	2.96285E-12
B3GALT5	1.466806477	3.30174E-12
ADRB1	-2.487501461	3.30174E-12
ADD3	1.280180859	3.36685E-12
UBE2QL1	-1.571338991	3.78164E-12
GDPD5	-1.746077869	4.64031E-12
KCNK9	-2.243579859	5.12897E-12
SLC25A25	-1.113362558	5.13745E-12
ZC3H12C	1.233895254	5.18129E-12
LCOR	1.131537186	5.21011E-12
MARCH3	-1.599489806	5.21011E-12
TSHZ3	-1.868857349	5.627E-12
CNTNAP2	1.526591651	6.28785E-12
LGALS3	-1.9403598	6.29471E-12
KRT33B	-3.500151694	7.06345E-12
IGF2BP1	1.22902062	7.90029E-12
CSF1R	-1.576729901	8.05227E-12
MYL4	-2.189925097	8.77987E-12
RHCG	-1.932235479	9.02773E-12
LHX9	2.101447191	1.04035E-11
RNF113A	-1.263894342	1.05318E-11
MGAM	1.461266409	1.14704E-11
FAM196A	-1.568621365	1.20969E-11
HEPACAM	-2.879262838	1.23245E-11
HMGN5	1.349115257	1.35957E-11
PCDH11Y	-2.10475491	1.4912E-11

L1TD1	1.943111035	1.6178E-11
LARP6	-1.005320705	1.84884E-11
TRIM66	1.499279415	2.1746E-11
CCDC14	1.120656137	2.28486E-11
EVA1C	-1.86785476	2.3722E-11
NKX2-2	-2.144896738	2.62651E-11
DSEL	-1.346588268	2.79698E-11
ALS2CR12	1.190349976	3.09678E-11
HHIPL1	-1.108253586	3.12081E-11
CLMN	1.575820013	4.10801E-11
KLHL31	1.444083098	4.14337E-11
SEMA3C	-1.279306443	4.23438E-11
PIK3CD	-1.220634494	4.97654E-11
ADAMTS19	1.768334633	5.34754E-11
CMTR2	1.035337739	5.60829E-11
TEX15	1.764082704	6.25376E-11
HLA-B	-1.760599387	6.25376E-11
ABCC2	1.262102656	6.57974E-11
ZNF518A	1.074205262	7.3716E-11
AOAH	-1.187621733	7.98364E-11
SELPLG	-1.517286364	8.64134E-11
CDC42EP1	-2.173740896	8.70251E-11
FOSL2	-2.080639434	8.8706E-11
PDXK	-1.166511554	9.16804E-11

Supplementary Table 3.3 List of 81 differentially expressed genes that overlap between 9 and 12 PCW using highest fold change vs smallest P value

Gene Symbol	Log 2 fold	P adjusted
ACY3	-2.991134193	1.0051E-24
ADAMTS5	2.108111086	3.18695E-16
ADRB1	-2.487501461	3.30174E-12
ATP2C2	-2.685291763	1.82576E-17
BCAS1	-4.21646826	1.61969E-31
C1orf95	-1.919960968	1.35004E-17
CCL3	-2.567332649	6.32138E-13
CCL3L3	-2.770310379	1.30666E-12
CCND1	1.97263006	2.25718E-13
CDC42EP1	-2.173740896	8.70251E-11
CHRM5	-3.135105025	5.69478E-13
CIDEA	-4.035745122	2.63098E-28
CRYM	-3.163538495	1.81394E-20
CYR61	-1.886434695	3.3257E-21
DIRAS3	2.436985305	4.14963E-17
DYNC1I1	-1.937347079	5.30468E-38
ENOSF1	1.812963208	2.11301E-14
ERICH5	-2.323898407	1.49827E-12
EVA1C	-1.86785476	2.3722E-11
EYA2	2.308299509	1.86441E-15
FAP	2.747006322	2.42919E-12
FOS	-1.882460041	2.51179E-12
FOSL2	-2.080639434	8.8706E-11
FPR1	-1.769487583	2.37489E-12
GABRP	2.086788024	1.04123E-13
GADD45B	-2.461291738	1.15003E-21
GPR50	2.855073641	2.53589E-13
GPX3	-3.330593594	2.98985E-25
HEPACAM	-2.879262838	1.23245E-11
HLA-C	-2.045282181	2.45145E-12
HMGA2	2.42882644	1.01582E-13
HS3ST4	-2.048937259	1.21654E-18
HSPA1A	-3.059663682	2.62294E-21
HSPA1B	-2.63453646	7.48666E-17
HSPA6	-4.789756785	3.02686E-26
HTR1A	-3.810910701	3.02686E-26
HTR2B	-2.826589998	1.82462E-15
IFI44L	2.000428647	1.97527E-12
INHBA	-3.050150611	1.21654E-18

ITGB5	-2.797474446	9.36529E-20
KCNK9	-2.243579859	5.12897E-12
KIF26B	-2.026416883	8.33892E-13
KRT31	-4.120775404	1.52382E-16
KRT33B	-3.500151694	7.06345E-12
KRT34	-4.016801799	2.53216E-15
L1TD1	1.943111035	1.6178E-11
LGALS3	-1.9403598	6.29471E-12
LHX9	2.101447191	1.04035E-11
LIMCH1	-1.872800611	1.41115E-23
LIN28B	2.865437872	9.06073E-28
LPL	-2.473995173	4.36254E-21
MYL4	-2.189925097	8.77987E-12
NDRG1	-2.002482897	5.39501E-23
NDST4	2.446380774	3.46265E-19
NHS	1.846459295	1.10352E-19
NKX2-2	-2.144896738	2.62651E-11
NR4A2	-1.959099345	8.01416E-14
NXPH3	-2.421847619	8.37792E-13
OTOR	-3.637188584	8.38909E-17
PCDH11Y	-2.10475491	1.4912E-11
PLAG1	1.794090145	1.89089E-12
PTGDR2	-2.128118729	1.10302E-12
PTPRQ	4.295245357	1.47248E-20
RASL10A	-3.855068264	1.07419E-30
RGS6	-2.422529019	1.03774E-19
RHCG	-1.932235479	9.02773E-12
SCNN1G	-2.124410007	2.83834E-13
SEMA7A	-1.925202754	5.50184E-17
SERPINE1	-2.320634042	1.08846E-23
SH2D2A	-1.931472479	2.51845E-15
SHISA9	-1.881593626	2.96285E-12
SLC35F2	-2.327910539	3.67506E-19
SLCO1C1	-3.035869614	6.57456E-26
SOWAHB	-1.960087246	2.51845E-15
SPINK5	3.144914762	9.09149E-15
SV2C	2.546574702	8.46655E-16
SYT10	2.253876006	3.0857E-14
TAP1	-1.955396556	1.69388E-12
TMOD1	-2.903499997	9.06073E-28
TSHZ3	-1.868857349	5.627E-12
TTLL2	-2.705766923	1.66424E-18

Supplementary Table 3.4. List of genes differentially expressed between anterior and posterior cortex at 9PCW

Gene Name	Log 2 fold	P adjusted
ZIC3	3.316820934	4.63E-21
DCT	3.209577832	5.32E-12
GADL1	3.116578311	5.69E-16
CYP26A1	3.033623659	3.75E-12
MIR217HG	2.586740936	4.78E-08
LAMP5	2.494707745	2.16E-07
MYBPC1	2.487607005	2.07E-07
DCHS2	2.465400441	1.16E-11
AE000661.37	2.291916082	6.61E-07
LRRTM3	2.251127881	1.07E-07
MOXD1	1.972086114	0.000234356
TRDC	1.926029601	0.000234356
HKDC1	1.888456551	0.000520509
ACTC1	1.887124989	0.00065774
SPATA13	1.873128127	0.000218255
ANKRD34B	1.781708416	0.000714798
GOLGA2P5	1.774802282	7.57E-11
CTD-2140G10.2	1.773493427	0.000162022
OTOG	1.770753975	0.00286286
KCNK9	1.715955148	2.28E-05
PIP5K1B	1.693719647	4.78E-08
RORB	1.691912163	3.28E-05
CYP26B1	1.690272228	3.67E-05
GIPR	1.674604017	0.007743684
LINC00643	1.6541064	0.003310335
HILS1	1.641858318	0.009556352
CNIH3	1.637172011	2.68E-05
FAM196B	1.63377138	0.008870445
RP11-159K7.2	1.633666305	6.78E-05
RLBP1	1.631551174	0.010110274
CTB-12A17.2	1.617698181	2.16E-07
RP11-844P9.2	1.615644149	0.000974515

ARAP2	1.613256644	0.003659133
HTR2A	1.600195345	0.00028881
NRK	1.598215971	0.002140384
PTER	1.590798504	8.82E-06
SALL3	1.585766195	0.000187245
CDH7	1.580273883	1.03E-08
IL1RAPL2	1.565916233	0.017203942
RP11-331K15.1	1.524803787	0.024192831
SMCR2	1.519849323	0.026541759
IMPA1P	1.519264368	0.005962805
TEC	1.505161482	0.003384429
RP1-137H15.2	1.503100791	0.028686765
RCAN3	1.493077424	2.24E-07
HHIP	1.489927198	0.032019824
USH1C	1.464187669	0.007627456
RP11-849I19.1	1.461171468	0.013848172
LINC01285	1.45307862	0.022919544
ETV1	1.444949634	4.28E-06
ROBO1	1.433814992	1.45E-07
CXXC4	1.418928923	0.008983871
RP11-517P14.7	1.412157586	0.003450351
SALL4	1.410132006	0.006568306
LRRC9	1.409562211	0.008442412
SOCS2	1.403587248	0.000520509
IGSF11	1.402476565	0.000218255
GRB14	1.400588027	0.007214822
RIT2	1.394773327	0.014235703
RP3-466P17.1	1.384298357	1.29E-06
COL6A4P2	1.37120209	0.009556352
PCDH17	1.36930736	0.018578223
MYLK	1.3647404	0.01015303
WNT3	1.361005614	6.67E-05
C11orf63	1.347683637	0.02891569
SLC17A6	1.345440183	0.00457877
NUS1P2	1.344425207	0.007727407
CCDC175	1.344298448	0.03037099

C18orf42	1.337492654	0.0466742
POU3F2	1.328316267	0.010029015
NTN4	1.323537562	0.000234356
ANKRD29	1.317713457	6.15E-07
EYS	1.312153194	0.033268716
FOLH1	1.292304635	1.18E-07
RP11-384O8.1	1.291022683	0.035095866
IGDCC4	1.290775688	1.72E-06
RGAG1	1.288926273	0.000858828
ZDHHC14	1.273409838	6.82E-06
HOPX	1.268426033	0.036219784
SYNJ2	1.243243967	0.005558463
ME1	1.229041476	0.016128379
VAT1L	1.213489668	0.007327158
CA8	1.20485907	0.016208501
PHYH	1.20342625	6.15E-07
CCDC36	1.192401559	0.018773181
SYNM	1.189627814	0.02935281
KITLG	1.186059617	0.048343662
SPAG1	1.165229985	0.019732216
RP11-1109F11.3	1.164827062	0.007743684
KCNV1	1.164327401	0.011400965
PCDH8	1.164217503	0.036219784
ST8SIA3	1.162692981	0.035977285
ADCY1	1.161217488	0.000206635
CRNDE	1.161129637	0.00105812
RHOJ	1.153188308	0.00286286
LINC01152	1.14973179	0.007214822
TOX	1.13750851	0.034350746
NFIA-AS2	1.13592409	0.015618075
NPY5R	1.133032484	0.033268716
RFTN2	1.126468384	0.034119921
SP8	1.123387038	0.007743684
RNF125	1.121703665	0.023689998
RP3-428L16.2	1.095433337	0.005410852
SOCS2-AS1	1.08176746	0.014311841

BCAT1	1.067781707	0.036219784
TENM2	1.0512605	0.008563057
PDE1C	1.045669936	2.49E-05
AC068057.1	1.040251561	0.011908999
AF186192.5	1.029889466	0.002269008
FRK	1.003643089	0.042375124
NPTX1	-1.026722118	0.034350746
SYN2	-1.030678148	0.03466247
KIAA1324	-1.083999781	0.005378528
FZD7	-1.090133906	1.87E-07
ATOH7	-1.091872893	0.013848172
CD27-AS1	-1.122652691	4.43E-05
PTPN3	-1.184900742	0.002393295
PPARG	-1.244819902	0.000351408
DPP10-AS1	-1.253273096	0.01586224
CNGA3	-1.269763447	3.28E-05
DIRAS3	-1.272498218	0.006195153
FBLN7	-1.315110144	0.00105812
MICAL2	-1.364668498	0.033558184
SHISA6	-1.381317652	2.19E-09
RP11-469H8.6	-1.442205554	0.013848172
HRK	-1.451894452	0.035095866
RP11-215G15.5	-1.46059807	0.003659133
DCLK3	-1.467524864	1.32E-07
ECEL1	-1.479798852	0.036726237
MKX	-1.480895438	0.028478615
CHST15	-1.501754861	0.000120101
SPINK5	-1.509970831	0.00105812
TAC3	-1.511082221	0.021722228
CHRNA3	-1.539331212	8.82E-06
NECAB1	-1.592348869	0.015618075
AC004862.6	-1.618431954	0.002843333
PDPN	-1.675994324	3.25E-09
CHODL	-1.681906874	7.21E-06
RP1-212P9.2	-1.821408372	0.000267376
DGKK	-2.029295282	0.000234356

NR2F1	-2.388162835	3.77E-07
CDH9	-2.413055811	6.15E-07
NR2F1-AS1	-2.430720945	5.60E-09
OCA2	-2.686241692	1.08E-20
MAS1	-3.115027474	9.56E-24
FGFR3	-4.827595278	1.05E-39

Supplementary Table 3.5 List of genes differentially expressed between anterior and posterior cortex at 12 PCW

Gene Name	Log 2 fold	P adjusted
SLC17A8	1.986488835	2.84E-05
PDZRN3	1.806706571	4.56E-05
GIPR	1.799005991	0.00020008
RGS8	1.798780444	5.74E-05
WIF1	1.757220095	4.56E-05
ST8SIA5	1.7517511	5.74E-05
CA8	1.744838065	0.000125193
SYNDIG1L	1.70635595	0.000382599
TLL1	1.705672593	0.000404269
ADAM33	1.663302763	0.000382599
GRIN2C	1.646699383	0.000404269
MYBPHL	1.635050469	0.000382599
GABRQ	1.618568721	0.000302197
GRM4	1.617734366	4.56E-05
BRINP2	1.560318881	0.000733991
CNTN6	1.554833905	0.000738993
RP11-742D12.2	1.537572916	0.001974768
ZBTB7C	1.526508466	0.000757096
NPY5R	1.526108755	0.001127388
KCNMB2	1.512664046	0.000302197
LAMP5	1.510964811	0.002313725
KRT19	1.499720273	0.001154186
RP4-555D20.2	1.499231581	0.001081937
RP11-13K12.1	1.498550696	0.002148024
COL12A1	1.494859875	4.56E-05
SLIT3	1.463999332	0.002148024
LRFN2	1.454279151	0.001550305
THRB	1.447695737	0.00093725
UNC5C	1.445913097	0.004359524
ATP5F1P5	1.439007069	0.004667426
SERTM1	1.438674621	0.000302197
ERBB4	1.405202114	0.002272964

GPR1	1.40425059	0.00388003
SCGN	1.400443331	0.003990401
SLC25A45	1.395775832	0.004667426
DLX6-AS1	1.388336881	0.007336626
DPF3	1.384870383	0.001064948
NAALAD2	1.382831785	0.001154186
SPON1	1.376416154	0.008428975
NPAS1	1.37235405	0.00093725
PTGS2	1.37180351	0.003395648
PIP5K1B	1.365153768	0.001321734
ARAP2	1.3617944	0.001465728
EYA1	1.359974198	0.001664042
NRIP3	1.358255781	0.007162924
SYNJ2	1.355165116	0.00043179
ABI3BP	1.352077848	0.006096373
RCAN2	1.350648634	0.001013927
KCNC2	1.349368036	0.008101362
KCNIP4	1.347029962	0.004359524
CLDN1	1.345659783	0.008901471
MDGA1	1.343475313	6.22E-06
ADAMTS14	1.340986243	0.003656903
ABCA1	1.339803582	0.001038083
ZNF385A	1.33852827	0.00093725
COLEC12	1.337523218	0.001974768
KIRREL3	1.327766115	5.74E-05
RARB	1.325682687	0.012219823
C11orf63	1.322195873	0.005681219
BEST4	1.31798197	0.004359524
CDCP1	1.313878539	0.012993273
ANXA2P2	1.311476069	0.01085732
TRIM71	1.30893981	0.000652415
CPNE8	1.305105285	0.012361349
MGAT4C	1.301265076	0.000733991
CARD10	1.295989474	0.001570893
NRXN3	1.29295984	0.005648568
ITPR2	1.289733894	0.000685751

RPH3A	1.285288319	0.007167369
KLHL4	1.277852901	0.000652415
PRLHR	1.277759967	0.00595939
TUNAR	1.277719206	0.012391233
GPNMB	1.277082488	0.018339787
RP11-796G6.1	1.275441259	0.01779328
TAC1	1.266885811	0.018339787
CAMK1G	1.265333112	0.008901471
LGI4	1.264412158	0.003789042
ALCAM	1.263662702	0.000154585
RP11-679B19.1	1.260783522	0.00093725
SYTL5	1.259641977	0.014099385
NPY1R	1.255279837	0.002743176
PLCXD3	1.249421627	0.005408338
VWA5B1	1.248784622	0.008101362
GAD1	1.247676744	0.014099385
UST	1.236920339	0.007187283
SLC32A1	1.233817326	0.02207139
GHR	1.233353486	0.00041953
YPEL2	1.231013973	0.005648568
AE000661.37	1.229042327	0.025233298
GRM7	1.227786053	0.022323189
TBX21	1.227605344	0.016841981
DLX1	1.226381448	0.023500504
BCYRN1	1.213800674	0.018339787
ADARB2	1.212824829	0.018683871
FBLN5	1.211648662	0.028460906
PTPRR	1.205809841	0.000712745
CNIH3	1.204293076	0.019277258
CBLN2	1.195416344	0.024641462
GRIP2	1.194855054	4.09E-05
TGFB2	1.19266424	0.025029895
ZCCHC12	1.191655913	0.012440962
PTPRT	1.191547352	0.01147044
PLXNB3	1.189170959	0.01640914
RP11-449L23.2	1.184536112	0.031722112

GABRA1	1.183901767	0.028544847
DNAH10	1.180590732	0.025182785
CSMD3	1.177546325	0.019668238
PRRT4	1.177169542	0.00954253
RGS11	1.170624572	0.000733991
PART1	1.165997051	0.03819006
LINC00277	1.165282932	0.013293208
CACNA1D	1.162130738	0.000644946
DLX5	1.161394367	0.035536552
FAM222A	1.159587395	0.030011208
BCL6	1.159560332	0.003656903
CDH6	1.150651388	0.010297013
SYNM	1.14944412	0.003112694
GPR17	1.142744109	0.031733934
COL15A1	1.139621181	0.004667426
CYP26A1	1.136857201	0.042761997
RP11-108M9.3	1.135853334	0.039254064
ANGPT1	1.124975184	0.025828658
LGR4	1.124563712	0.030426116
TC2N	1.116463449	0.036424633
FGF1	1.11483867	0.041315732
FREM1	1.104543942	0.030271439
CYGB	1.10252841	0.029063441
NTN1	1.098538215	0.032962239
LMO4	1.094950781	0.023900177
RP11-272L13.3	1.092305026	0.019496855
STK32B	1.092082722	0.043585879
GREB1	1.090450413	0.048499065
MIR137HG	1.086801138	0.01779328
HR	1.086247373	0.030639231
CHRM2	1.086077409	0.030503459
LINC01305	1.079637519	0.046526097
PLA2R1	1.07685542	0.035536552
RP11-517P14.7	1.074278956	0.013835774
ABCA12	1.071101725	0.038121398
RP11-351J23.2	1.067483975	0.030271439

LRRTM3	1.061567548	0.017627886
ZMAT4	1.059851036	0.017611929
HCRTR2	1.057633472	0.030579987
PLS3	1.056349203	0.013468674
PCDH19	1.055149208	0.035536552
RP11-9G1.3	1.04989341	0.010345895
FZD4	1.044892984	0.00509972
SYN3	1.0447323	0.027018371
COLGALT2	1.044474814	0.038121398
KLHL29	1.041304475	0.012901845
IFIT3	1.038473943	0.034889917
ITPR3	1.037854642	0.013293208
LRRC9	1.036517003	0.00746993
MLIP	1.035815363	0.000605117
RP11-713C5.1	1.034886821	0.047739216
TMEFF2	1.034677096	0.031193902
LYPD6B	1.031651118	0.045362743
ZNF114	1.029678508	0.007675266
MIR770	1.025060559	0.031733934
KRT13	1.023614141	0.048048637
ANXA2	1.023136221	0.002587916
TCEA3	1.022755603	0.042761997
RP11-490G2.2	1.020095362	0.042761997
PPP2R1B	1.014332573	0.012391233
C2orf72	1.010211126	0.04938823
OLIG2	1.007006495	0.038507579
RASGRF2	1.005397282	0.012325442
ETS2	1.001307274	0.000520869
PTGES3P2	-1.002540735	0.000382599
PAPPA2	-1.009824411	0.030627497
CHRNA3	-1.015743977	0.017991722
RPL12L3	-1.027108261	0.036862095
CTD-2620I22.7	-1.076004754	0.035536552
SLC7A10	-1.094301561	0.044338822
RP11-932O9.10	-1.096634206	0.031132905
CYP26B1	-1.120025742	0.019668238

SNORD15B	-1.122684366	0.043099727
COX7A1	-1.133388921	0.005680874
KIF25-AS1	-1.14935424	0.026428148
MDFI	-1.167748363	0.004113725
RPS12P26	-1.263538249	0.008882413
LLNLF-158E9.1	-1.271713817	0.003990401
RP5-1024C24.1	-1.3538704	0.000600792
CARTPT	-1.365887388	0.001664042
RP11-677M24.1	-1.418327114	0.002952903

List of publications & abstracts

Harkin, L.F., Gerrelli, D., Gold Diaz, D.C., Santos, C., Alzu'bi, A., Austin, C. & Clowry., G. (2016) Distinct expression patterns for type II topoisomerases IIA and IIB in the early foetal human telencephalon. *Journal of Anatomy* **228** pp452-463

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.

Lindsay, S.J., Xu, Y., Lisgo, S.N., **Harkin, L.F.**, Copp, A., Gerrelli, D., Clowry, G., Talbot, A., Keogh, M.J., Coxhead, J., Santibanez-Koref, M., Chinnery, P.F. (2016) HDBR Expression: A unique resource for global and individual gene expression studies during early human brain development. *Frontiers of Neuroanatomy* (In print)

HDBR Expression: A Unique Resource for Global and Individual Gene Expression Studies during Early Human Brain Development

This paper describes a new resource, HDBR (Human Developmental Biology Resource) Expression, for studying prenatal human brain development. It is unique in the age range (4 post conception weeks [PCW] to 17PCW) and number of brains (172) studied, particularly those under 8PCW (33). The great majority of the samples are karyotyped. HDBR Expression is also unique in that both the large-scale data sets (RNA-seq data, SNP genotype data) and the corresponding RNA and DNA samples are available, the latter via the MRC-Wellcome Trust funded HDBR1(Gerrelli *et al*., 2015). There are 557 RNA-seq datasets from different brain regions, the majority between 4 and 12PCW. During this time the major brain regions are established and the early stages of cortex development occur (Bystron *et al*., 2008; O'Rahilly and Muller, 2008). In addition, there are 42 RNAseq data sets from spinal cord and 29 from cerebral choroid plexus. There are also 243 additional tissue specimens in paraffin wax blocks available for individual gene expression studies. For almost all of the brains and specimens in wax blocks there are corresponding SNP genotype data.

Large-scale/high-throughput studies, such as next-generation sequencing, are providing raw material in a wide variety of research fields (for review of concepts and methodologies of RNA-seq, see Shin et al., 2014). Studies of human development are hampered by difficulties in obtaining tissue which means that publicly available large-scale data sets are particularly useful because data can be used and re-used (Kang et al., 2011; Zhang et al., 2011; Fietz et al., 2012; Miller et al., 2014; Darmanis et al., 2015).

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

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 NRXNs 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.