THE EFFECT OF GROWTH RESTRICTION AND POSTNATAL FLUCTUATING OXYGEN ON THE DEVELOPING BRAIN

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Doctor of Medicine

The University of Edinburgh 2012-2013

For Doug,

with whom I am blessed to share life.

Abstract

Background: Preterm birth is the most common cause of childhood mortality and morbidity in the United Kingdom. Placental insufficiency is significant in the aetiology of preterm delivery and implicit to fetal growth restriction. Preterm growth restricted infants show reduced cerebral cortical volume which correlates with poor neurodevelopmental outcome. Respiratory support in the form of supplemental oxygen or mechanical ventilation is inevitably required by preterm infants with acute and chronic respiratory morbidities which are commonly experienced by growth restricted preterm infants. Postnatal oxygen therapy is also associated with markers of cortical injury in the developing brain.

Aims: To define the independent and combined effects of growth restriction and a clinically derived postnatal fluctuating oxygen profile on markers of cortical integrity including (1) laminar thickness (2) cortical transcription factor expression and (3) cerebral growth factor expression, within a rodent model.

Methods: Sprague-Dawley rat dams received a normal or protein restricted diet to induce growth restriction. Litters were reared in air or fluctuating hyperoxia for 7 days. Pup brains were weighed, sections through the motor cortex stained with cresyl violet and the thickness of the corpus callosum, superficial (II-IV) and deep (V-VI) cortical layers were measured. Neuronal subtypes (callosal, superficial, corticospinal and corticothalamic) were immunofluorescently stained using neuronal subtype-specific transcription factors (Satb2, Cux1, Ctip2, Tbr1 respectively). Cerebral expression of trophic factors Insulin-like growth factor-I (IGF-I), Brain derived neurotrophic factor (BDNF), Fibroblast growth factor-2 (FGF-2) and Vascular endothelial growth factor (VEGF) were analysed and circulating serum IGF-I determined. Comparisons were made between study groups.

Results: Growth restricted pups had smaller brain weights than normally grown pups but cortical thickness was preserved. Fluctuating hyperoxia did not affect brain weight but cortical thickness was reduced in both normally grown and growth restricted groups. Cortical thinning was observed in the later born superficial cortical layers of both fluctuating oxygen study groups and in the deep layers of pups who were also growth restricted. Cell density and corpus callosum thickness was preserved. Fluctuating oxygen did not affect proportions of neurons expressing Satb2, Ctip2, or Tbr1 but was associated with a reduced proportion of superficial neurons expressing Cux1, intrinsic to cortico-cortical connectivity within the superficial cortex. Circulating IGF-I was reduced in all study groups in comparison to control, most significantly in the growth restricted group exposed to fluctuating oxygen. Cerebral expression of IGF-I and FGF-2 were conserved across the groups. VEGF expression was reduced in the dual group. An increased expression of BDNF was observed in the brainstem in all study groups, most significantly the growth restricted group exposed to oxygen.

Conclusions: The developing brain is dynamically influenced by the antenatal and postnatal environment. In a rodent model, normal cortical growth is inhibited by in utero growth restriction and postnatal oxygen fluctuation, in part associated with adverse effects on transcription factors and trophic factors instrumental to normal development. In utero growth restriction may permanently alter elements of cerebral development and postnatal oxygen fluctuation, a modifiable factor in neonatal care, may further impair cerebral growth with a preferential disadvantage to later born superficial neurons. Specific effects on neurons intrinsic to cortical circuitry suggest cortical development exposed to pre- and postnatal growth restriction may be particularly vulnerable to oxygen induced injury.

Declaration

Except where due acknowledgment is made by reference, the studies undertaken in this thesis were the unaided work of the author. The work described in this thesis has not been previously accepted for, or currently submitted in candidature for another degree. I acknowledge Mr Ronnie Grant for his invaluable help in creating many of the figures in this work.

Chapters 2 – 7

I acknowledge Mrs Jean Wade, Senior Technician at the Queens Medical Research Institute, Edinburgh who taught me to use the Edinburgh rat model equipment and the vast majority of laboratory techniques including: brain tissue collection and processing, collection and processing of rodent serum, use of ELISA assays, how to extract RNA, convert to cDNA and use of Taqman for RT-PCR. Mrs Jean Wade ordered and made the required stock solutions and liaised with the animal house in planning experiments. I acknowledge the animal house staff at Little France for mating the animals and for daily husbandry.

Chapters 5 & 6

I acknowledge Dr Petrina Georgala, Genes and Development Group, Hugh Robson Building, University of Edinburgh who taught me the technique of immunofluorescence and provided the primary and secondary antibodies. I acknowledge Dr Trudi Gillespie, Impact Imaging Facility, University of Edinburgh who helped capture the confocal images.

My gratitude is extended to each of these people, without whom I could not have completed this work.

Elaine Woodrow

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Acknowledgements

I am indebted to Dr Julie-Clare Becher and Dr Ian Laing for their excellent supervision, support and encouragement. It has been a privilege to be so generously supported by the Jennifer Brown Research Fund of the charity Piggybankkids who provided financial support to allow this work to be undertaken.

I am extremely grateful to Mrs Jean Wade for all her help and support in teaching me the techniques required to complete the animal work. I am so very grateful for her kindness and friendship. I have had the privilege of working with a great team in the Jennifer Brown Laboratory and many thanks go to Dr Elizabeth Pilley, Dr Vicky King, Dr Ellie Golightly and Dr Sarah Barr who have helped make the last 2 years so enjoyable.

The Edinburgh Neonatal Unit is an inspiring place and my extended thanks go to the committed, compassionate medical and nursing staff that made the shifts I worked times of great learning and excellent team-work.

Finally, a huge thank you to Doug for his endless support, love and encouragement and to my parents Alan and Linda, for everything they do.

Proceedings from this work

Presentations:

1 – 'Cortical brain growth is impaired by postnatal fluctuating hyperoxia in a preterm model of growth restriction', European Society of Paediatric Research, Newcastle, UK, October 2011.
2 – 'Fluctuating hyperoxia impairs cortical brain growth and Cux1 expression in a preterm model of growth restriction', The Neonatal Society, Institute of Child Health, London, November 2011.

3 - Invited speaker to Castang Foundation by Professor Anne O'Hare, 'The effect of oxygen & poor intrauterine & postnatal growth on cortical brain development', Castang Foundation, Royal College of Physicians, Edinburgh, March 2012.

Prize:

Award for best trainee presentation at the Neonatal Society Autumn meeting, November 2011.

Publication:

Woodrow E, Georgala P et al, Cortical growth is impaired by fluctuating hyperoxia in a preterm model of growth restriction, Pediatric Research, 2011;70(5):126 – *proceedings from the European Society of Paediatric Research, Newcastle, 2011.*

Manuscript in Preparation:

Woodrow E, Wade J, Georgala P, Gillespie T, Becher JC, 'Thinning of the motor cortex and disruption of neuronal transcription factor expression occurs in response to growth restriction and oxygen fluctuation in a model of preterm brain injury'.

Abbreviations

ADHD:	Attention Deficit and Hyperactivity Disorder
AGA:	Appropriate for gestational age
ANOVA:	Analysis of variance
BAPM:	The British Association for Perinatal Medicine
BDNF:	Brain Derived Neurotropic Factor
BPD:	Bronchopulmonary Dysplasia
cDNA:	Complementary DNA
CNS:	Central Nervous System
CP:	Cortical Plate
Ct:	Threshold cycle
Ctip2:	Chicken ovalbumin upstream promoter transcription factor-2
Cux1:	Cut-like-1 transcription factor
°C:	degree Celsius
DEHSI:	Diffuse excessive high signal intensity
dNTPs:	Nitrogenous bases; adenine, guanine, thymine, cytosine
Dual:	Dual study group exposed to growth restriction and fluctuating oxygen
ELBW:	Extremely Low Birth Weight
FiO2:	Fractional inspired oxygen concentration
FGF:	Fibroblast Growth Factor
FFP:	Fresh-frozen plasma
FGR:	Fetal Growth restriction
FGFR:	Fibroblast Growth Factor Receptor
GABA:	Gamma-Amino-Butyric Acid

GH:	Growth Hormone
GR:	Growth restriction study group
IGF:	Insulin-like Growth Factor
IGF-IR:	Insulin-like Growth Factor-1 Receptor
ITU:	Intensive Care Unit
IUGR:	Intrauterine Growth Restriction
IV:	Intravenous
LBW:	Low Birth Weight
LPS:	Lipopolysaccharide
MRI:	Magnetic Resonance Imaging
NCPAP:	Nasal continuous positive airway pressure
NEC:	Necrotising Enterocolitis
NT 3/4/5:	Neurotrophins 3/4/5
ΔO2:	Fluctuating oxygen study group
P0/7:	Postnatal day 0/7
PaO2:	Arterial oxygen partial pressure
PAX6:	Paired-box-6
PNS:	Peripheral Nervous System
PPROM:	Preterm Premature Rupture Of Membranes
PVL:	Periventricular Leukomalacia
qPCR:	Quantitative polymerase chain reaction
RDS:	Respiratory Distress Syndrome
rRNA:	ribosomal RNA
ROP:	Retinopathy of Prematurity
Satb2:	Special AT-rich sequence-binding protein 2
SD:	Standard Deviation

SEM: Standard Error of Mean

SGA: Small for gestational age

SP: Subplate

SVZ: Subventricular Zone

Tbr1: T-box brain1

TcO2: Transcutaneous oxygen measurement

VEGF: Vascular Endothelial Growth Factor

VLBW: Very Low Birth Weight

VZ: Ventricular Zone

Chapter 1:

General Introduction

1.1 Premature Birth

The definition of human infant prematurity is birth before 37 completed weeks of gestation. In the UK, prematurity affects between 6-8% of pregnancies (Bliss, National Charity for the newborn, UK). Preterm birth is the leading cause of perinatal morbidity and mortality in developed countries (Marlow et al. 2005) and the challenges increase as gestational age decreases. Although late preterm infants born between 32 and 37 weeks have a more favourable outcome, there is emerging evidence of subtle deficits in cognition and other health morbidities in this group (Odd et al. 2012). A significant proportion of preterm neonates born at less than 32 weeks gestation face a range of challenges in the ante- and postnatal period which may have lifelong impact. When evaluated at 30 months corrected, 50% of infants born at 25 weeks gestational age or less have identifiable functional impairments (Wood, Marlow et al. 2000). In the UK, 1-2% of infants are born <32weeks gestation including very preterm births at 28+0 to 31+6 (incidence 0.7%) and extremely preterm births at 23+0 to 27+6 weeks (incidence 0.4%) (McParland et al. 2004). The risk of poor outcome is also proportional to birth weight. Low birth weight (LBW) is defined as less than 2500g and this can be subdivided into very low birth weight (VLBW, less than 1500g) and extremely low birth weight (ELBW, less than 1000g).

1.1.1 Causes of Prematurity

Infants may be born prematurely following either spontaneous labour with intact or ruptured membranes, accounting for two thirds of preterm deliveries (McParland et al. 2004) or following induction / Caesarean delivery for fetal or maternal indications which accounts for approximately one third (McParland et al. 2004). Common fetal and maternal indications for preterm delivery include intrauterine growth restriction and pregnancy induced hypertension. Spontaneous preterm births result from a number of causes, the most common being intrauterine inflammation and infection which are often clinically asymptomatic. Multiple gestation carries a significant risk of preterm delivery and accounts for 15-20% of preterm births (Goldenberg et al. 2008).

1.1.2 The Economic cost of Prematurity

There are economic implications of providing neonatal care to an increasing number of infants, and as a consequence of the social and economic cost of preterm-related disabilities, there is an ongoing debate regarding whether intensive care is justified for infants of borderline viability. Total public sector costs per survivor to 18 years are estimated at £40,000 for term infants, £104,000 for <33 week gestation infants, £140,000 for <28 weeks and £210-£275,000 for 23-24 week gestation infants (Mangham et al. 2009). Improving understanding of how the intrauterine and postnatal environments interact to impact on development may lead to modification of care practices and new therapies to minimise long term morbidity and reduce the

economic burden of prematurity. Social costs exist in providing ongoing medical care, special needs schooling, financial and psychological support to parents caring for a child with disabilities and / or additional support needs. As a society, some question the use of resources to resuscitate and care for increasingly premature infants over channelling resources to improve prenatal care and prevention of prematurity (Moster 2008).

1.2 Consequences of Prematurity

Advancing knowledge in the field of perinatology continues to improve the care provided to preterm infants before and after birth. The disturbance to fetal development caused by removal from the in utero environment is demonstrated by the significant number of survivors who suffer consequences of their prematurity. Such consequences affect many organ systems, most significantly the brain, the respiratory system, retinal maturation and gastrointestinal function (Saigal et al. 2008). The ultimate goal in caring for each preterm infant is intact survival without neonatal morbidity or future impairment. However, significant mortality and morbidity rates continue to be associated with preterm birth.

1.2.1 Neonatal Mortality

The neonatal mortality rate in England and Wales has fallen from 7.7 per 1000 live births in 1980 to 3.1 deaths per 1000 live births in 2009 (Office for National Statistics, UK, 2011). This is a combined effect of advances in technology and the collaborative efforts of obstetric and neonatal care. The preterm birth rates in the United States and Europe have been reported as 11% and 5-7% respectively (Goldenberg et al. 2002; Goldenberg et al. 2008). The EPICure study considered mortality and outcomes specifically for extreme preterm infants born before 26 completed weeks of gestation in the United Kingdom and Republic of Ireland in 1995. This study reported a survival rate at discharge from in-patient neonatal care at 39%. Within this cohort, survival to discharge improved with increasing gestation; 19.9% at 23 weeks, 33.6% at 24 weeks and 52.1% at 25 weeks gestation (Costeloe et al. 2000). The EPICure 2 study launched in 2006 includes babies born between 22 and 26+6 weeks and demonstrated improved survival rates to discharge at each gestation for current neonatal care; 26% at 23 weeks, 47% for 24 weeks and 67% for 25 week infants (Costeloe et al. 2008, Moore et al. 2012). The EPIPAGE study carried out in France in 1997 reported on survival of preterm infants born between 22 weeks and 32 weeks (Larroque et al. 2004). Survival was 67% for all births between 22 and 32 weeks and increased with gestational age: 31% at 24 weeks, 78% at 28 weeks and 97% at 32 weeks. Survival was lower for male infants and infants born small for gestational age.

1.2.2 Neonatal Morbidity

Despite the improvements observed in preterm infant survival, rates of many of the morbidities associated with preterm birth have remained static (Fanaroff et al. 2003). It is well recognised that morbidity is inversely related to gestational age (Doyle 2004). The most significant morbidities that impact long-term outcome for preterm infants, in particular neurodevelopmental outcome, include: bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), necrotising enterocolitis (NEC), infection and inflammation, poor growth and brain injury. Extra-cerebral morbidities are first considered and then brain injury and subsequent neurological outcomes are discussed in the following sections (1.2.3 & 1.2.4).

1.2.2.1 Bronchopulmonary Dysplasia (BPD)

BPD is defined as an oxygen dependency at 36 postmenstrual weeks. It most commonly affects ELBW infants less than 26 weeks gestation (Coalson 2003). The pathogenesis of BPD includes oxygen-induced injury, volume trauma from mechanical ventilation, impaired alveolarisation and pre- and post-natal inflammatory response (Coalson 2006). The EPICure study reports that 74% of their cohort required oxygen at 36 weeks and no significant difference was noted between gestational ages. However at 40 weeks postmenstrual age, 51% of infants required oxygen and this was inversely related to gestational age; at < 23 weeks gestation, 78% of infants required oxygen, whilst at 25 weeks, 44% of infants required oxygen (Hennessy et al. 2008). In the same study, 36% of infants were discharged home

with supplemental oxygen and this was continued for a median of 2.5 months. Infants who develop significant BPD are more likely to suffer ongoing respiratory morbidity in childhood including recurrent hospital admissions for infection, wheeze and in some cases pulmonary hypertension (Fawke et al. 2010).

In addition to respiratory disease, preterm infants who develop BPD are more likely to experience other complications including infection (Hack et al. 2000), inadequate nutrition and poor postnatal growth (O'Shea et al. 1996). Infants with BPD, by definition are exposed to ongoing oxygen therapy which may be damaging to multiple body systems including the brain (Askie et al. 2003). Episodes of hypoxia are more frequent in infants with BPD (Garg et al. 1988) and the combination of these factors place these infants at higher risk of brain injury (Volpe, 2001) and neurodevelopmental impairment (Anderson et al. 2006). Infants with BPD are more likely to have a grade III or IV intraventricular haemorrhage but BPD is itself an independent risk factor for cerebral palsy (Skidmore et al. 1990). Children with BPD have poorer fine and gross motor skills than preterm infants without BPD (Short et al. 2003). Some studies report that school-aged children with BPD have lower IQ than children without BPD (Short et al. 2003; Gray et al. 2004) however other studies have found no significant difference (Bohm et al. 2003). It has also been reported that preschool BPD children have receptive and expressive language skills which are less developed compared to preterm children without BPD (Singer et al. 2001) and have increased problems with articulation which, as mentioned above, may be part of a general motor deficit (Lewis et al. 2002). Finally, BPD has been

suggested as a strong predictor for attention deficit and hyperactivity (ADHD) behaviours (Farel et al. 1998; Short et al. 2003).

1.2.2.2 Retinopathy of Prematurity (ROP)

ROP is caused by disorganised growth of retinal blood vessels mediated by vascular endothelial growth factor (VEGF). ROP is more prevalent in lower gestations and in lower birth weight and growth restricted infants (Palmer et al. 1991; Dhaliwal et al. 2009). Liberal oxygen exposure and fluctuations in oxygen are established risk factors in the development of ROP (York et al. 2004). ROP can lead to retinal scarring, detachment and blindness. The EPICure study reported that 14% of infants in the 1995 cohort required treatment for ROP. Blindness as a consequence of ROP has fallen from 8-10% in the early 1990s to 3% in current practice (Chow et al. 2003; Hintz et al. 2005). This is a consequence of greater awareness of the harm caused by oxygen therapy and the success of cryotherapy and, most recently, laser therapy in treating threshold disease (Gilbert 2008). However, the complications of these therapies include clinically significant visual field loss and myopia caused by the permanent destruction of the peripheral retina in the pursuit of eradicating VEGF-producing cells (Smith 2008). Recent studies have considered the use of anti-VEGF agents, primarily intravitreal bevacizumab in treating ROP as a monotherapy or in combination with conventional laser therapy and some promising results have been reported (Mintz-Hitter et al. 2011).

1.2.2.3 Necrotising Enterocolitis (NEC)

The prevalence of NEC in infants of birth weight 500-1500g is approximately 7% (Holman et al. 2006) and the associated mortality rate is between 20-30% (Rowe et al. 1994; Fitzgibbons et al. 2009). The exact pathogenesis of NEC remains to be fully elucidated, however a combination of intestinal immaturity, abnormal bacterial colonisation, increased immunoreactivity of the intestinal mucosal cells and abnormal microvascular tone are all considered contributory factors (Neu et al. 2011). NEC is an inflammatory process and, in preterm infants with minimal reserve, can rapidly become a systemic inflammatory process with implications for other organs such as the brain. In particular, infants who require surgery for management of NEC, as opposed to medical management, are at increased risk of adverse neurodevelopmental outcome (Hintz et al. 2005).

1.2.2.4 Inflammation and Infection

Maternal and intrauterine infection is long-established as playing a significant role in neonatal brain injury, irrespective of gestational age. Raised levels of pro-inflammatory cytokines measured in amniotic fluid and umbilical cord blood have been associated with white matter lesions in the neonate (Dammann et al. 1997). The source of the circulating cytokines is thought to be ascending micro-organisms from the vagina and cervix which have invaded the amniotic cavity, induced inflammation of the chorioamniotic membranes and led to the production of pro-inflammatory cytokines which then reach the fetal membranes (Rees et al. 2005).

The fetus develops an inflammatory response through an alteration of pro-inflammatory cytokines (Svigos 2001). Elevated levels of circulating cytokines have been linked to dramatic increases in the risk of periventricular leukomalacia and cerebral palsy (Nelson et al. 1999). Recent evidence has also suggested that intrauterine inflammation results in fetal neuronal injury in animal models (Burd et al. 2010) and in humans, the fetal inflammatory response mediated by raised cytokines has been implicated in neurodevelopmental delay and disorders such as autism and schizophrenia (Meyer et al. 2006; Goines et al. 2011).

1.2.2.5 Postnatal Growth

Extreme preterm infants are at an increased risk of poor postnatal growth (Yanney et al. 2004). When the EPICure cohort of children was examined at 30 months, they were found to have significantly lower weight and smaller head circumference than the full term control children (Wood et al. 2003). Factors exacerbating this risk include BPD, nutrition problems secondary to NEC, related surgery and infection (Cooke et al. 2004). Studies have observed a positive correlation between reduced head circumference of VLBW infants at school age and adverse neurodevelopmental outcome (Tan et al. 2008). The benefit of catch up growth however is controversial (Thureen 2007). It has been shown that infants with low birth weights, particularly those that have been growth restricted in utero, have a particular predisposition to gain weight rapidly in the first two years of life (Ong et al. 2002). At school age, such infants have reduced adipose tissue in their extremities but increased central adiposity. This altered fat distribution is thought to underlie the link between low

birth weight and the increased risk of developing cardiovascular disease, hypertension and glucose intolerance in adulthood (Gianni et al. 2008).

1.2.3 Effects of Preterm Delivery on the Brain

Up to 50% of preterm infants born less than 25 weeks gestation have neurological deficits that affect motor, visual, behavioural and cognitive function (Wood et al. 2000). There is an established but overly simplistic view that motor deficits correlate with white matter damage whereas cognitive deficits correlate with grey matter damage (Ajayi-Obe et al. 2000; Abernethy et al. 2002; Nosarti et al. 2002). Traditionally, brain injury associated with preterm delivery was considered a discrete lesion causing damage to the affected brain region and deficit in the associated body part (Volpe 2009b). Historically, the two major brain lesions considered to cause such discrete injury have been (1) periventricular hemorrhagic infarction secondary to germinal matrix or intraventricular haemorrhage and, (2) periventricular leukomalacia. The most established neuropathology associated with preterm delivery is spastic diplegic cerebral palsy, caused by non-progressive damage to the motor tracts of the developing brain and affecting 5-10% of VLBW infants (Platt et al. 2007). The neonatal imaging correlate of this form of cerebral palsy is cystic periventricular leucomalacia observed in 5% of very preterm infants (Volpe 2009a). Despite the advancements made in reducing mortality, the prevalence of cerebral palsy has remained largely unchanged at 8-10% (Hintz et al. 2005), although reports on this do vary in both directions depending on the definitions used (Vincer et al. 2006; Platt et al. 2007). There is emerging evidence that more subtle brain injury associated with preterm birth may underlie the milder deficits in neurological function. Improvements in imaging techniques have allowed more detailed examination.

Preterm brain injury was traditionally assessed by cranial ultrasound scan. The presence of Grade III and IV intraventricular haemorrhage, hydrocephalus or periventricular leukomalacia were associated with a higher likelihood of subsequent neurodevelopmental concerns (Hack et al. 2000). The presence of a normal cranial ultrasound scan however, does not exclude long-term disability.

The increasing use of MRI imaging of preterm infants has identified a range of features noted on the preterm infant brain which are not noted in term born controls and therefore provide crucial insights of the effects of premature delivery and neonatal care on the developing brain (Counsell et al. 2003). Such features include diffuse white matter abnormalities known as diffuse excessive high signal intensity (DEHSI), occurring most commonly around the ventricles but also seen throughout the white matter and affecting the majority of preterm infants (Maalouf et al. 1999). DEHSI is thought to represent white matter microstructural damage (Volpe 2003) and is associated with astrogliosis and microgliosis (Volpe 2009a). In addition, DESHI is associated with a reduction in premyelinating oligodendrocytes (Riddle 2011, Volpe 2009a) in keeping with observations made in preterm neonates where episodes of oxidative stress appear to selectively target premyelinating oligodendrocytes (. MRI studies examining the effect of lipopolysaccharide (LPS) on white matter of fetal sheep who are gyrencephalic, demonstrate a decrease in white matter volume and corpus callosal thickness at 10 days post LPS exposure (Looij 2012). Other work confirms this post-LPS diffuse white matter injury in fetal sheep is accompanied by reduced cortical volume on MRI and histological

confirmation of reduced numbers of subplate and cortical neurons (Dean 2011). The long term clinical correlate of such diffuse injury is not fully established but diffusion tensor imaging studies measuring apparent diffusion coefficient have suggested associations with neurodevelopmental impairment in preterm infants (Counsell et al. 2008; Boardman et al. 2010). Anisotropy is the term used to describe directional dependence of water in tissues and represents the crux of diffusion MRI. Quantitative MRI imaging allows volumetric analysis and it has been shown that preterm infants with PVL have reduced cortical grey matter at term compared to infants without PVL (Inder et al. 1999, Nosarti et al. 2002, Inder et al. 2005). This suggests that PVL affects cerebral cortical development and may indicate why PVL is associated with deficits in cognitive as well as motor function.

Although the mechanism of preterm neurological deficit has been traditionally regarded as secondary to brain injury, there have been recent shifts in opinion which focus on the disruption caused by the interruption of normal development. Volpe proposed the term "Encephalopathy of Prematurity" (Volpe 2009b) to describe the association between focal and diffuse white matter damage, found in 50% of very low birth weight infants (Volpe 2001), and decreased grey matter volume identified by imaging and neuropathology of the thalamus and basal ganglia (Inder et al. 2005; Pierson et al. 2007). In addition, the "Encephalopathy" may include the associated disruptive effects on myelination of developing axons (Deguchi et al. 1999) and reduced volume and complexity of the cerebral cortex beyond the preterm period (Ajayi-Obe et al. 2000; Nosarti et al. 2002). Despite the widely accepted findings of

cortical volume effects into childhood and adolescence, it is reported that gross cerebral volume is not generally reduced during the neonatal period (Boardman et al. 2007). Recent imaging studies support the concept that preterm birth has specific effects on developing neural systems such as the thalamocortical system where volume reduction in the thalamus is inextricably linked to volume loss in the cortex and hippocampus (Ball et al. 2011). Specific effects may be linked to damage in cortical proliferating zones and damage to the subplate synaptic compartment, both of which are areas undergoing significant developmental maturation at the time of preterm birth (Volpe 2009b). Cortical development will be discussed in more detail in section 1.5.

1.2.4 Neurodevelopmental Outcome following preterm birth

As described above, each of the major morbidities associated with preterm birth including bronchopulmonary dysplasia, retinopathy of prematurity, necrotising enterocolitis, poor postnatal growth and brain injury, have all been shown to impact negatively on infant neurodevelopment. Data from follow-up studies across Western countries suggest approximately 25% of infants born less than 28 weeks develop significant neurodevelopmental impairments in early childhood (Saigal et al. 2008) including cerebral palsy, developmental delay and sensory impairments of vision and hearing. For infants at the lowest gestations, the EPICure study reported that 19% of infants born less than 25 weeks had severely delayed development at 30 months corrected with developmental scores more than 3 standard deviations (SD) below the mean (Wood et al. 2000). This is supported by studies showing a strong trend of increased special educational need with lower gestation at birth (MacKay et al. 2010).

There is evidence from follow-up studies that even when preterm infants have progressed through early childhood without manifesting any of the major associated morbidities, there are often underlying neurological effects of their prematurity which become apparent during childhood, particularly at school age. In comparison to their peers, children born preterm have reduced scores on general cognitive tests (Marlow et al. 2005), impaired executive function skills (Anderson et al. 2004; Taylor et al. 2004), memory deficits and lower academic performance (Bhutta et al. 2002; Marlow et al. 2005; Johnson et al. 2009). Furthermore, these children have an

increased prevalence of mild dysfunction of neuromotor and coordination skills (Saigal et al. 2003), behavioural problems including inattention and hyperactivity (Reijneveld et al. 2006), and psychiatric disturbance including autism and anxiety disorders (Grunau et al. 2004; Johnson et al. 2010).

The adverse outcomes associated with late prematurity are often underappreciated. Research demonstrates that a third of children born at 32-35 weeks gestation have increased difficulties with motor skills, speaking, writing, mathematics and behaviour (Huddy et al. 2001), again indicating that subtle effects on brain function are associated with premature delivery. Furthermore amongst term infants (37-40 weeks gestation), the risk of special educational need is lowest at 40 weeks compared to 37-39 weeks (MacKay et al. 2010).

In summary the effect of preterm birth can be life-long with significant effects on neurodevelopment. Active reproductive biology research continues to explore the mechanisms underlying preterm birth (Iams et al. 2008) whilst key aims in perinatal research are to prevent or modify brain injury from exogenous processes (sections 1.3 and 1.4) and minimise the interruption of normal brain development and growth (sections 1.5 & 1.6). It is important to identify at-risk infants, understand pathophysiology of organ damage, identify early biomarkers of injury and investigate strategies to improve outcome.

1.3 Growth Restriction and the Preterm Infant

Intrauterine growth restriction secondary to placental failure is one of the major precipitants of preterm delivery (Goldenberg et al. 2008). Fetal growth restriction is reported to occur in approximately 5% of the general obstetric population (Neerhof et al. 1995). Placental insufficiency is significant in the aetiology of preterm birth as it causes chronic hypoxia, deprivation of nutrients and reduced perfusion of the fetus. Infants delivered preterm are ten times more likely to have suffered intrauterine growth restriction than infants delivered at term (Gilbert et al. 2003).

1.3.1 Growth restriction definitions

Intrauterine growth restriction (IUGR) can be defined as a reduced fetal growth velocity causing a fetus to fail to attain its genetic growth potential (de Bie et al. 2010). This is a prenatal clinical diagnosis made by serial ultrasound scans (Bertino et al. 2007). IUGR can lead to infants being small for their gestational age (SGA), meaning birth weight less than a predefined centile for gestation. The British Association for Perinatal Medicine defines SGA as birth weight below the 5^{th} centile for gestational age. There are, however, a wide range of definitions used in the literature, including a birth weight less than the 10^{th} centile, a birth weight greater than two standard deviation below the mean and a birth weight less than 2500g (Hokken-Koelega et al. 1995). Importantly, not all SGA infants will be IUGR, as up to 20% (Mamelle et al. 2001) will be constitutionally small, in accordance with their genetic growth potential. In addition, some infants who are deemed appropriate

weight for gestation may have experienced curtailment of their growth potential in utero. Growth restriction may be symmetrical, where onset is much earlier in the course of pregnancy and weight, length and head circumference are equally affected, or more commonly, asymmetrical, where there is relative brain-sparing and head circumference is preserved whilst weight and possibly length are reduced. Associated morbidity tends to be higher in the symmetrical group where there is an innate fetal abnormality or a prolonged inhibitory insult to fetal growth (Lin et al. 1991). A variety of physiological mechanisms may interact to cause IUGR, including:

Table 1 – Causes of intrauterine growth restriction

Maternal Factors	Uteroplacental Factors	Fetal factors
Cardiovascular Disease	Reduced uteroplacental flow	Infection
Anaemia	Pre-eclampsia	Chromosome abnormalities
Renal Disease	Diabetes Mellitus	Confined placental mosaicism
Drugs: recreational / therapeutic	Connective tissue disorders	Isolated malformations
	Abruption	
	Thrombosis / infarction	
	Chorioamnionitis	

1.3.2 Growth restriction and neonatal outcome

It is likely that fetal growth restriction of different aetiologies have different outcomes. Overall however, preterm SGA neonates have a higher fetal and neonatal mortality rate at each gestational age in comparison with 'appropriate' weight for gestational age (AGA) infants (Piper et al. 1996; Simchen et al. 2000). Some studies report that below 32 weeks, the effects of morbidities associated with prematurity

outweigh the effects of growth restriction (Yanney et al. 2004). The risk of cerebral palsy is higher in growth restricted infants after 33 weeks gestation but there is no clear risk posed by growth restriction at earlier gestations (Blair et al. 1990; Topp et al. 1996). The confounding factor in many studies examining the effect of growth restriction is the use of neonatal as opposed to fetal growth standards which use intrauterine growth curves to define growth restriction (Ley et al. 1997). Using neonatal standards, 10% of the 'normal' population will have a birth weight below the 10^{th} centile. When fetal growth standards that track in utero growth were used in one study, the proportion of infants meeting the criteria for growth restriction in the cohort increased from 11.6% to 23.3% (Zaw et al. 2003). This group found that the growth restricted infants had an increased risk of acute respiratory distress syndrome, bronchopulmonary dysplasia and retinopathy of prematurity. A recent retrospective study examining growth restricted infants born between 25 and 36 weeks gestation, found that in addition to the increased risk of respiratory compromise, preterm growth restricted infants were also at increased risk of thrombocytopenia, neonatal sepsis and NEC. The increased risk of sepsis was the most significant underlying factor accounting for the associated increased mortality rate in this cohort (Engineer et al. 2010).

1.3.3 Growth restriction, the developing brain and neurodevelopment

Infants who are born SGA are reported to have long-term cognitive impairments, lower intelligence, poorer memory, lower academic attainment and increased behavioural problems (O'Keeffe et al. 2003; Noeker 2005; Geva et al. 2006). Other groups have reported reduced motor scores, impaired short-term memory but preservation of recognition memory (Leitner et al. 2007). Notably, research suggests that whilst SGA infants score lower on cognitive testing, extreme prematurity and neonatal complications have a greater detrimental effect on a child's neurodevelopmental outcome than IUGR alone. Such studies have included control groups matched both for gestation and birth weight (Sung et al. 1993; McCarton et al. 1996; Gutbrod et al. 2000).

Preterm growth restricted infants were evaluated postnatally by MRI and were found to have specific structural and functional effects on brain development (Tolsa et al. 2004). In comparison to gestation matched controls, the growth restricted infants had reduced intracranial and cerebral cortical grey matter volumes. These changes were associated with reduced attention-interaction scores on behaviour testing in comparison to gestation matched appropriately grown preterm infants (Tolsa et al. 2004). The hippocampus is particularly vulnerable to volume loss following placental insufficiency induced intrauterine growth restriction in preterm infants. This volume loss is reported in association with altered behavioural score as

evaluated by the Assessment of Preterm Infants' Behaviour at term corrected (Lodygensky et al. 2008). Quantitative MRI studies have shown that preterm growth restricted infants have abnormalities in molecular diffusion values in the posterior limb of the internal capsule and altered regional anisotropy in the thalamus and cortical grey matter in comparison to appropriately grown preterm infants, thereby suggesting altered development in these regions (Thompson et al. 2007).

The main method of monitoring fetal wellbeing in utero is by using Doppler ultrasound to measure fetal blood flow velocity. As stated above, the most common type of growth restriction seen is asymmetrical growth restriction, suggestive of third trimester placental insufficiency and relative brain-sparing (Lackman et al. 2001). Brain-sparing is a continuum where there is initial increased brain perfusion followed by a decrease in the cerebroplacental ratio (Hernandez-Andrade et al. 2008). A recent study documented that 55% of intrauterine growth restricted fetuses had abnormal middle cerebral artery (MCA) Doppler scores and that neurobehavioural disruption at term was more prevalent in growth restricted infants with abnormal MCA Doppler (Figueras et al. 2011). A large cohort study showed that brain-sparing during the third trimester was associated with a 23% higher prevalence of behavioural problems at 18 months in comparison to infants with normal MCA Doppler (Roza et al. 2008). A further study that included preterm infants found significant cognitive differences between infants with and without brain-sparing at age 2, however these differences were not significant after adjustment for brain volume (Leppanen et al. 2010). This suggests that the effect on brain structure, as opposed to absolute blood flow, may be most important in determining outcome.

1.3.4 Growth restriction and metabolic effects

Postnatal growth is generally poor in growth restricted preterm infants with approximately 15% of infants demonstrating any degree of catch up growth at 3 months of age (Hokken-Koelega et al. 1995). Beyond infancy into childhood and adulthood, growth restricted infants have been shown to have shorter heights and reduced weights (McCarton et al. 1996). In addition such infants have an increased risk of insulin resistance, metabolic syndrome, type 2 diabetes mellitus, cardiovascular disease and end-stage renal failure (Hales et al. 1991; Gluckman et al. 2005), in keeping with the Barker hypothesis:

> "Environmental influences that impair growth and development in early life may be risk factors for metabolic disease".

(Barker et al. 1989; Hales et al. 1991).

1.4 The Effect of Oxygen Therapy

The primary cause of preterm neonatal death is respiratory failure due to a combination of surfactant deficiency, interrupted anatomical lung development and immaturity of other organ systems. In addition to the use of oxygen, there are a number of key respiratory interventions in neonatal care which have contributed to the reduction in mortality and these will be briefly summarised before the discussion focuses on the use of oxygen therapy.

The use of a single course of antenatal steroids for threatened preterm delivery has become widespread practice since the late 1990s and been shown in a Cochrane review to reduce rates of: neonatal mortality, respiratory distress syndrome (RDS), cerebrovascular haemorrhage, necrotising enterocolitis (NEC), requirement for respiratory support, intensive care unit (ITU) admission and onset of systemic infection in the first 48 hours of a preterm infant's life (Roberts et al. 2006). There is no evidence that multiple courses of antenatal steroids have a beneficial effect, however multiple antenatal doses are often given in practice (Banks et al. 1999). Although this is associated with beneficial effects on lung function, there are concerns from animal data that multiple courses may lead to a reduction in brain volume (Carlos et al. 1992), which in turn, has implications for neurodevelopment (Peterson et al. 2000).

The use of exogenous surfactant, administered following intra-tracheal intubation, was first shown in the 1970s to reduce the incidence of respiratory distress syndrome (RDS), intraventricular haemorrhage (IVH) and neonatal mortality (Liggins et al. 1972). The beneficial effect and safety for preterm infants was reinforced by the Ten Centre Trial which reported a 30% reduction in RDS and a 48% reduction in neonatal mortality in comparison to control infants (Ten Centre Trial 1987). A recent Cochrane review suggests for preterm infants who are stabilised on nasal continuous positive airway pressure (NCPAP), selective as opposed to prophylactic use of surfactant, is associated with reduced risk of chronic lung disease and death (Rojas-Reyes et al. 2012). This suggests intra-tracheal intubation of such stable infants for the purposes of administering prophylactic surfactant is not required. Studies have looked at nebulised surfactant administration; however a Cochrane review has suggested further trials are required to ascertain any beneficial effect (Abdel-Latif et al. 2012).

The use of oxygen in neonatal care began in the 1950s and improvements in assisted ventilation and the development of intensive care practice of preterm infants led to a significant increase in survival in the mid-1990s (Gultom et al. 1997). Respiratory support in the form of supplemental oxygen or mechanical ventilation is inevitably required by preterm infants with respiratory morbidity. These therapies are associated with the development of bronchopulmonary dysplasia (BPD) which is characterised by ventilation/perfusion mismatch, variable oxygen saturations and fluctuations of inspired oxygen titrated to a target oxygen saturation range (Doyle et

al. 2006). The presence of BPD is an independent predictor of adverse neurodevelopmental outcome (Short et al. 2003) although the mechanism is unclear. Recent MRI studies in preterm infants indicate that supplemental oxygen is an independent risk factor for impaired brain growth and loss of brain tissue volume at term (Boardman et al. 2007). Even preterm infants who do not receive supplemental oxygen are exposed to partial pressures of oxygen ex utero which are four times higher than the intrauterine environment (Felderhoff-Mueser et al. 2004).

Oxygen was first used in newborn resuscitation by Chaussier in 1780 (Silverman 2004). Its use became more widespread in newborn care over the 20th century and early 21st century. Until the 1950s, 100% oxygen was regularly administered, and between the 1930s and the 1950s, there had been a steady decline in neonatal deaths (Bolton et al. 1974). In the 1950s, a number of units in both the USA and the UK noted that a significant proportion of preterm survivors developed visual problems linked to the use of high oxygen concentrations (Bolton et al. 1974). This eye disease was termed retrolental fibroplasia to describe the pathological process now more commonly known as retinopathy of prematurity (ROP). There was then a trend toward reducing oxygen use in neonatal care during the 1950s and 1960s. However this was met by an increase in neonatal deaths between birth and day three of life secondary to hypoxia (Bolton et al. 1974). This was the beginning of the ongoing major dilemma in neonatal care; the balance between giving sufficient oxygen to prevent hypoxia-driven mortality and giving excess oxygen causing hyperoxia-related morbidity. It is now clear that oxygen therapy is not benign when used in

resuscitation or in the ongoing management of term or preterm infants (Saugstad et al. 1998). Recent evidence suggests that maintenance of oxygen saturations within a higher targeted range (90-95%, equivalent to 5.9-8.9 kPa) is associated with significantly less mortality than a lower targeted range (Stenson et al. 2011).

There is a substantial medical literature across specialties noting the organ damage caused by high concentrations of oxygen (Kones 2011; Brenner et al. 2012). Reactive oxygen species are formed as a natural by-product of oxygen metabolism and can damage all components of cells including protein, DNA and enzyme systems (Maltepe et al. 2009). Studies have demonstrated that short exposure of preterm infants to oxygen during resuscitation increases circulating oxidative stress biomarkers, potentially harmful to all body systems, not least the developing brain (Vento et al. 2003; Vento et al. 2005). There is evidence that preterm infants have lower antioxidant reserves (Taglialatela et al. 1998; Kuster et al. 2011).

In animal studies, a fluctuating oxygen profile increases short and long term indices of brain injury (Felderhoff-Mueser et al. 2004; Loeliger et al. 2006; Ratner et al. 2007). Oxygen fluctuations have been studied extensively in animal models of retinopathy of prematurity where exposure of newborn rats to fluctuating oxygen was found to be a more effective stimulus for proliferative, and therefore more severe retinopathy, than constant hyperoxia (Penn et al. 1993). The proposed underlying mechanism for the proliferation is that hyperoxia causes an initial cessation of retinal

vessel development by down-regulating VEGF. Retinal neurons continue to develop but the impaired blood vessel development is unable to supply adequate oxygen to these developing neurons. The resulting hypoxia induces the release of hypoxic-inducible factor (see 1.4.3), causing VEGF expression and rapid formation of new, less robust vessels which are prone to leakage and therefore bleeding. The hyperoxic fluctuations then switch off this mechanism again leading to cessation of vessel development and further retinal hypoxia (York et al. 2004). There is evidence that this HIF-1alpha / VEGF mechanism is active in protecting the brain from injury in mice (Sheldon et al. 2009). These oxygen fluctuations and subsequent aberrant retinal vascularisation can be replicated in animal studies where effects on myelination of the optic nerve and white matter of the brain are also observed (Bhushan et al. 2007; Sedowofia et al. 2008; Pilley et al. 2010).

The partial pressure of oxygen in arterial blood can fluctuate rapidly in preterm infants causing alternating episodes of hyperoxemia and hypoxemia despite clinical staff adjustments of inspired oxygen in an attempt to maintain saturations within a target range (Quine et al. 2009). Preterm infants experiencing fluctuating PaO2 are at higher risk of developing more severe proliferative ROP (York et al. 2004) and therefore, fluctuations between hyperoxia and hypoxia may have specific effects on the brain.

1.5 Cortical brain development

Both brain injury and disrupted brain development both play a role in explaining why many preterm infants have neurological impairment. During the third trimester in intrauterine development, cortical grey matter volume increases fourfold and white matter volume increases fivefold (Huppi et al. 1998). For infants born preterm, this development is ongoing whilst the infant fights for survival in the neonatal unit. The major phases of brain development occurring during the third trimester include dendritic and axonal growth, synaptogenesis, glial proliferation, differentiation and myelination (Volpe 2001). These processes are therefore vulnerable to the effects of neonatal care and the effects of other morbidities experienced by preterm infants.

1.5.1 Human Cortical Development

> "The cerebrum is the primary seat of the rationale soul in man, and of the sensitive soul in animals. It is the source of movements and ideas."
>
> Thomas Willis, 1664

In the 17th century, the eminent anatomist and physician, Thomas Willis, conjectured that human higher cortical function originated in the folds of the cerebral cortex. He had noted the significant size difference between the cortex of various animals such as sheep and cats in comparison to the human and believed this explained why an animal "thinks or remembers scarce anything but what the instincts and needs of nature suggest" (from Willis' *Cerebri Anatome* translated in 1681 by Samual

Pordage). He also believed the smooth, lissencephalic cortices of birds and fish explained why such creatures have a limited capacity for learning and comprehension.

The human cerebral cortex is a complex convoluted structure, yet it is highly organised into six distinct layers. Each layer contains neurons with specific morphological features to support their individual function. There are significant uncertainties regarding the intricacies of cortical development, therefore the regulation and underlying mechanisms continue to be a focus of current scientific research. The summary given below represents current understanding of the major developmental events in humans. In all mammals, there is a common basic pattern of cortical development. The growing brain becomes progressively populated with neurons by neuronal progenitor proliferation, differentiation, migration, maturation and neuronal cell survival. Differences do exist between humans and other more extensively researched animal models such as rodents. These differences will be discussed in section 1.5.2.

Cortical Embryology

By the third week of development, the three major regions of the forming brain are distinguishable in the neural tube: the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). From the prosencephalon, the telencephalon and diencephalon arise. The dorsal telencephalon has two lateral

outpouchings, the cerebral vesicles, which later form the cerebral hemispheres, see Figure 1.

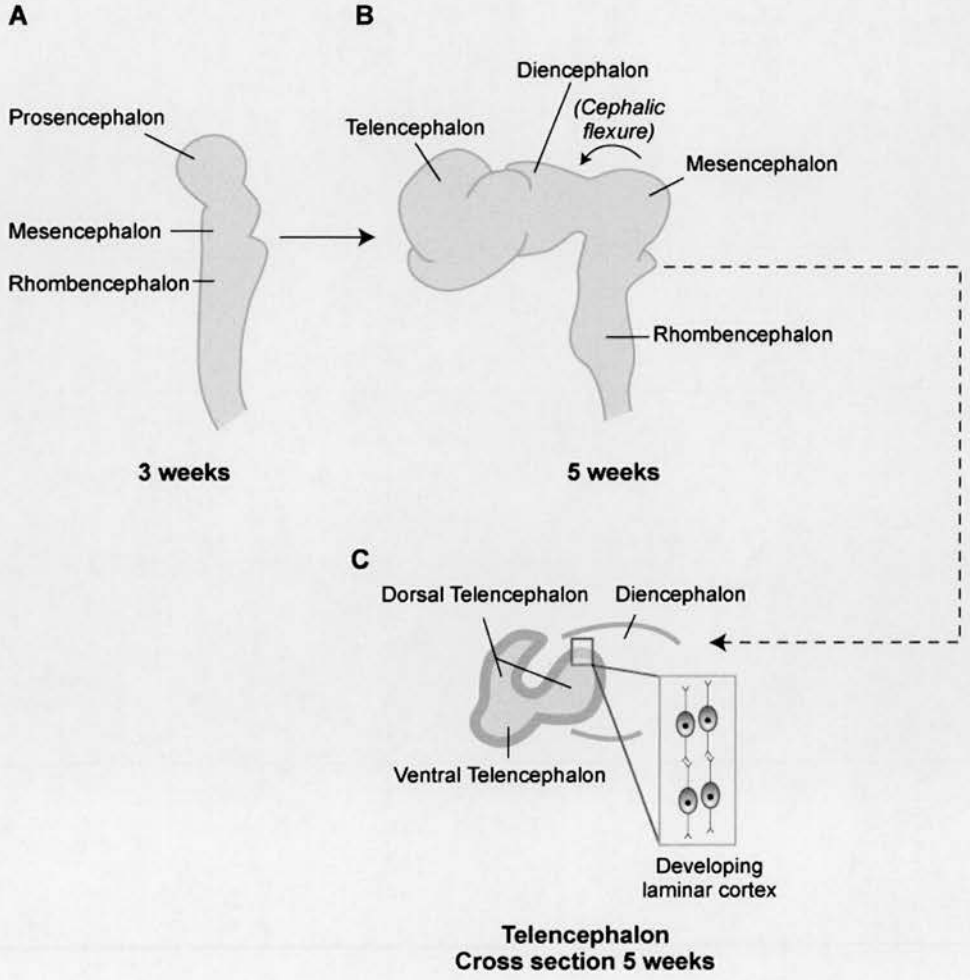

Figure 1 – Early development of the Human Brain, adapted from Price et al, 2011.

A – Schematic of the neural tube at 3 weeks showing developing prosencephalon, mesencephalon and rhombencephalon.

B – By 5 weeks of gestation the cephalic flexure at the level of the mesencephalon has driven the shape of the developing brain. The two lateral outpouchings at the rostral end of the telencephalon are the cerebral vesicles, the primitive cerebral hemispheres.

C – Cross-section through the telencephalon at 5 weeks shows cells of developing neocortex.

The human cerebral cortex, or neocortex as it is phytogenetically known, develops by proliferation of cells at the outer surface of the cerebral vesicles. Initially the telencephalic cells are all neuroepithelial cells, derived from the neural plate. They continue to proliferate and increase both the surface area and depth of this initial layer of the cerebral cortex which is called the ventricular zone (VZ). At this point, all replications are symmetrical and the VZ is composed entirely of pseudostratified epithelial cells (Bystron et al. 2008).

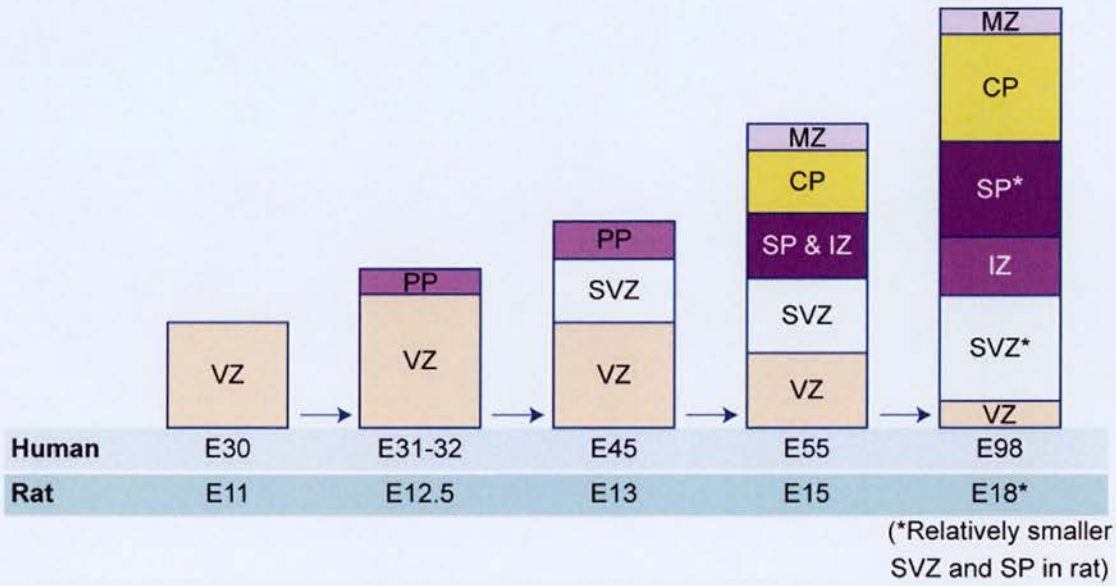

Figure 2 – Cerebral Cortical Expansion
VZ – ventricular zone. PP – preplate. SVZ – subventricular zone. SP – subplate. IZ – intermediate zone. CP – cortical plate. MZ – marginal zone. Embryonic day in humans determined from first day of mother's last menstrual period. Embryonic day in rats determined from day of conception.

The Preplate (PP)

As shown in Figure 2, in humans at embryonic day 31 (E31), there is evidence of a complex layer of cells developing dorsal to the VZ. This is called the preplate and the function of the cells in this layer is largely unknown. There is evidence that these

cells may provide a guidance scaffold for developing cortical cells. In addition, a specific subtype of preplate cells, called Cajal-Retzius cells, produce a protein called reelin, thought to regulate the formation of the cortical layers (Tissir et al. 2003). At E50-51 the cortical plate, the precursor to neocortex, develops within the preplate and splits the preplate into an upper zone called the marginal zone which becomes layer I of the cortex, and a lower zone which develops into the subplate (SP).

The Subplate (SP)

The SP is initially a thin region with a small number of cells present before the cortical plate forms. It thickens considerably after 9 weeks gestation. The SP has 2 main functions: 1) a temporary waiting and synaptic interaction compartment for thalamic axons intending to transmit sensory information to the cortical neurons once they have completed development in the cortical plate, and 2) SP cells have long axons which act as guides to other afferent and efferent axons in the developing cortex. The network formed by these axons is particularly elaborate in primates. The SP reaches its maximum thickness at approx. 24 weeks gestation when it is four times thicker than the CP (Kostovic et al. 2002a). Once thalamic afferents have moved on from the SP into the cortical plate, programmed cell death is initiated within the SP. The SP can still be identified at 6 months postnatal age, but only as scattered cells in subcortical white matter.

Clearly insults from 24 weeks gestation which affect the integrity of the SP synaptic interaction compartment, may have profound consequences for neural development and there are indications that hypoxic-ischaemic insults which cause selective damage to subplate neurons contribute significantly to cognitive dysfunction in preterm infants (McQuillen et al. 2003).

Neurogenesis

In humans at 5 weeks gestation, there is a pivotal molecular-driven switch directing the onset of neurogenesis (Iacopetti et al. 1999). From this point, divisions of the VZ cells first start to become asymmetrical and produce one daughter cell which remains a progenitor and another which is a postmitotic differentiated cell, committed to becoming a cortical neuron or a glial cell (Pasko 1995; Iacopetti et al. 1999) as shown in Figure 3. The cells which remain as neural progenitors (also called neural stem cells) have multipotent as well as self-renewing capabilities (Price 2011).

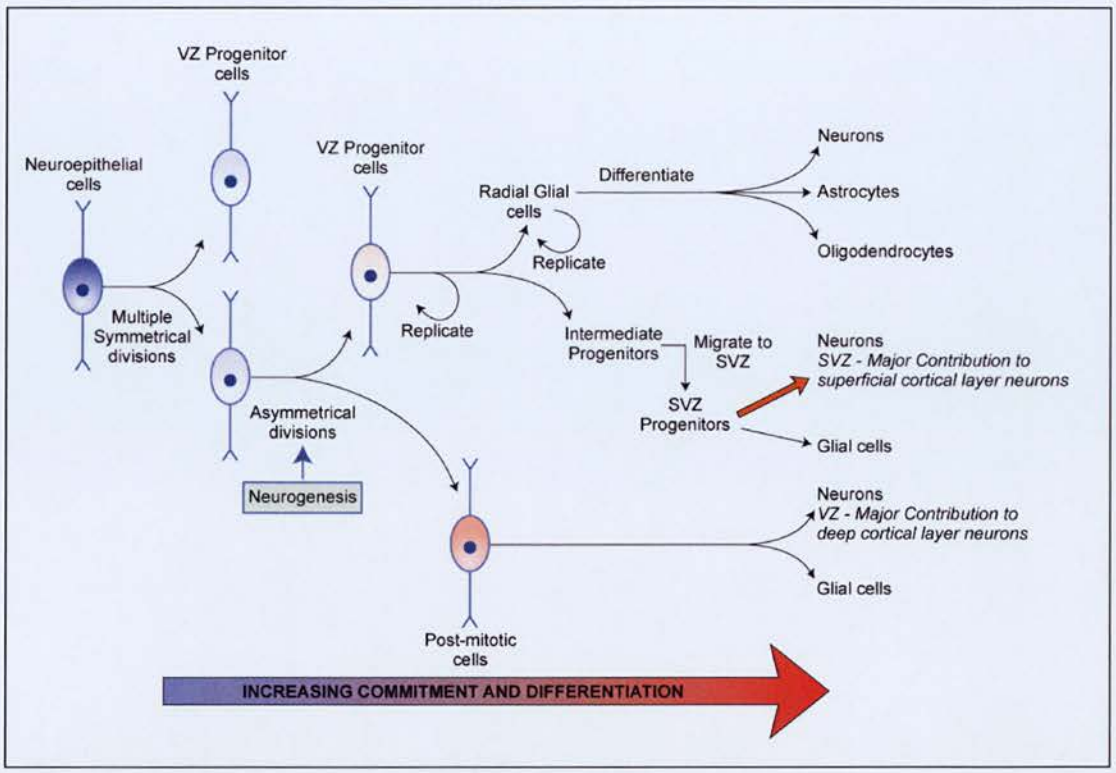

Figure 3 – Neuroepithelial cell differentiation

Schematic diagram showing the proliferating cells within the VZ and SVZ and the origins of the major cell types of the brain. This illustrates some of the lines of development, however the lineage process is not fully understood. The VZ progenitors take a number of forms including Radial Glial Cells (RGCs) and Intermediate Progenitors (IPs).

Radial Glial Cells (RGCs) are an important subtype of neural progenitor in the VZ. These cells divide to produce both further precursors and a number of differentiated postmitotic cells (Mo et al. 2007) as shown in Figure 3. In addition it has been demonstrated that these cells may also function as a 'migratory scaffold' to direct cortical neurons to their laminar fates (Rakic 2003).

The Intermediate Progenitor (IP) cell also develops from VZ progenitor cells at E40-41 (Bystron et al. 2008). These IP cells migrate to the dorsal aspect of the ventricular zone and form a new layer of cells called the Subventricular Zone (SVZ) at 7-8 weeks gestation, refer Figure 2. These cells proliferate and also function as progenitors for postmitotic neurons and glial cells. The SVZ grows to become particularly thick and complex in humans in comparison to other species. Proliferation continues until 26-27 weeks gestation by which time the VZ is a thin layer of cells. In humans, the SVZ is the major source of cortical neurons, particularly those neurons that are later born and migrate to the superficial layers (Zecevic et al. 2005; Bystron et al. 2008).

The Cortical Plate

The first cells accumulating to form the cortical plate are visible in the rostral telencephalon preplate from 7-8 gestational weeks (Bystron et al. 2008; Sidman RL, Rakic P, 1973). These neurons migrate radially from the ventricular zone and take their position in an 'inside out' pattern such that neurons destined for layer VI take position first and then successively committed neuronal subtypes from the VZ and progressively the SVZ, destined for layers V, IV, III and II take their positions in a deep to superficial direction, see Figure 4.

Neurons within the deep layers of the cortex, layers V and VI, are large projection neurons whose axons project to subcortical targets such as the thalamus, striatum,

pons, spinal cord and the other cerebral hemisphere (Molnar et al. 2006). Layer IV neurons receive the major sensory information from the thalamus. The superficial cortical layers (II-IV) contain smaller neurons that form more localised intra-cortical connections (Gilbert et al. 1979). Each cortical layer can contain several neuronal subtypes, expressing specific marker transcription factors (Molyneaux et al. 2007), discussed further in section 1.5.3.

The peak period of cortical neuronal migration is between 12 and 20 weeks gestation. However migration for the most superficial layers continues during the third trimester (Uylings 2000). As shown in Figure 4, migration is succeeded by further neuronal cell development including synaptogenesis which involves the development of dendritic spines and the formation of synapses between neural cells (Kostovic et al. 2002; Kostovic et al. 2007). After synapsing on subplate neurons, axon terminals from thalamic, callosal, and association fibres enter the cortex and together with the neuronal cell development, contribute to a four-fold increase in cerebral cortical volume between 28 and 40 weeks gestation (Huppi et al. 1998).

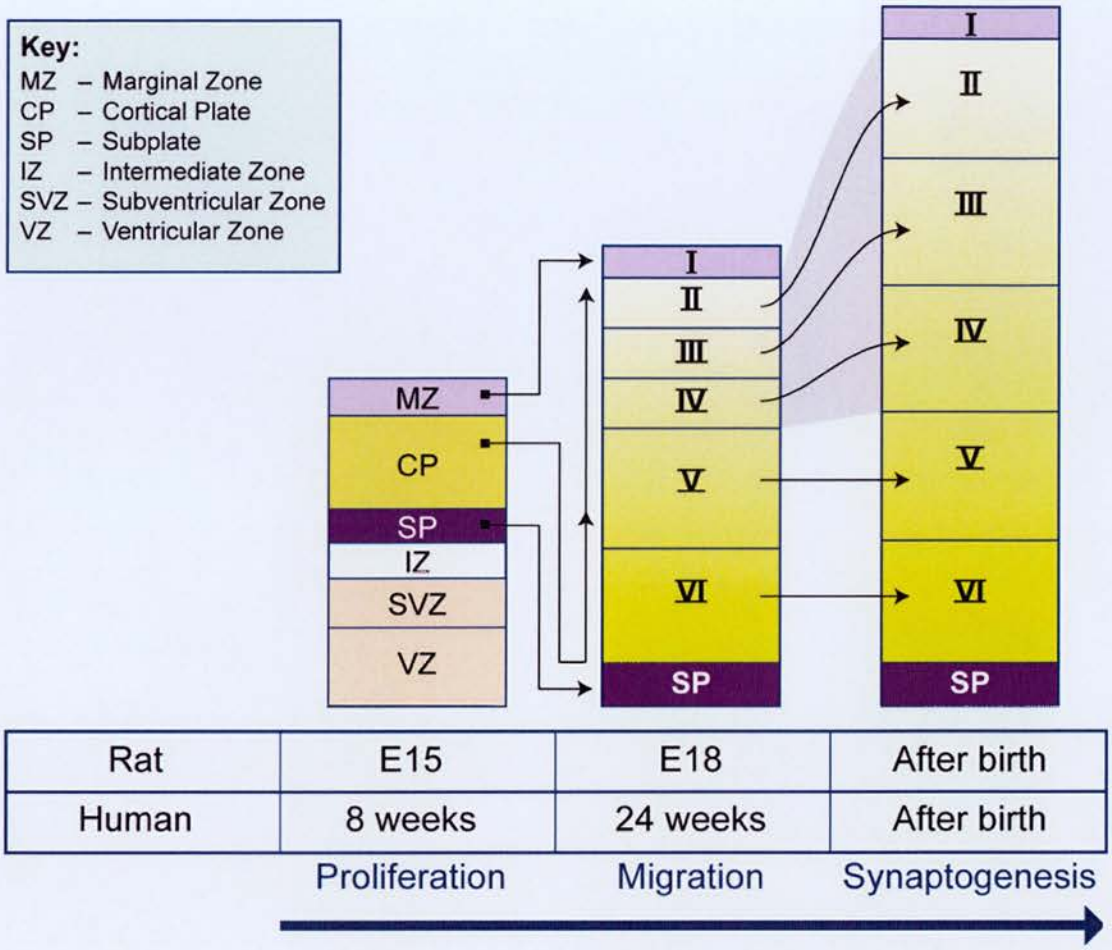

Figure 4 – Schematic Illustration of cortical development in rats and humans

Migration to deep layers V and VI is complete by 24 weeks gestation and synapses begin to appear as thalamocortical axons exit the subplate and enter the cortex at this time (Kostovic et al. 2006; Bystron et al. 2008). The superficial layers undergo a disproportionate increase in thickness in humans and this is partly due to late arriving GABA-ergic interneurons, critical for cortico-cortical communication (Letinic et al. 2002; Tan 2002). Concurrently, neurons within superficial layers II to IV continue to develop dendrites and synapses. Furthermore, arealisation of the cortex and final differentiation of specialist neuronal cell subtype function will also occur during the third trimester.

It therefore follows that for an infant born preterm at 23-24 weeks gestation, significant ongoing cortical developmental processes may be vulnerable to disruption caused by premature birth and neonatal intensive care practices.

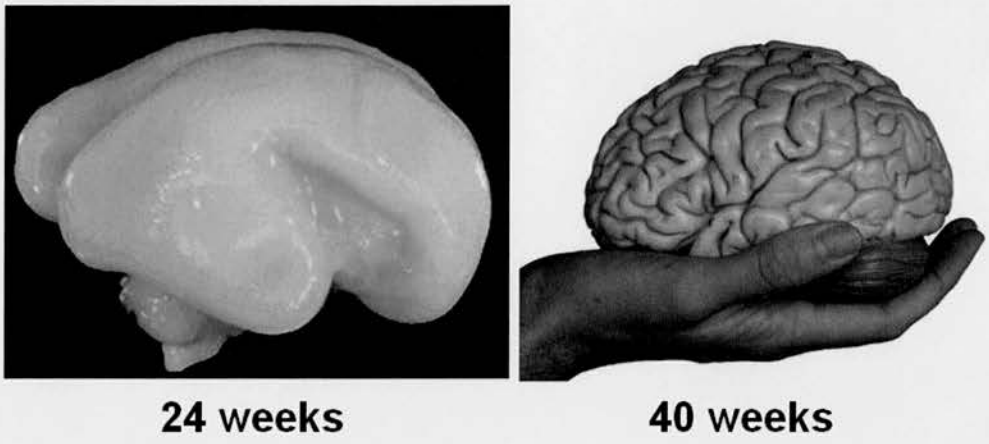

Figure 5 – Human brain at 24 weeks gestation and 40 weeks gestation

The cortex of the human 24 week gestation brain is smooth. The expansion of the superficial layers in the third trimester underpins the extensive increase in surface area and the development of gyri and sulci, illustrating the significant degree of development and maturation between 24 and 40 weeks which is vulnerable during neonatal care. The brain at 40 weeks morphologically resembles the adult brain.

Photographs supplied with kind permission from Dr JC Becher.

1.5.2 Similarities and differences in rodent cortical development

The species where cortical development has been most well described and understood is the rodent. The main sequence of events is broadly similar to humans; the cortex develops from proliferating ventricular zone cells at the outer surface of two lateral vesicles of the telencephalon and the six layered cortical plate forms in a deep to superficial direction. Significant similarities and differences are discussed below.

A - The SVZ and SP

Both the SVZ and SP are not as large in rodents as in the human. In general, rodent precursors for the deep cortical layers originate in the ventricular zone (VZ) and precursors for the superficial layers originate in the subventricular zone (SVZ). In humans, the SVZ is the main cell proliferating layer from mid-gestation and so gives rise to the vast majority of the late born superficial layer neurons which are involved in localised intracortical connections, as described above. It is also however, thought to be more complex than this in humans (Bystron et al. 2008). The SVZ also gives rise to subplate neurons and some interneurons throughout the third trimester (Zecevic et al. 2005). By contrast, all cortical interneurons in rodents originate in the medial ganglionic eminence. Furthermore, oligodendrocytes, glia and astrocytes are also generated, proliferate, migrate and differentiate within the SVZ, providing significant opportunities for interaction between all these cell types (Jakovcevski et al. 2005). The significance of the large SVZ containing numerous different cell

types means that insults to this area during prenatal development may have significant effects on a number of neuronal functions.

The large SVZ in humans generates the large SP. As described above, the SP acts as a temporary synaptic compartment and the SP neuronal axons guide other afferent and efferent axons. The smaller rodent SP may reflect fewer requirements for complex connectivity.

B - Neurogenesis

Gestation in a rat is 21-22 days from day of conception. The onset of neurogenesis in rodents is E10 (Garcia-Moreno et al. 2007; Rakic 1995), midway through gestation and continues until E18. Neurogenesis in humans begins at around 5-6 weeks gestation and continues throughout gestation, slowing around 18 weeks (Uylings 2000). This reflects an extended period of neuronal formation during human fetal development. Each cortical progenitor undergoes more divisions in the human, amplifying cellular numbers and generating a much larger cortex (Uylings 2000).

C - The Developed brain: Rodent Vs Human

The adult rat brain is lissencephalic whilst that of the term neonate has extensive gyri and sulci to support a much enlarged cortical surface area. This occurs concurrently with substantially expanded underlying upper cortical layers in all mammals, most impressively humans, and it is thought these thicker superficial cortical layers underpin enhanced cognitive function.

In the adult mammalian brain, including humans, there are small populations of neuronal precursor cells in the remaining subventricular zone and in the dentate gyrus of the hippocampus (Gage 2000) that can give rise to new neurons and glia. These neuronal precursor cells are important to learning and memory and contribute to recovery from neuronal injury.

1.5.3 The Control of Cortical Development

Newly generated multipotent precursor cells will eventually commit and develop into a neuronal subtype as shown in Figure 3. Particular transcription factors, specific to neuronal subtype and function, are expressed in neural cells (Molyneaux et al. 2007). Transcription factors are proteins that bind to specific regions of DNA (promoters and enhancers) and alter transcription of mRNA and therefore alter gene expression. They may achieve this by, for example, recruiting histone-modifying enzymes that alter chromatin and make conditions more favourable for transcription, or, by recruiting kinases that phosphorylate RNA polymerase, activating RNA synthesis (Price et al. 2011).

Some transcription factors may only be expressed in a particular projection neuron or interneuron at a discrete developmental time and therefore direct a specific developmental event. A differentiated neuron has restricted potential from birth as the subtype it will become is influenced by the transcription factors expressed in its progenitor (Molnar et al. 2011). These early transcription factors influence the key cellular events in cortical development; proliferation, migration, differentiation, dendritic morphology, synaptogenesis and neurotransmitter release. Different transcription factors can be expressed in neural progenitors at different times in development, allowing such progenitors to give rise to a number of different post-mitotic neural types including projection and interneurons, astrocytes and oligodendrocytes (Dominguez et al. 2012). As in Figure 3, early progenitors have a higher multipotency and late born progenitors are more restricted in their fate. As

indicated previously, many of the intricacies of cortical development are not yet understood and remain the focus of ongoing research.

1.6 Fetal brain growth

The growth and development of the brain from conception to birth involves a complex interplay of neuronal proliferation and migration, growth and extension of axons toward target zones and development of dendrites, synapses and communication networks between neurons. Neuronal development of this nature is highly dependent on the surrounding environment and in particular on the presence or absence of trophic factors. Many trophic/growth factors are involved in overall brain growth. Four key factors have been selected for this study; (1) Insulin-like growth factor-I, (2) Brain Derived Neurotrophic Factor, (3) Fibroblast Growth Factor and (4) Vascular Endothelial Growth Factor. The following review summarises the literature surrounding the role of each of these factors in brain development. Further details of literature surrounding the effect of specific perinatal factors on each of the factors are given in the introduction to Chapter 7.

1.6.1 The Insulin-Like Growth Factor (IGF) system

The Insulin-Like Growth Factor (IGF) system is key to several aspects of nervous system development and is expressed in the rodent brain from embryonic day 14 (E14). Peak levels in the cerebral cortex are seen at postnatal days 8 and 13 (Bach et al. 1991). The two peptides of the IGF system, IGF -I and IGF-II act predominantly through the IGF-I receptor (IGF-IR) and their actions are modulated by six IGF binding proteins that can either inhibit or augment IGF ligand activity (Rajaram et al. 1997). Animal experiments involving null mutations in transgenic mice have

demonstrated that the IGF system controls overall growth during intrauterine development (Baker et al. 1993). In such experiments, IGF-I and IGF-IR knock-out mice have birth weights 60% and 45% of normal, respectively (Lui et al. 1993). Within the brain, IGF-I stimulates the proliferation of neural progenitors. IGF-I overexpression results in an increased number of progenitors in the VZ and SVZ, an increased number of neurons in the cortical plate of the telencephalon at E16 and an increased number of neurons and glia in the cortex postnatally (Popken et al. 2004). It is thought to achieve this by reducing G1 phase length, thereby reducing cell cycle length and increasing cell cycle re-entry of the progenitor cells (Hodge et al. 2004). In addition, IGF-I has a neurotrophic effect in maintaining survival and inhibiting apoptosis of neurons and oligodendrocytes in rodents, therefore representing a second mechanism by how IGF-I increases neuronal cell number (D'Ercole et al. 1996; D'Ercole et al. 2002). Furthermore, IGF-I supports neuronal development by stimulating neuritic outgrowth, dendritic branching and synaptogenesis in rodent cerebral cortex (Niblock et al. 2000). Brain IGF-I overexpression results in an increased brain size (Popken et al. 2004) and IGF-I null mutant mice have restricted body and brain growth of around 60% and 30% respectively (Beck et al. 1995). The hippocampus, cerebellum and cerebral cortex are reduced in the null mutant mice in comparison to control.

In humans, deletions or mutations in IGF-I cause severe intrauterine growth restriction (Walenkamp et al. 2005), microcephaly and severe learning difficulties (Woods et al. 1996). Microcephaly also occurs in patients with IGF-I insensitivity

secondary to a heterozygous IGF-IR mutation and learning impairment ranges from severe to no impairment (Walenkamp et al. 2006). This suggests that different IGF-IR mutations cause variable quantities of functional IGF-IR.

IGF-I is produced in the liver and released into the circulation. IGF-I is able to cross the blood-brain barrier (Reinhardt et al. 1994; Pulford et al. 1999), but it is thought that local production of IGF-I is the primary source for neural cells (Russo et al. 2005). This is supported by studies confirming that IGF-I is produced by cortical and other CNS neurons (Niblock et al. 1998) and by endothelial cells of the cerebral vasculature (Sonntag et al. 1997).

Control of Insulin-like Growth Factor-I

Postnatally in humans, IGF-I influence on somatic growth is under the control of Growth Hormone (GH), the major regulating hormone of postnatal growth. However, prenatally during brain development, IGF-I is significantly less dependent on GH but is influenced by a number of growth factors which modulate biological activity and cellular responses to IGF-I as described below.

1.6.2 Brain-Derived Neurotrophic Factor (BDNF)

BDNF is part of the neurotrophin family of peptides which also includes nerve growth factor (NGF) and Neurotrophins 3, 4 and 5. Within the developing brain, BDNF plays a key role in neuroprotection. It also contributes to cell proliferation, migration, differentiation, axonal outgrowth, regulation of neurotransmitter secretion and synaptic plasticity (Rao et al. 2009).

BDNF is produced predominantly by several neuronal and glial cell populations (Radka et al. 1996). Several other tissues including immune cells and vascular endothelium produced smaller amounts of BDNF (Noga et al. 2003). All neurotrophins exert effects by binding with the tropomyosin receptor kinase family (Trk) of tyrosine kinase receptors. Specifically BDNF and NT4 interact with the TrkB receptor. BDNF is also known to increase VEGF secretion (Nakamura et al. 2006).

Preterm infants have lower circulating levels of BDNF than term infants and levels increase with increasing gestational age (Malamitsi-Puchner et al. 2004). Serum levels of BDNF increase into adulthood. Recent research suggests that serum BDNF concentration correlates with a number of antenatal and postnatal factors that influence neurodevelopmental outcome (Rao et al. 2009). This includes increased measured levels of BDNF in association with antenatal steroid use, linked to improved outcome (Roberts et al. 2006) and reduced levels of BDNF measured in

association with postnatal steroid use which, at some doses, have been linked to detrimental effects on the developing brain (Murphy et al. 2001).

Within the developed brain, BDNF plays important roles in learning and memory (Hashimoto et al. 2008). Furthermore, there is literature to support reduced circulating BDNF in patients with depression (Karege et al. 2002) and schizophrenia (Durany et al. 2001; Toyooka et al. 2002).

1.6.3 Fibroblast Growth Factor (FGF)

There are 22 FGF ligands in both humans and rodents. The ligands signal via one of 4 transmembrane receptor tyrosine kinases, Fibroblast Growth Factor Receptors 1-4, FGFR 1-4 (Johnson et al. 1993; Mason 2007). The fibroblast growth factors (FGF) have key roles in early brain patterning, cell proliferation, differentiation and synapse organisation. Members of the FGF family are expressed very early in the developing brain from involvement in neural induction (Streit et al. 2000). The FGF family of trophic factors are also involved in cortical patterning, a crucial aspect of cerebral development not studied in this thesis. The cerebral cortex of all mammals is patterned so that each area connects to a specific area of the body and within the cortex (Rakic 1988). FGF signalling is key to midbrain / hindbrain region specification as demonstrated by experiments where tissue from the midbrain-hindbrain boundary is grafted into the posterior forebrain, and FGF signalling re-specifies the forebrain tissue as midbrain tissue (Crossley et al. 1996; Chi et al. 2003; Zervas et al. 2005). Later in brain development, one of the ligands, FGF-2 has proliferative effects on precursor populations in the SVZ and hippocampus (Kuhn et al. 1997; Tao et al. 1997). FGF-2 is reported in a number of early studies to be inhibitory to differentiation of cortical progenitors and the hippocampus (Gritti et al. 1996; Qian et al. 1997). This inhibitory property could however be overridden by the addition of another tropic factor, Neutrophin-3 (NT3) in vitro which blocks cortical proliferation, enhancing cell differentiation (Ghosh et al. 1995), thus supporting the concept that extracellular trophic factors interact in regulating neuronal development. Recent studies confirm that in certain brain regions, FGF signalling directs cell differentiation. It has been shown that during rodent forebrain

development, FGF signalling stimulates release of the Foxg1 transcription factor from the nucleus leading to cell differentiation within the forebrain (Regad et al. 2007). In the absence of FGF signalling, Foxg1 remains in the nucleus and the forebrain neurons continue to proliferate undifferentiated (Regad et al. 2007). The roles FGF-2 and FGF-22 play in synaptogenesis is now appreciated as these ligands lead to presynaptic vesicle clustering and organisation in the developing synapse (Li et al. 2002).

1.6.4 Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factor (VEGF) is a crucial signalling protein involved in new blood vessel formation (vasculogenesis) and subsequent vascular development (angiogenesis) (Ruhrberg 2003). VEGF acts through one of three VEGF receptor (VEGFR) subtypes, all tyrosine kinase receptors. VEGF-stimulated blood vessel development is required for normal nerve growth in fetal development. Low VEGF expression in the developing rodent brain causes decreased angiogenesis, major defects in blood vessel formation and reduced neuronal proliferation, increased apoptosis and aberrant neuronal migration in the VZ and SVZ reflected in a decreased cortical thickness (Haigh et al. 2003).

Studies reveal that VEGF has significant nonvascular functions within the developing brain. In explanted brain cortex, VEGF enhanced neuronal survival and neurite outgrowth (Rosenstein et al. 2003). In hypoxic tissues, VEGF transcription was induced by hypoxia-inducible-factor-1 (HIF-1), activated by the dimerization of subunits HIF-1α and HIF-1β and binding to the VEGF receptors (Neufeld et al. 1999). Cerebral VEGF expression is up-regulated in hypoxic driven ischaemia in rodents (Jin et al. 2000) and in vitro, VEGF reduced associated cell death in cortical and hippocampal neurons in a model of cerebral hypoxic-ischaemia, demonstrating an important neuroprotective role (Jin et al. 2000). VEGF also promotes the survival of primary motor neurons following oxidative stress (Oosthuyse et al. 2001). Evidence also exists that blood vessel branching influences axon guidance and vice versa (Eichmann et al. 2005).

1.7 Summary

In summary, the preterm infant's brain is undergoing significant maturational processes at a point when antenatal factors may expedite preterm delivery and postnatal therapies may adversely impact this process and ultimately impair long term neurodevelopment. There is evidence that preterm infants with pre- and postnatal growth restriction are at risk of long term adverse neurodevelopmental outcome. Preterm infants who have ongoing oxygen requirements or who suffer from chronic respiratory morbidity such as BPD, have abnormal imaging at term which correlates with abnormal neurodevelopmental outcome.

1.8 Research Objectives

This study aims to examine the independent and combined effects of growth restriction (GR) and postnatal fluctuating hyperoxia ($\Delta O2$), on cerebral cortex lamination, transcription factor quantities and on the expression of growth factors within the developing rodent brain.

Chapter 2:

The Animal Model

The animals involved in this work were studied in accordance with institutional guidelines and UK Home Office legislation under the Animal Scientific Procedure Act 1986 and the Code of Practice for the Housing and Care of Animals used in Scientific Procedures, HMSO, 1989 (Project Licence PPL60/12624) and following approval by the Local Ethics Committee. All animal studies were conducted under an Animal Handling Licence (PIL 60/12624) awarded to EW (author) by the Home Office following successful completion of training and assessment by the Scottish Accreditation Board.

2.1 Introduction

There is a common agreement in the scientific world that no single animal model can replicate the human condition. The use of animals in research is however important to the development of new and more effective methods for understanding mechanisms and diagnosing and treating diseases that affect humans. Small animals such as the rat are easily housed and cared for in appropriately sized cages, have short gestation lengths, are relatively economical and are capable of producing multiple offspring. Rats have an equivalent maturation period and can be studied over a short time period. Rats share 98% of their genes with humans, equivalent to that of the mouse and pig, also common choices in studying neonatal disease.

The gross anatomy of the rat and human brain are very different. The rat brain has a smooth lissencephalic surface, whereas the human brain is gyrencephalic. At birth

the rat brain, like the human brain, is at a relatively early stage of maturation and this classifies both species as postnatal brain developers. Dobbing and Sands published the much referenced paper that illustrated the perinatal brain growth spurt of seven mammalian species (Dobbing et al. 1979). In humans, total brain weight gain as a percentage of adult weight peaks around birth. In the rat, where length of gestation is 21-22 days, the peak brain growth spurt is at postnatal day 7 (P7). By P7, all 6 layers of the cortex are complete in the rodent. The cortical anatomy at this stage has been compared as developmentally equivalent to a 35 - 36 week gestation human infant (Rice et al. 1981; Romijn et al. 1991). Similarities and differences have been discussed in section 1.5.2.

2.2 Growth Restriction

Several models have been described to induce growth restriction in rodent offspring to assess effects of intrauterine growth restriction and fetal programming, including various nutrition manipulation models, mid-gestation surgical procedures e.g. uterine artery ligation and models administering growth inhibiting substances e.g. glucocorticoids (Snoeck et al. 1990; Woodall et al. 1996; Holemans et al. 1998; Holemans et al. 2003). A nutritional manipulation model of protein restriction was used from day 15 of gestation. In each study of this work, Sprague-Dawley rat dams were fed either 18% or 9% casein isocaloric protein diet from embryonic day 15 (E15) of gestation until postnatal day 7 (P7). Both diets were of the same colour and texture. Diet compositions were supplied by Dr Simon Langley-Evans, University of Nottingham. Special Diet Services, Edinburgh University, then composed and supplied the diets. The casein protein content was checked in each batch. The properties of the diets are summarised in the following table:

Table 2 – Compositions of diets used in the animal model

Ingredients	18% casein diet (g/kg of diet)	9% casein diet (g/kg of diet)
Casein (dry acid free)	180	90
Corn oil	100	100
Cornstarch	425	485
Cellulose	50	50
Sucrose	213	243
Vitamins	5	5
Minerals	20	20
D,L-Methionine	5	5

Dam intake of the 9% casein protein isocaloric diet has been previously shown to result in significantly smaller offspring pups (Langley-Evans et al. 1994), who developed hypertension at 4 weeks of age (Langley-Evans et al. 1998) secondary to fetal exposure to higher levels of maternal glucocorticoids. Glucocorticoids are well established to impair skeletal growth (Avioli 1993; Ahmed et al. 2002; Mushtaq et al. 2002). Gestational protein restriction in rodent models also impairs uterine artery blood flow possibly through an increase in circulating angiotensin (Gao et al. 2012). In addition poor maternal nutrition of the mother in pregnancy causes reduced substrate delivery to the fetoplacental unit and a reduction in total milk volume during lactation (Rosso et al. 1980; Holemans et al. 1998). The combination of these factors therefore hampers fetal and neonatal growth of the pups in response to low maternal protein intake.

This model has also been validated in previous studies by investigating retinopathy of prematurity where pups from dams fed on the 9% diet, were on average 5% lighter at birth and 36% lighter at postnatal day 14 (Dhaliwal et al. 2011). The method is therefore simple and effective at inducing growth restriction but does not cause increased early mortality in the pups. The 9% diet during pregnancy has however been associated with reduced adult lifespan (Aihie Sayer et al. 2001) linked to a higher resting blood pressure in the 9% pups at 4 weeks of age (Langley-Evans et al. 1998). A further advantage of this method is that postnatal growth restriction can be induced by continuing the diet postnatally. This is relevant to the clinical situation where growth restricted preterm infants often fail to grow adequately (Hokken-

Koelega et al. 1995). This method induces asymmetrical growth restriction with relative brain-sparing, again reflective of the most common type of growth restriction seen in the clinical situation.

The main limitation of this model is that the aetiology underlying the growth restriction is not typical of the clinical correlate of placental vascular insufficiency although the low protein diet has been associated with decreased uteroplacental flow secondary to impaired uterine artery blood flow as described above (Gao et al. 2012).

The body and brain weight results obtained from this animal model are discussed further in the context of the literature in Chapter 4.

2.3 Postnatal Oxygen Exposure

The use of oxygen in neonatal care has been subject to much controversy over the last decade, as described in Chapter 1. Due to the nature of the lung disease associated with prematurity, the amount of oxygen required by a preterm neonate will often fluctuate. The severity of a particular infant's lung disease will influence the amount of oxygen absorbed into the circulation and transported to the brain. Preterm infants on the neonatal intensive care unit have their circulating oxygen levels monitored, directly, by arterial blood gas sampling and indirectly, by pulse oximetry or by transcutaneous pO2 monitoring (Quine et al. 2008). The oxygen delivered can then be titrated accordingly.

Many of the experimental oxygen profiles used to study the effects of oxygen on developing body systems employ high, constant levels of oxygen or step-wise fluctuations in oxygen which are not accurately reflective of what preterm infants experience in the clinical situation. An established fluctuating model was therefore used to deliver more clinically relevant doses of oxygen and examine its effects on the developing brain.

2.3.1 Background to the Oxygen Profile

The profile delivered to the rat pups in this study was adapted from the transcutaneous oxygen profile of a preterm infant cared for in a tertiary neonatal unit, Edinburgh. This oxygen profile has been used in previous work in studies of retinopathy of prematurity (ROP) and white matter injury (Cunningham et al. 1995; Cunningham et al. 2000; Sedowofia et al. 2008; Dhaliwal et al. 2011). The details regarding the particular infant have been anonymised, however, the infant had the most marked variability in oxygen levels from a cohort of 31 infants (Cunningham et al. 1995), was between 26 and 27 weeks gestation with respiratory distress syndrome and had severe ROP. The profile was recorded over the first 7 days of life and shows a fluctuating pattern of transcutaneous oxygen. Recordings were made every second and averages taken to give a value for each minute as illustrated in Figure 6. The minute-to-minute fluctuations were then adapted for use in the rodent (see below).

Many studies examining the effect of oxygen on the developing brain in the context of neonatal care use high levels of inspired oxygen, e.g. a FiO2 of 80-100%, which result in high oxygen saturations in the organism studied (Taglialatela et al. 1998; Gerstner et al. 2008; Yis et al. 2008). These studies allow conclusions to be drawn regarding the detrimental effect of high levels of oxygen on the developing brain, including the generation of reactive oxygen species and increased apoptosis observed in response to high levels of oxygen. Such profiles no longer reflect current clinical practice where a more targeted use of oxygen is employed.

Transcutaneous oxygen measurements (TcO2) correlate well with arterial oxygen partial pressure (PaO2) in very low birth weight infants (Sandberg et al. 2011). In the clinical setting, TcO2 values can be used as a measure of PaO2 and calibrated against the infant's arterial blood gases. An electrode placed on the upper right area of the thorax avoids the effect of ductal shunting and measures the TcO2. Safety concerns relating to epidermal burning (Poets et al. 1994) and relative ease of pulse oximetry as an alternative monitoring method has resulted in widespread adoption of the pulse oximeter in neonatal intensive care units. Recent studies have demonstrated that pulse oximetry readings often show a significant discrepancy with target PaO2 by underestimating the PaO2. Pulse oximetry measurements can be also be associated with a higher degree of variability than TcO2 (Quine et al. 2008).

2.3.2 Adaptations for the Rodent Model

The oxygen profile applied to the rat litters was adapted from transcutaneous recordings of the oxygen profile from the infant who developed severe retinopathy of prematurity (ROP) and has been validated in animal studies of ROP as described above. The rat pups were born at term and therefore have mature lungs in contrast to preterm infants receiving oxygen. To adjust for the difference in rat physiology, the profile was adapted as previously published (McColm et al. 2000); a rat breathing room air (21% oxygen) has a circulating PaO2 of 12.9kPa. Infants requiring oxygen have their PaO2 maintained within a range of 6 - 10 kPa; mean 8kPa, 4.9kPa lower than rat pups. Therefore, to each minute-to-minute fluctuation of the infant transcutaneous profile, 4.9kPa was added to simulate fluctuations in the rodent.

There is a linear relationship between rat pup PaO2 and inspired oxygen concentration (FiO2) (Penn et al. 1995). The transcutaneous profile was transformed into FiO2 values, see Figure 6. By altering the inspired oxygen concentration in this way, the blood oxygen profile recorded in the preterm infant who developed severe ROP can be replicated in the rat pups within a specialised oxygen chamber.

Studies of ROP using this model have shown that the greatest changes in retinal vessel formation were found when the mean of the simulated profile was raised from 8kPa to 10kPa (McColm et al. 2004), the upper limit accepted in the neonatal clinical setting. The mean of the fluctuating profile applied to the rat pups was therefore 14.9kPa and this hyperoxic profile was the one used in this work. The mean inspired oxygen concentration delivered was 24.9% and ranged from 12.57% to 44.86%. The litters spent 85% of the 7 days receiving additional oxygen, 4.1% in air and 11% hypoxic.

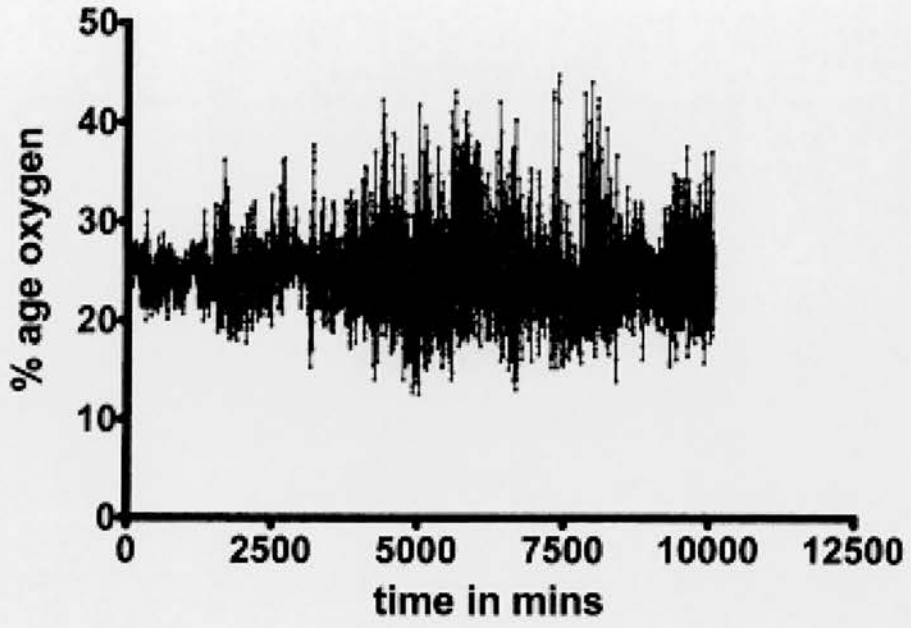

Figure 6 – Oxygen Profile

Y axis shows the percentage oxygen delivered to the rodent chamber. X axis shows time in minutes for 7 days (10,080 minutes). Recordings were made every second and plots show average value for each minute.

The main strength of this oxygen profile is that it is physiological and developed from a preterm infant with significant neonatal morbidity. Previous work has shown that the fluctuations can be more damaging to the developing brain than constant hyperoxia (Loeliger et al. 2006; Ratner et al. 2007). In addition, this profile has been validated for study in the brain where the fluctuating profile was associated with markers of cerebral injury (Sedowofia et al. 2008).

A weakness of the model is that the profile used may rest in higher oxygen concentrations than typically observed in the clinical situation. Recent evidence however suggests that maintenance of oxygen saturations within a higher targeted range (90-95%, equivalent to 5.9-8.9 kPa) is associated with significantly less mortality than a lower targeted range and there is evidence that this has already influenced neonatal practice (Stenson et al. 2011). Where TcO2 monitoring is used in neonatal units, published recommendations advise limits of 6.7 – 10.7kPa (American Academy of Pediatrics Guidelines, 1997). In addition, arterial oxygen saturations were not confirmed during the experiments. Previous researchers had confirmed that rat PaO2 and FiO2 were linear (Penn et al. 1995) and the rodent pups were term born with no reason to suspect abnormal lung function.

2.3.3 The Complete Experimental Circuit

The complete experimental circuit used in the studies is shown below. The computer controlled delivery system for the oxygen profile, illustrated in Figure 7 was designed by McColm and Cunningham (McColm et al. 2000) and developed by Biospherix, Redfield, New York, USA.

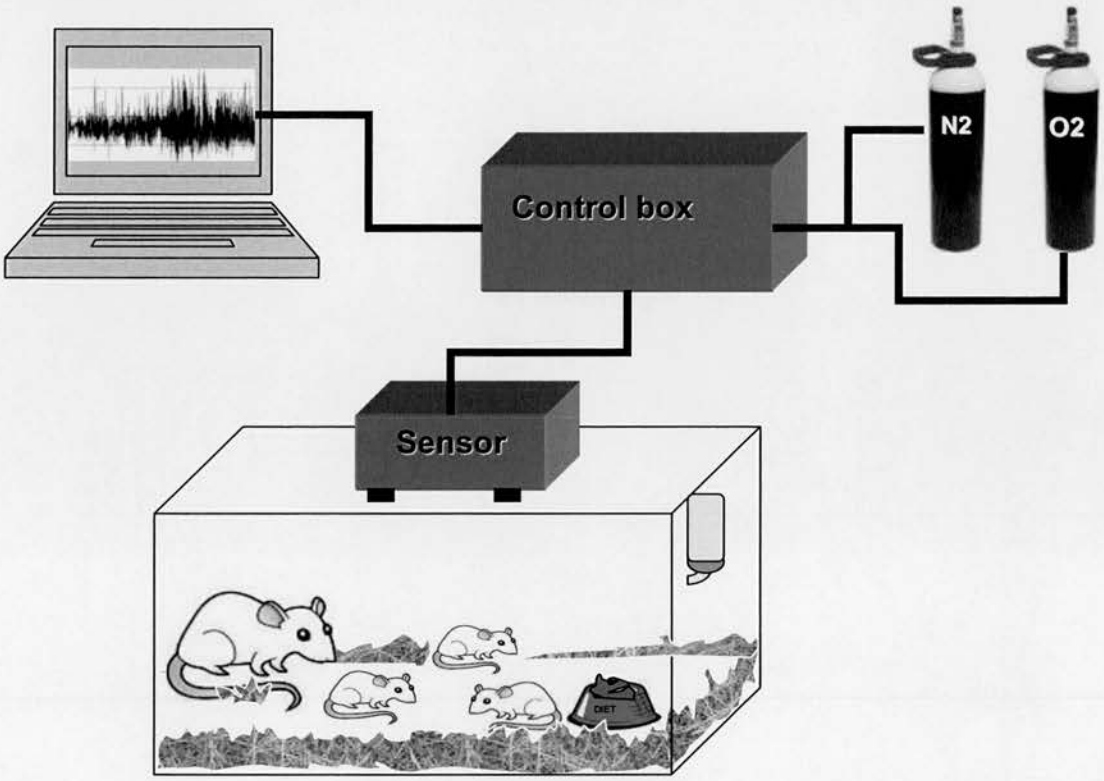

Figure 7 – The computer controlled system for delivery of the oxygen profile.

The oxygen profile determined how much atmospheric oxygen was delivered to the rats in a closed circuit, monitored cage. Oxygen and nitrogen gas were delivered to the chamber in quantities determined by the oxygen profile and the control box.

Oxygen and nitrogen gases are released into the specially designed closed animal chamber on a minute-by-minute basis to cause the required change in atmospheric oxygen as prescribed by the oxygen profile. Accurate changes up to 50% oxygen

within 1 minute were possible in the rat cage (McColm et al. 2000). To maintain the carbon dioxide at normal atmospheric levels, soda lime (Medisorb GE healthcare, UK) was placed in the oxygen chamber.

2.4 Study Groups

From E15 of gestation until P7 rat dams were fed 18% or 9% casein protein diet. From birth until P7, litters were allocated randomly and reared in air or fluctuating hyperoxia. Four study groups were formed and the conditions of each group are summarised in Table 3.

Table 3 – The Study Groups

Group	Dam protein feed (E15-P7)	Pup Growth	Environment Litter Reared
1. Control	18%	Normal	Room Air
2. Oxygen Fluctuation (ΔO2)	18%	Normal	Fluctuation Oxygen
3. Growth Restricted (GR)	9%	Restricted	Room Air
4. Dual Exposed (ΔO2+GR)	9%	Restricted	Fluctuating Oxygen

The following diagram summarises the animal model, incorporating both the growth restriction and fluctuating oxygen elements of the study:

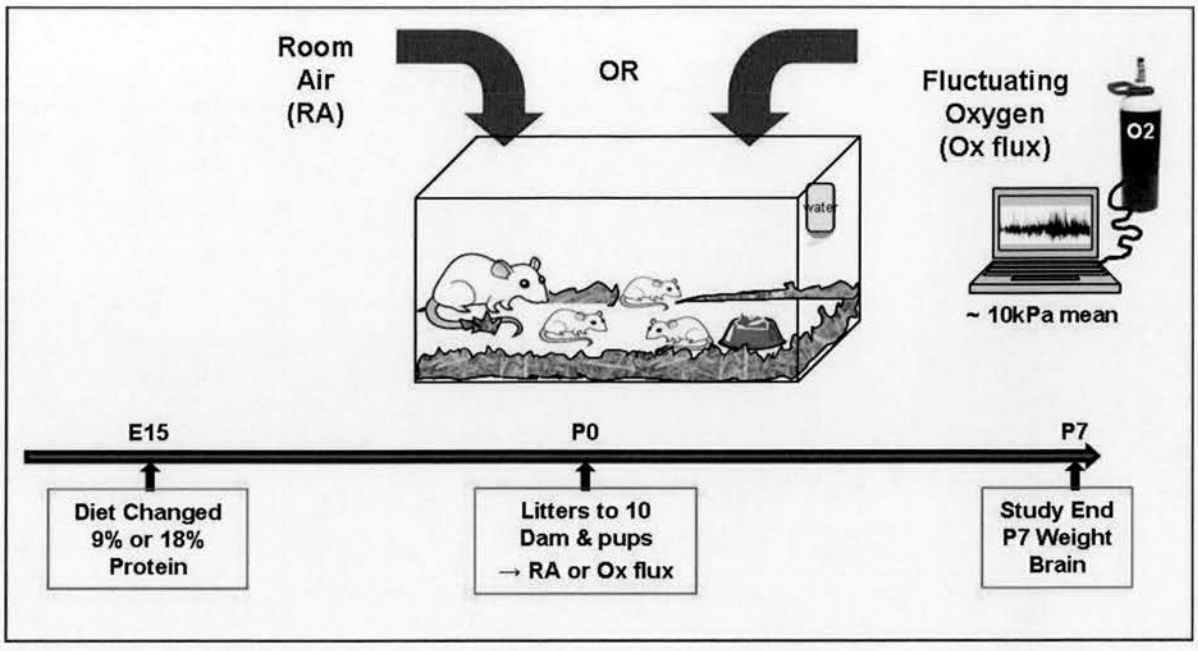

Figure 8 – The Animal Model

Until E15 all rat dams were maintained on standard animal house chow. Diet was changed at E15 and continued until the experimental end at P7. Litters of 10 pups with dam were placed in room air or oxygen chamber immediately after birth until P7.

The room air chamber was placed next to the closed circuit oxygen chamber to ensure that noise exposure was similar and all animals underwent 12 hour light/dark cycle. The room temperature was maintained at 21°C. Immediately after birth, litters were limited to 10 pups of equal numbers of males and females. The pups were weighed; their paws tattooed with Indian ink for identification and placed with their mother in the room air or oxygen chamber.

2.5 Collection of Tissue

2.5.1 Serum Collection

At P7, all pups were weighed and anaesthetized by intraperitoneal injection of 0.2ml Sodium Pentobarbital, 54.7mg/ml (CEVA Santa Animal). The thoracic cavity was opened by a vertical incision and blood 30-40 microlitres (μl) was aspirated from the left ventricle using a 25 gauge orange needle and a 1ml syringe. Blood was pushed into a labelled eppendorf with the needle removed to avoid inducing haemolysis.

2.5.2 Brain Tissue Collection for Immunohistochemistry

Five pups from each litter then underwent perfusion with 20ml 4% Paraformalydehyde fixative (Sigma Aldrich) and then an intracardiac injection of 0.2ml Sodium Pentobarbital 200mg/ml (CEVA Sante Animal). The head was removed and whole brain was dissected from the skull with olfactory bulbs attached and rotated in 15mls of 4% Paraformaldehyde for 24hours at 4°C. The brains were later used in staining and immunohistochemistry studies of the cerebral cortex (Chapters 5 & 6).

2.5.3 Brain Tissue Collection for Molecular Biology

The other five pups from each litter were perfused with 20 mls of saline/PBS (phosphate buffered saline 1:1000). These pups had an intracardiac injection of 0.2ml Sodium Pentobarbital 200mg/ml (CEVA Sante Animal) before removal of the brain from the skull. These brains were placed on a clean, RNAse free cold surface and cut into 5 sections: the olfactory bulbs, anterior and posterior cerebrum, brainstem and cerebellum as shown in Figure 9. These were placed in pre-labelled sterile eppendorfs and kept on dry ice (approx. -60°C). These brains were later used for molecular biology studies of cerebral growth factors (Chapter 7).

2.6 Tissue Processing

2.6.1 Serum Samples

Whole blood was allowed to clot at room temperature and then centrifuged at 3000rpm for 20mins. Serum was carefully pipetted into clean eppendorf and the centrifuge cycle was repeated. Supernatant serum was again carefully pipetted into clean eppendorf and stored at -80°C until analysis.

2.6.2 Brain tissue for Immunohistochemistry

The 4% paraformaldehyde perfused brains for immunohistochemistry were rotated in 15mls 4% paraformaldehyde at 4°C for 24 hours. The brains then underwent 2 washes in PBS and were cryoprotected by initially soaking in 10% sucrose for approximately 1 hour until the brain sank. The lower concentration of sucrose was initially used, and then brains were soaked in 20% sucrose for 24 hours. The cryoprotection in sucrose prevents the formation of ice crystal artefact which is important in the brain where the cellular grey matter freezes at a different rate to the lipid white matter. The initial soak in the lower concentration of 10% sucrose prevents osmotic shock caused by high water content secondary to a high diffusion gradient. The brains were then frozen in 2-methylbutane at -30°C. Temperature was monitored by a low reading thermometer and careful titration with dry ice. Once frozen, the brains were stored at -80°C until use.

2.6.3 Brain Tissue for Molecular Biology

The saline perfused brains which were cut into the 5 sections shown below were stored at -80°C until thawed for molecular biology analysis (see Sections 3.1-3.4). Attempts had been previously made to dissect out discrete regions of the brain, e.g. hippocampus, thalamus, hypothalamus. Due to the excessive friability of the P7 brains, these dissections could not be accurately replicated.

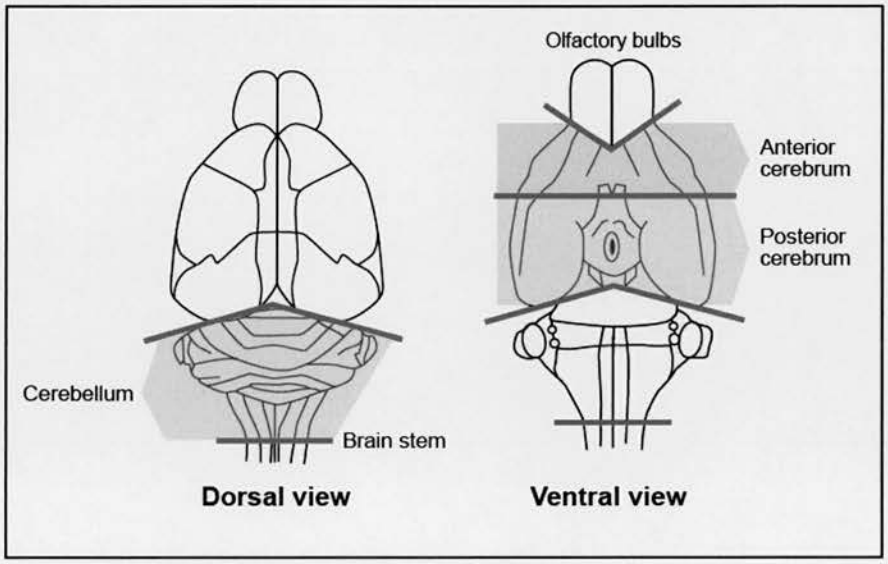

Figure 9 – Cut positions in sectioning brain tissue for molecular biology

The red lines represent the cuts made from a dorsal and ventral perspective to obtain the 5 sections: olfactory bulbs, anterior cerebrum, posterior cerebrum, brainstem and cerebellum.

The brains were carefully cut with a fresh blade per brain into 4 sections for further analysis:

1. *Anterior Cerebrum* – containing rostral cerebral cortex, partial dorsal hippocampus, and rostral corpus callosum.
2. *Posterior Cerebrum* – containing caudal cerebral cortex, thalamus, hypothalamus, partial midbrain, majority of hippocampus and caudal corpus callosum.
3. *Brainstem* – containing partial midbrain, pons and medulla.
4. *Cerebellum*.

Chapter 3:

General Methods

3.1 Ribose Nucleic Acid (RNA) Extraction

Obtaining high quality intact RNA is the first step in successful quantitative real-time Polymerase Chain Reaction (qPCR) for detection and measurement of specific gene expression.

The four brain sections described in Section 2.6.3 were stored at -80°C until analysis, and then thawed gradually to room temperature.

Anterior Cerebrum – containing rostral cerebral cortex, partial dorsal hippocampus, and rostral corpus callosum.

Posterior Cerebrum – containing caudal cerebral cortex, thalamus, hypothalamus, partial midbrain, majority of hippocampus and caudal corpus callosum.

Brainstem – containing partial midbrain, pons and medulla.

Cerebellum.

To ensure optimal RNA yield, the tissue was weighed and the anterior cerebrum cut into 2 sections and the posterior cerebrum, 3 sections, to ensure each section of tissue weighed less than 100g, the limit of the RNeasy spin column binding capacity. RNeasy Lipid Tissue Minikits (Qiagen Sciences, Maryland, USA) were used to perform phenol-choloform RNA extraction using the Qiagen standard protocol.

3.1.1 Disruption and Homogenisation

Simultaneous disruption and homogenisation of the tissue was achieved by adding QIAzol Lysis Reagent (Qiagen Sciences, Maryland, USA) and stainless steel beads to the tissue which was placed into the TissueLyser (Qiagen, manufactured Retsch) at 25Hz, 2x2 minutes to ensure complete disruption of cell plasma membranes and organelles. The lysis buffer and beads homogenize high molecular weight cellular components including genomic DNA. The QIAzol Lysis Reagent (Qiagen Sciences, Maryland, USA) is composed of guanidine thiosulphate and phenol to optimise lysis of fatty tissues including the brain. Guanidine thiosulphate is a chaotropic agent and therefore disrupts and denatures the structure of proteins including RNAses. Following lysis, bromochloropropane was added to each tissue sample before centrifugation. This resulted in phase separation of an upper aqueous phase, a lower organic phase and a middle white interphase. The upper aqueous phase contains RNA and the lower organic phase contains protein dissolved in phenol and lipid dissolved in chloroform.

3.1.2 Isolation of RNA

To provide the conditions such that RNA will stick to the RNeasy membrane, ethanol is added to the aqueous phase before transferring the solution to the RNeasy mini spin column. On-column DNase digestion was performed to ensure removal of residual DNA from the membrane. The RNA is then eluted from the membrane by the addition of RNase free water.

3.2 RNA Quantification and Quality Check

3.2.1 Spectrophotometer - Nanodrop

All RNA samples were analysed using the Nanodrop Spectrophotometer (Nanodrop products, Thermo Fisher Scientific, 3411 Silverside Rd, Wilmington, DE 19810 USA) to quantify the concentration of RNA and assess the purity of the sample. Readings were taken at wavelengths of 260nm and 280nm. The 260nm reading allows calculation of the nucleic acid concentration within the sample and the 280 reading indicates the amount of protein. Pure preparations of RNA have 260/280 ratios of >2.0. Samples with ratios below 2.0 may have been contaminated with protein or phenol and were excluded from further analysis.

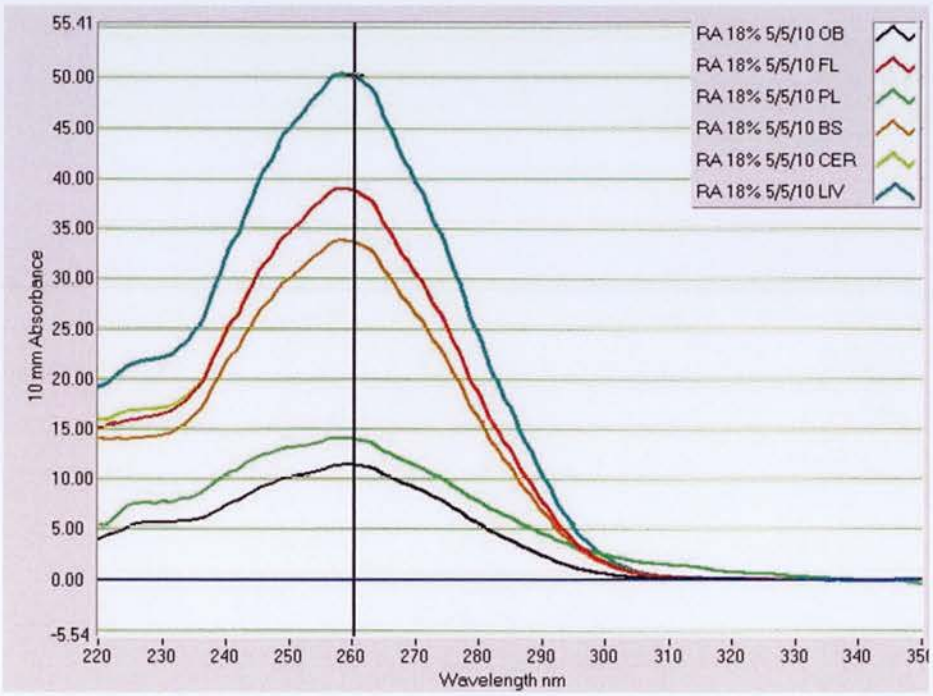

Figure 10 - Graph plot of Nanodrop

The four pieces of brain from which RNA was extracted are shown by a different colour. The olfactory bulbs (OB) and liver (LIV) were also extracted and used for optimising methodology only. The absorbance at wavelengths 260nm and 280nm are recorded to give a 260:280 ratio.

3.2.2 Agilent bioanalyser

Gene expression data can only be informative if the initial RNA to be transcribed is intact. To assess RNA integrity, the Agilent 2100 Bioanalyzer (Agilent Technologies) was used. Random RNA samples were selected from each RNA preparation batch for integrity analysis. For each Bioanalyser run, the RNA 6000 LabChip (Caliper Technologies Corporation), was first loaded with RNA 6000 Nano gel matrix, labelled with fluorescent RNA 6000 Nano dye. Thereafter, 1μl of RNA from selected samples was loaded onto the chip with Agilent RNA 6000 Nano marker. To assess the integrity of extracted RNA, the integrity of ribosomal RNA (rRNA) fragments 18S and 28S were examined in each sample as rRNA is one of the only gene products present in all cells (Smit et al. 2007). An RNA ladder with concentrations and sizes of individual base pairs preset within the assay was used as a mass and size standard. In reading the chip, the Bioanalyser uses electrophoresis to separate RNA which is then detected by laser induced detection. This gives an electropherogram (Figure 11) and a densiometry plot gel image (Figure 12). The area under the electropherogram is determined and the ladder information is used to transform the area values into concentration values.

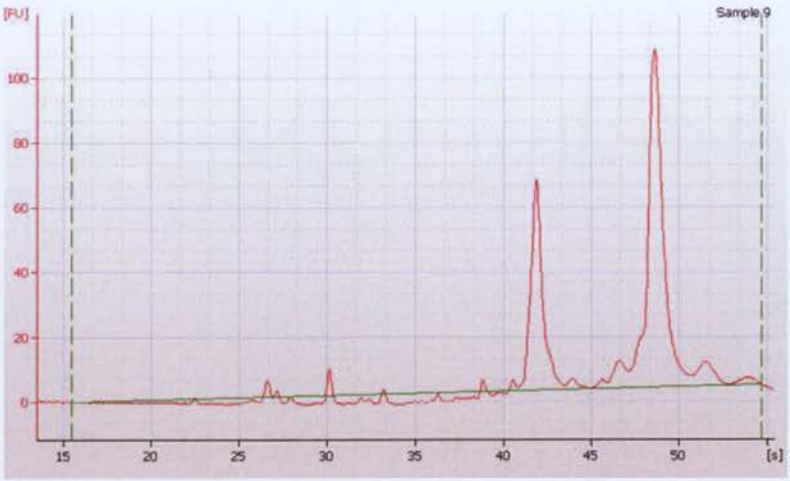

Figure 11 - Electropherogram

Fluorescence intensity against migration time (seconds). Marker, 18S fragment and 28S fragment peaks shown respectively. The 18S and the 28S fragment represent the small and large subunits of ribosomal RNA respectively. This electropherogram shows 2 clear peaks at 18S and 28S, representing intact fragments.

This ratio of fragments is calculated to give a RNA Integrity Number (RIN). A RIN score of 1 represents a degraded sample and a RIN of 10, an intact sample.

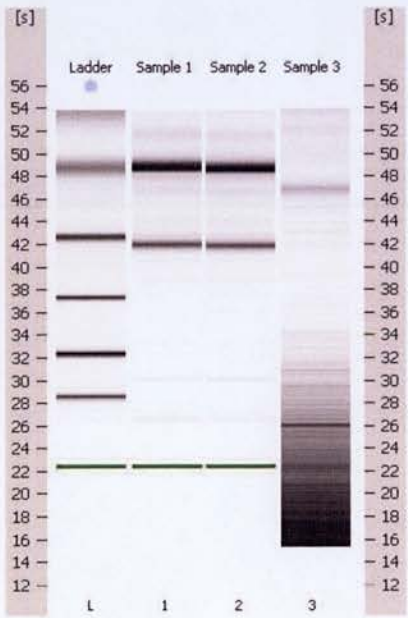

Figure 12 - Densiometry gel image

From left to right: RNA ladder, Sample 1 – RIN score 10, Sample - RIN score 10, Sample 3 - RIN score 1, degraded sample which was excluded from further analysis.

Only samples with RIN scores of 9 or 10 were used in the experiments.

3.3 Complementary DNA (cDNA) Synthesis

Reverse transcription of the extracted RNA was performed using a standard Applied Biosystems protocol (Life Technologies Corporation, 5791 Van Allen Way, California 92008).

The process of Reverse Transcription involves:

- mRNA mixed with components of Master Mix (Table 4).
- Oligo (dT) primer and random primers attach to mRNA.
- In the presence of reverse transcriptase and deoxyribonucleotide triphosphates (dNTPs), single stranded cDNA forms.

3.3.1 Laboratory Technique

Each RNA sample was diluted to 300ng/10μl using the concentration data from the Nanodrop Spectrophotometer (See section 3.2.1). For each sample, 10μl of RNA was added to an eppendorf with 6μl nuclease free water (Qiagen) and 4μl of High capacity RNA-to-cDNA Master Mix (Applied Biosystems, Life Technologies Corporation, 5791 Van Allen Way, California 92008). This Master Mix contained the components listed in Table 4. In batches of 96, eppendorfs containing the sample RNA, Master Mix and water were loaded into the same G-storm thermal cycler (Gene Technologies Ltd, Braintree, Essex, UK) and the underwent:

- 5 minutes at 25 degree Celsius (°C),
- 30 minutes at 42 °C,
- 5 minutes at 85 °C and held at 4 °C until samples were removed.

Table 4 – Components of RNA-to-cDNA Master Mix

Applied Biosystems RNA-to-cDNA Master Mix	
Component	**Role in RT Reaction**
Magnesium Chloride	Affects annealing of the primers to the template and stabilises the replication complex.
Deoxyribonucleotide Triphosphates (dNTPs)	The building blocks of DNA. Each deoxyribonucleotide has 3 parts: A deoxyribose / pentose sugar, 1 or more phosphate groups, a Nitrogenous base: either Adenine, Guanine, Thymine or Cytosine.
Recombinant Ribonuclease Inhibitor	Required to inhibit any Ribonuclease contamination and therefore prevent breakdown of RNA and provide optimal conditions for reverse transcriptase.
Reverse Transcriptase Enzyme	This is an RNA-dependant-DNA polymerase. This enzyme catalyzes the transcription of single stranded RNA to single stranded cDNA.
Random Primers	Short segments of single-stranded DNA (ssDNA) called oligonucleotides. These consist of every possible combination of bases and can therefore bind to any section of DNA thus allowing the production of many short copies of every section of DNA.
Oligo (dT) Primer	A short sequence of deoxy-thymine nucleotides tagged as a complementary primer to bind to the poly-A tail providing a free 3'-OH end that can be extended by reverse transcriptase to create the complementary DNA strand.

Each batch of samples included the following negative controls:

1. RNA negative (RNA replaced with nuclease free water - 16μl water + 4μl Master mix).
2. RT negatives (no Master Mix – 10μl RNA, 10μl water). This negative was performed for a random representative of each of the 5 sections of brain tissue.

The cDNA was then stored at -20 °C until use.

3.4 Quantitative Real-Time Polymerase Chain Reaction

Quantitative Real-time Polymerase Chain Reaction (qPCR) was used to quantify gene-specific RNA and compare expression between the control group and three study groups. TaqMan qPCR quantifies transcription of a specific gene by detecting the release of a fluorescent reporter dye. For each gene of interest, validated primers and probes for rat specific to gene of interest were used (TaqMan Gene Expression Assays, Life Technologies). Each assay contained unlabelled forward and reverse primers that recognise the target DNA sequence, and a probe that recognises a sequence between the annealing sequences of the two primers. The probe is labelled with two dyes: a 5' fluorescent reporter dye and a 3' quencher dye (Figure 13). When the probe anneals to the target DNA, the fluorescence of the reporter dye is suppressed by the quencher dye. When the target sequence is amplified during the PCR reaction, the probe is cleaved by Taq polymerase and this separates the two dyes. The quencher can no longer suppress the reporter fluorescence and fluorescence can therefore be quantitatively measured and is representative of PCR product. Detection of nonspecific amplification is avoided because fluorescence is only detected if the probe's target sequence is amplified.

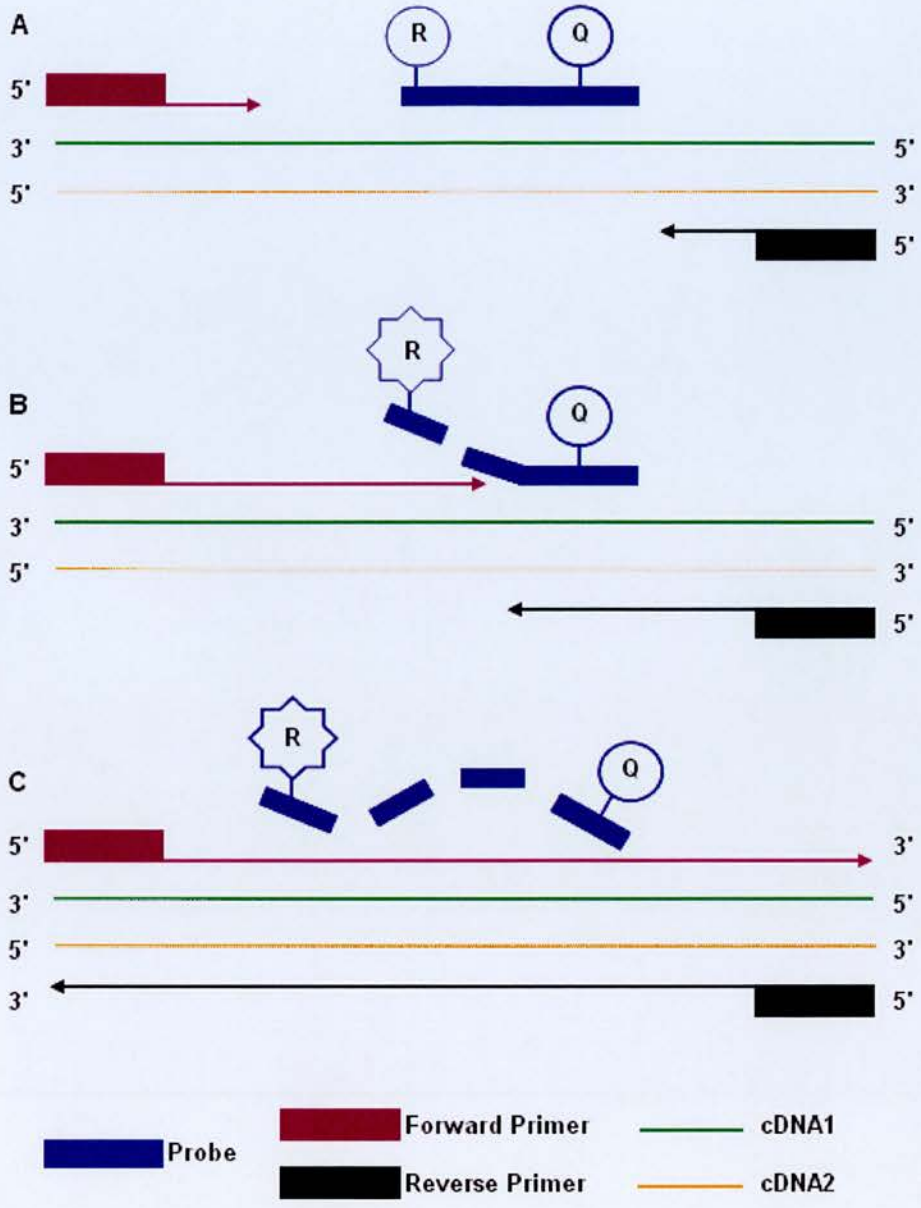

Figure 13 – Quantitative PCR reaction

1 – Polymerisation: Primers and probe polymerise to template cDNA. Quencher (Q) suppresses fluorescence of reporter dye (R).

2 – Displacement: Primer extends along template, displacing the probe.

3 – Cleavage: Probe is cleaved by Taq polymerase, separating the reporter dye from the quencher resulting in increased fluorescence of the reporter.

4 – Fluorescence: As fluorescence increases, this reflects the amount of PCR product generated and can be measured.

(Adapted from Taqman Real-time PCR Systems, Applied Biosystems).

3.4.1 qPCR Method

A reaction mix was made from Taqman Universal Master Mix containing Taq polymerase, dNTPs and optimised buffer components) with either Beta-Actin primers and probe or gene of interest (i.e. IGF-1, BDNF, VEGF, FGF) forward and reverse primers and specific hybridisation probe. Beta-Actin was used as the reference gene for each qPCR plate (further details in section 3.4.2). Samples were pipetted carefully in at least duplicate onto 384 well TaqMan Fast optical PCR plates (Applied Biosystems), with each replicate comprising of 5µl Universal Master Mix, 0.5µl gene probe, 1µl cDNA (15ng), 3.5µl RNA free water. Three negative controls were included on each plate; (i) An RNA-negative sample generated at reverse transcription (water in place of RNA) and was used to ensure specificity of reverse transcription, e.g. no contamination of the water, (ii) A RT-negative sample, also generated at the time of reverse transcription (no reverse transcriptase enzyme) was used to exclude genomic DNA contamination, and (iii) A Taqman reaction negative where cDNA was replaced with RNA free water to ensure no nonspecific amplification. Wells were covered and sealed with an optical cover (Applied Biosystems) and the PCR reaction run on ABI Prism 7900.

The PCR reaction consists of four kinetic stages Freeman et al. 1999). Firstly, a lag phase where exponential amplification is already ongoing within the PCR tube but no fluorescence signal above background is measurable. Secondly, in the logarithmic, exponential phase, there is doubling of PCR product every cycle and this is measured as a fluorescent signal released by the reporter dye. Thirdly, there is

a deceleration phase, where accumulation of PCR inhibiting factors and loss of enzyme and substrates, decelerates the reaction. Fourthly, the PCR reaches a steady state in the stationary phase and no more amplicons are produced. For data analysis, the second log phase is the most crucial.

3.4.2 qPCR Analysis

The amount of target mRNA was normalised to the amount of Beta-Actin and this value was related to the mean of control group (pups who were normally grown and exposed to room air) using the formula $2^{-\Delta\Delta Ct}$. The threshold cycle, Ct, is the cycle at which the PCR signal crosses a set threshold.

Beta-Actin was used as the reference gene for each qPCR plate. The amount of Beta-Actin in samples is constant relative to the amount of cDNA present. Beta-Actin is a highly conserved protein involved in cell motility, structure and integrity. Its use as an internal control was validated by demonstrating stable expression across the four groups (Figure 14). Concurrent measurement of the Beta-actin reference gene and gene of interest is possible by labelling with reporter dyes emitting different wavelengths. Quantification of the specific gene of interest can then be related to the abundance of the reference gene.

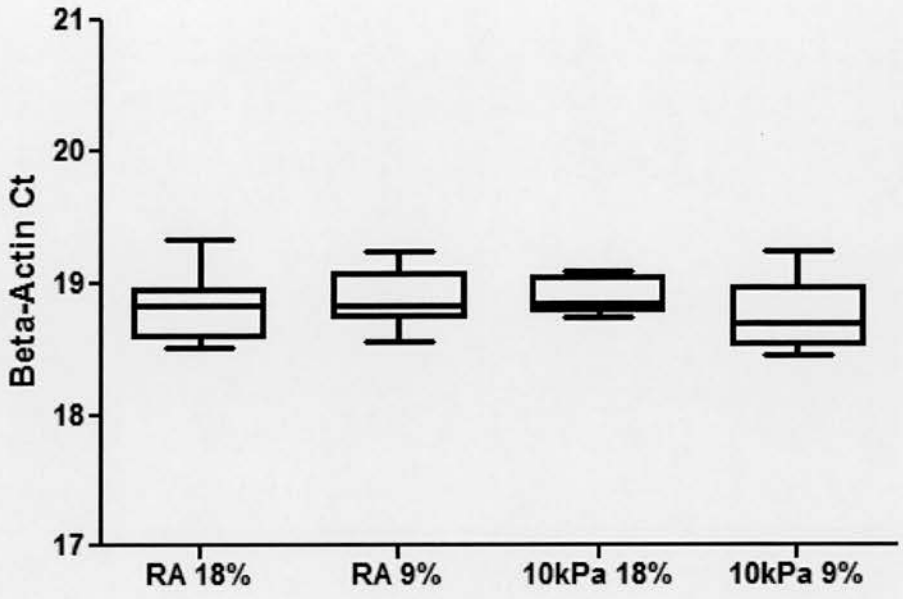

Figure 14 - Beta-Actin Ct in 4 study groups

Invariant expression of Beta-actin under each experimental condition, validating the use of Beta-actin as an internal control in all 4 regions of brain.

Another reference gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) had been considered, however this showed variation in the 10kPa 9% group as shown in Figure 15 and therefore could not be used.

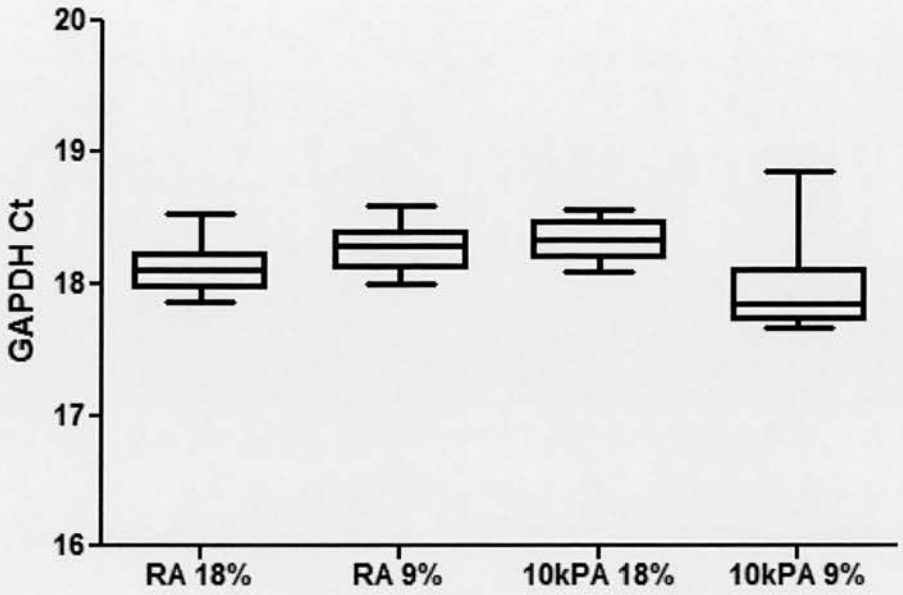

Figure 15 - GAPDH Ct in 4 study groups

Reduced expression of GAPDH in dual study group, therefore cannot be used as internal normalising control.

The difference between the Ct value for a specific amplicon and Beta-Actin is ΔCt and ΔΔCt refers to the difference between the ΔCt value of the sample and the average ΔCt of the control group. Therefore $2^{-\Delta\Delta Ct}$ is the value showing the fold difference in the amount of amplicon compared to the control group. Significance analysis was performed on raw ΔCt values. Data is plotted as fold change $2^{-\Delta\Delta Ct}$ in comparison to control group.

3.5 Enzyme-Linked Immunosorbance Assay (ELISA)

An Enzyme-Linked Immunosorbance Assay (ELISA) is used to quantify the amount of a selected antigen, in this case, protein, in a solution by comparison to a standard curve created from samples of known concentration of the protein. A sandwich ELISA was used to measure IGF-1 protein in 7d pup serum (IGF-1 ELISA kit for mouse / rat, Mediagnost, Germany) containing pre-coated plates, all reagents and buffers. All determinations were assayed in duplicate. To accurately measure total IGF-1 in the serum, an Insulin-Like Growth Factor Binding Protein (IGFBP) – blocked assay was used. Figure 16 summarises the technique.

3.5.1 Method

To dissociate IGF-1 from the IGFBP, serum samples, standards and controls were first diluted in an acidic buffer; 1 in 50 dilution, 5µl serum and 245µL buffer. A sandwich ELISA involves microtitre plates coated with immobilised 1st antibody raised against one epitope of the protein (IGF-1) to be measured. 50µl of standard, control or sample is added to each well. Following dissociation in the wells, the IGF-1 protein binds to the immobilised antibody. Wells were incubated for 1 hour at room temperature. The pH neutralises and this allowed IGF-II present in the serum to occupy the IGFBP binding sites. IGFBPs are therefore not removed but do not interfere with the assay. There is extremely low cross-reactivity between IGF-1 antibody and IGF-II. Protein not bound to the immobilised 1st antibody was then removed by washing five times on an automated plate washer with wash buffer. 50µl of the detector 2nd antibody labelled with biotin was added and binds to IGF-1 by a different epitope. Further washes removed unbound antibody-enzyme reagents.

100µl of Streptavidin bound to Horseradisch-peroxidase (HRP) was added to each well and binds to the biotin. Further washes were performed. 100µl HRP substrate (Tetramethylbenzidine, TMB) was then added to each well to detect the HRP bound to Streptavidin and biotin. During the incubation periods of 30 minutes, the Streptavidin-HRP reacted with the TMB to produce a colour reaction. The colour develops in proportion to the amount of IGF-1 bound to the immobilised 1st antibody. The reaction was stopped with sulphuric acid solution and the absorbance measured by spectrophotometer (ELISA Plate reader, Softmax Pro software).

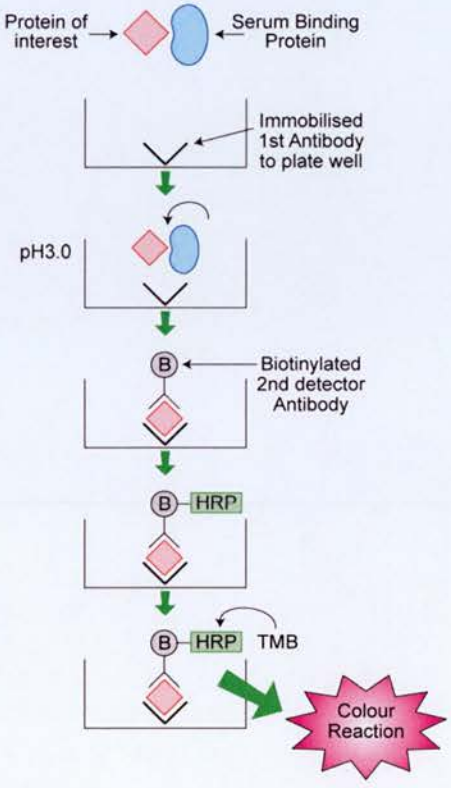

Figure 16 - IGF-I sandwich ELISA

ELISA plate wells are coated with immobilised 1st antibody to IGF-1. Standard, control or sample (IGF-I bound to serum protein IGFBP) is added to each well. Following dissociation from IGFBP, the IGF-1 protein binds to the immobilised antibody. Unbound protein is removed by washing. The biotinylated 2nd detector antibody is added and binds to IGF-I. Streptavidin bound to Horseradish-peroxidase (HRP) is then added and binds to the biotin. (Tetramethylbenzidine, TMB (HRP substrate) is then added to detect the HRP bound to Streptavidin and biotin. The Streptavidin-HRP reacts with the TMB to produce a colour reaction. The colour develops in proportion to the amount of IGF-1 bound to the immobilised 1st antibody and this can be quantitively measured.

Standard curves were constructed for each plate. Test and control samples were loaded onto the microtitre plate and analysed in duplicate. Controls were at top and bottom of the standard curve. Assays were validated by performing serial dilutions of a sample and confirming when absorbance was plotted, a linear response was obtained which was parallel to the standard curve.

3.6 Immunohistochemistry

The technique of immunohistochemistry involves localisation of an antigen within a section of tissue by a specific primary antibody. Fluorescently labelled secondary antibodies specific to the primary antibody can then be applied. Immunohistochemistry with fluorescent labelling of secondary antibodies (immunofluorescence) was used to identify specific transcription factor proteins within sections of brain tissue from the animal model.

3.6.1 Cryostat Sections

Following fixation and freezing (see sections 2.3.1 and 2.3.2), whole P7 pup brains were serially sectioned in the coronal plane at 20 micron thickness using a Leica CM 1900 Cryostat, chamber temperature -25°C, block temperature -23°C. Sections were mounted flat onto Superfrost Plus slides (Thermoscientific, Menzel-Glaser), 5-6 sequential coronal sections were mounted per slide, allowed to air dry at room temperature for 30 mins and stored at -20°C until use.

3.6.2 Immunofluorescence

Four transcription factors were selected as markers of the major neuronal subtypes in the motor cortex:

1) Transcription factor Cut-like-1 (Cux-1),

2) Special AT-rich sequence-binding protein 2 (Satb2),

3) Chicken ovalbumin upstream promoter transcription factor-2 (Ctip2), and

4) Transcription factor T-box brain1 (Tbr1).

These transcription factors and the neuronal subtype they represent are fully described in section 6.1. Cryosections at the level of the motor cortex corresponding to immediately before (for Ctip2/Tbr1) and just at the level of the corpus callosum decussation (for Cux1/Satb2) were selected and matched for each of the animal groups. Figure 17 shows the location of the motor cortex in the rodent brain.

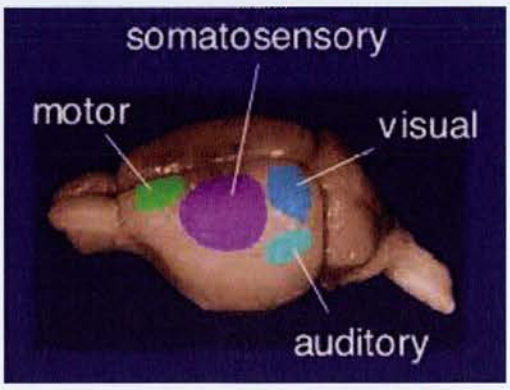

Figure 17 - Arealisation of the rodent cortex.

The P7 brain cryosections were air dried at room temperature for 30 mins and then hydrated by washing twice in phosphate buffered saline (PBS) for a total of 30 mins.

For the purposes of antigen retrieval, the sections were first placed in 0.01M sodium citrate, pH6.0 for 10 mins on a rocker platform before being microwave-treated in the same solution for a total of 20 mins to achieve optimal retrieval (1x 5 min 50% power, 1x 5min 30% power and 2x 5 min 10% power). A range of microwave power levels and timings were investigated during the optimisation of this technique. The sections were allowed to cool for 20 mins at room temperature, then washed with PBS, followed by PBS-Triton X (1:1000) for 15 mins on the rocker platform. A PAP pen was used to outline a hydrophobic circle around the sections. Blocking serum, either 20% normal goat serum or 20% normal donkey serum (Vector Labs) in PBS Triton X-100 (1:1000, Sigma-Aldrich) was applied to the sections for 1 hour at room temperature on a flat metal slide holder. Primary antibodies were as follows:

- Goat polyclonal antibody to Cux-1 (Santa Cruz Biotechnology, 1:50 concentration in donkey serum).
- Mouse monoclonal antibody to Satb2 (Abcam, 1:25 in donkey serum).
- Rat monoclonal antibody to Ctip2 (Abcam, 1:250 in goat serum).
- Rabbit polyclonal antibody to Tbr1 (Abcam, 1:100 in goat serum).

Sections were incubated overnight with primary antibody in cold room at 4°C. Following this, the sections were washed with PBS Triton X (1:1000).

To achieve fluorescent staining, the following secondary antibodies were used:

- Donkey anti-goat (Alexa Fluor-568, 1:200 in donkey serum for Cux1).
- Donkey anti-mouse (Alexa Fluor-488, 1:200 in donkey serum for Satb2).
- Goat anti-rat (Alexa Fluor-568, 1:200 in goat serum for Ctip2).
- Goat anti-rabbit (Alexa Fluor-488, 1:200 in goat serum for Tbr1).

Sections were incubated with the appropriate secondary antibodies for 1 hour. Tissue was dual stained for Cux 1 / Satb2 on the same slide and for Ctip2 / Tbr1 on the same slide and the interpretation of staining made possible due to the differing wavelengths of secondary antibody Alexa Fluor as listed above. Further washes with PBS-Triton X (1:1000) were performed with samples in darkness achieved by covering with foil. To-Pro3 nuclear counterstain (Molecular Probes, Invitrogen) diluted 1:1000 in PBS was applied to all samples for 20 mins. Cover slips were mounted with Vectashield Hard Set, Mounting Medium for fluorescence (Vector Labs). The slides were kept in darkness and stored at 4°C for at least 2 hours before transferring to the Leica Confocal Microscope (IMPACT imaging facility, Hugh Robson Building, University of Edinburgh).

3.6.3 Imaging of Immunofluorescent staining

Tissue sections images were acquired on a Leica TCS NT confocal laser scanning microscope (Leica Microsystems, Germany). FITC (Alexa Fluor 488), TRITC (Alexa Fluor 569) and Topro-3 were imaged sequentially to minimise bleed through. FTIC was excited at 488nm light and emitted photons were collected between 500-550nm. TRITC was excited at 568nm light and emitted photons collected between 570 and 625nm. To-Pro3 was excited at 647nm and signal collected above 650nm. For Ctip2 / Tbr1 deep layer nuclei, images were acquired using a x10 / 0.3 Plan Flourtar lens. Z sections were acquired at a distance of 4μm between each optical slice through the entire depth of the tissue section. For Satb2 images were acquired at x10 magnification for deep layer nuclei and x20 magnification for superficial cortical layer nuclei (Cux1 and Satb2) using the 20x/0.7 Plan Apochromat oil immersion lens with a distance of 1.2μm between optical slices in Z. Maximum intensity projections were made from each Z stack using the Leica Lite image analysis software. Adobe photoshop was used to overlay the Topro-3 nuclear staining and the nuclear staining for each of the 4 transcription factors of interest. Numbers of labelled nuclei were counted within a 100µm diameter box in the centre of the motor cortex. The height of the box was that of the cortical layers selected. A minimum of 3 sections from each brain were averaged to give each data point for the animal. The single observer (EW) was blinded to the study group. Accuracy of counts was confirmed on twenty blinded randomly selected recounts by a 2nd competent observer (PG). Inter-observer consistency was at least 95%. Cell counts and proportions were compared between each of the 4 study groups.

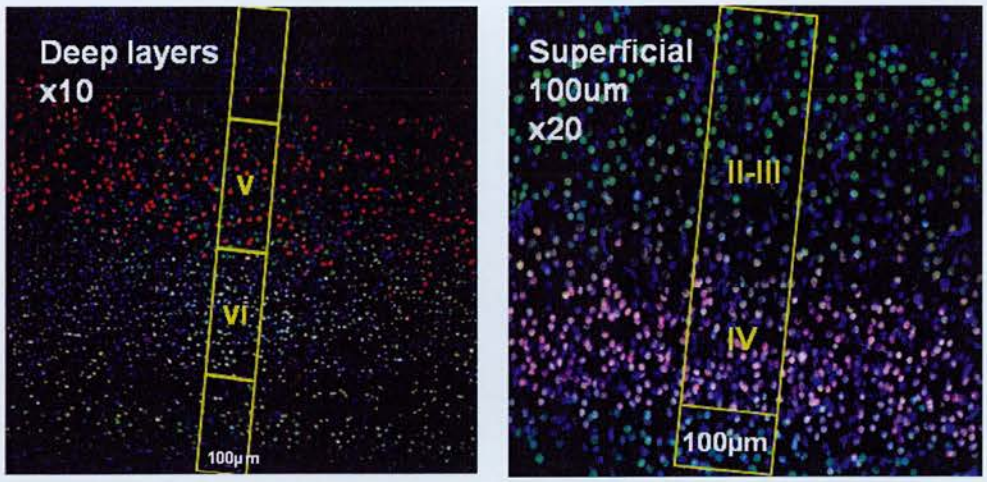

Figure 18 - Immunofluorescent labelling of rat brain sections

Deep cortical layers V & VI - images were taken at x10 magnification. 100µm box created in photoshop at x10 magnification and placed over centre of motor cortex. Blue staining is Topro3 nuclear counterstain, red is Ctip2 (layer V), green is Tbr1 (layer VI).
Superficial cortical layers II – IV - images taken at x20 magnification. 100µm box created in photoshop at x20 magnification and placed over the centre of the motor cortex. Blue is Topro-3 nuclear counterstain, red is Cux1 and green is Satb2.

3.7 Tissue Staining for Laminar & Corpus Callosal Thickness

3.7.1 Cresyl Violet Staining

Cryosections (sections 3.6.1) on slides immediately past the decussation of the corpus callosum were selected and matched for each of the animal groups. The slides were air dried at room temperature for 30 mins and then hydrated by washing twice in phosphate buffered saline (PBS) for a total of 30 mins. Cresyl Violet 0.125% solution was filtered 2 hours before the technique. The slides were placed into Cresyl Violet for 30-60 seconds then washed in running tap water for 5 mins. The brain sections were examined under light microscopy for quality of staining and returned to the Cresyl Violet if necessary. Alcohol dehydration was completed as follows:

70% alcohol – 1 min

80% alcohol – 30secs

95% alcohol with acetic acid – 15secs

100% alcohol – 2 mins with light microscope examination to ensure adequate staining. The slides were finally placed in Xylene for clearing of the dehydrant for 1-2 mins and then mounted with Pertex mounting medium (Histolab products AB, Gothenburg) and allowed to dry at room temperature.

3.7.2 Imaging of Cresyl Violet Stained Sections

Images were acquired on a bright field microscope (Olympus). Three sections per brain of the motor cortex and corpus callosum were taken at x5 magnification. Image J analysis was used to measure the thickness of the motor cortex and corpus callosum in the 3 images and an average taken to give each data point per animal for statistical analysis. Sections were blinded to a single observer (EW) and a random sample re-measured by a 2nd competent observer (JW). An inter-observer consistency rate >95% was accepted.

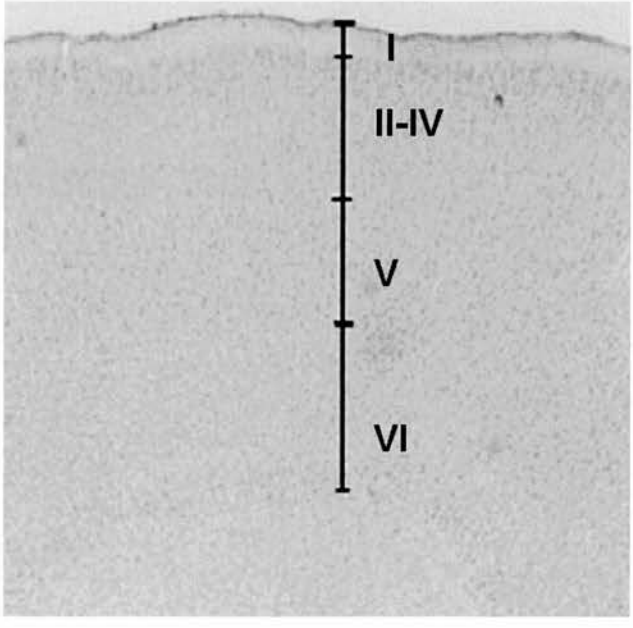

Figure 19 - Cortical thickness of motor cortex stained with cresyl violet

Left hemisphere.
Measurement line was applied in Adobe photoshop to identify accurately layers: VI, V, IV – II and I.
Measurements were made in Image J.

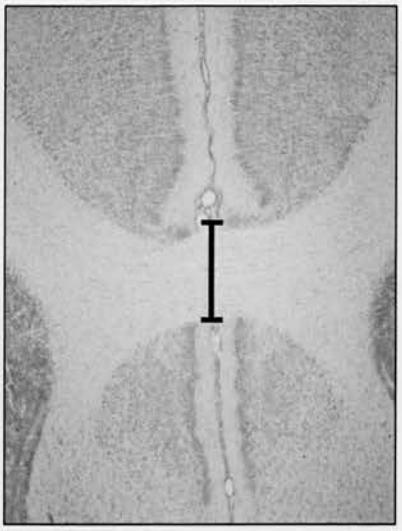

Figure 20 - Corpus callosum thickness stained with cresyl violet

Measurements obtained by image J analysis at mid-point of corpus callosum decussation.

3.8 Statistical Analysis

Continuous variables are reported as mean ± standard deviation (SD) and were analysed for normality by D'Agostino and Pearson omnibus normality test where n number would allow. Statistical analyses were performed using student unpaired t-test or 2-way analysis of variance (ANOVA) with Bonferroni post-test for multiple comparisons. The 2-way ANOVA was used to confirm the presence of variation within the dataset at statistical significance level of 5% ($p>0.05$). The Bonferroni correction for multiple comparisons was then applied. The Bonferroni correction lowers the p value considered significant to 0.05 divided by the number of comparisons, thereby ensuring that the 5% probability applies to the entire family of comparisons and not separately to each individual comparison. Non-parametric data is presented as median ± interquartile range. Calculations were made using Prism software, version 5.02.

Chapter 4:

Characterisation of the Experimental Model:

Birth weight, initial growth & brain weight

4.1 Introduction

Normal fetal growth in any mammalian species is a complex process dependent on the genetic profile of the embryo, adequate nutrient and oxygen supply via the placenta and a healthy feto-maternal environment.

A common cause of fetal growth restriction is fetal adaptation in response to an inadequate supply of oxygen and / or nutrients. A variety of animal models have been used in experiments investigating the genetic, cellular and metabolic events that determine fetal growth and the association between poor fetal growth and development of adult disease.

Nutritional models of inducing growth restriction affect the fetal nutrient supply. In this study we have used a low protein diet from E15 until P7. Used preconception, protein restriction adversely affects fertilisation and early egg development in mice (Munoz et al. 1979). When protein restriction is started mid-gestation, glucose, insulin and progesterone levels are significantly lower in mothers fed a low protein diet in comparison to control (Fernandez-Twinn et al. 2003). Litter size is not affected by low protein diet and the pups are consistently growth restricted in comparison to control. Previous work has also shown that a 9% diet is associated with a large placenta, higher circulating levels of maternal glucocorticoids and increased blood pressure in offspring at 4 weeks (Levy et al. 1993; Langley-Evans et al. 1994; Langley-Evans et al. 1998). This is consistent with observations made by

Barker in 1990 where small babies with large placentas were found to have the highest blood pressure in adulthood, suggesting circulatory changes in the feto-placental unit. This formed the basis of the Barker hypothesis where infants with low birth weights are at increased risk of developing cardiovascular disease in later life (Barker 1990). Other work has shown an association between 9% low protein diet and preterm retinal pathology where growth restricted rat pups had an increased area of avascular retina indicating that retinal vascularisation was delayed (Dhaliwal et al. 2011) in keeping with stage 1 of the pathological changes observed in preterm infants who develop ROP. Growth restricted preterm infants are more likely to develop ROP and more severe disease than appropriately grown infants (Dhaliwal et al. 2009).

The low protein diet used in this experimental model is isocaloric. Caloric restriction has been utilised in studies of intrauterine growth restriction. In rodents, caloric restriction from days E7 to E21 or from E15 to E21 does not affect length of gestation, litter size, or mortality rate but significantly reduces birth weight (Vickers et al. 2000; Ozaki et al. 2001; Jimenez-Chillaron et al. 2005). In contrast, caloric restriction from E7 to E14 does not affect pup weight (Garofano et al. 1998; Ergaz et al. 2005). Despite a reduction in body weight seen with calorie restriction continuing later in gestation, brain weight was not significantly different to the control pups by 60% calorie restriction (Agale et al. 2010). Studies have however noted that caloric restriction reduces the occurrence of late onset chronic disease and extends the lifespan in experimental animals (Fontana et al. 2004; Varady et al. 2008).

Maternal exposure to glucocorticoids has been associated with reduced birth weight and the development of hypertension in the offspring particularly when given in the latter stages of pregnancy (Nyirenda et al. 1998). As described in Chapter 2, one of the underlying mechanisms for the growth restriction caused by the low protein diet is fetal exposure to higher levels of maternal glucocorticoids which impair long bone growth and normal trabeculae formation (Avioli 1993; Ahmed et al. 2002; Mushtaq et al. 2002). These skeletal effects have been noted to normalise in the first postnatal month and catch up body weight achieved (Ornoy et al. 1972). The low protein diet has been found to cause a reduction in the placental enzyme 11B-hydroxysteroid dehydrogenase which usually regulates the transfer of maternal glucocorticoids to the fetus (Gardner et al. 1997).

Surgical interventions such as uterine artery occlusion have been used commonly for decades in animal models of growth restriction. Uni- or bilateral uterine artery occlusion induces hypoxia, hypoglycaemia and decreased growth factor availability and is associated with offspring mortality rates of up to 30% (Wigglesworth 1964). The fetuses will then undergo transient hypoglycaemia, hypoxia and acidosis in utero and long-term consequences for pups include obesity, hyperglycaemia induced by an observed reduction in pancreatic B-cell mass in postnatal life (Simmons et al. 2001) and hypertension caused by a reduction in glomerular filtration rate and effects on sodium tubular reabsorption (Alexander 2003).

The fluctuating oxygen profile used in the experimental model is described fully in Chapter 2. The main strength of this oxygen profile is that it is developed from a preterm infant with significant neonatal morbidity and replicates the rapid fluctuations commonly observed in the clinical setting. Previous work has shown that the fluctuations in oxygen are more damaging than constant hyperoxia (McColm et al. 2004; Loeliger et al. 2006; Ratner et al. 2007) and this profile has been validated for studies in the brain where the fluctuating profile was associated with markers of cerebral injury (Sedowofia et al. 2008). Reactive oxygen species are formed as a natural by-product of oxygen metabolism and can damage all components of cells including protein, DNA and enzyme systems (Maltepe et al. 2009). Highly metabolic organs such as the brain are most vulnerable to oxidative damage and preterm infants have lower antioxidant reserve (Taglialatela et al. 1998; Kuster et al. 2011). In addition, relevant to this study, growth restricted infants also have lower total antioxidant capacity (Toy et al. 2009).

It was hypothesised that antenatal and postnatal growth restriction would adversely affect body and brain weight of pups and a fluctuating hyperoxic profile would negatively impact brain growth.

4.2 Methods

The animal model, tissue collection and processing methods described in Chapter 2 were used. At P1 and P7 pups were weighed by placing in a weighing bowl and using the same calibrated digital scales (Kern 573 precision weight scale) for each measurement. Brain weights at P7 were achieved by weighing an allocated universal container of 15mls PFA only and then reweighing this container with the brain submerged.

4.3 Results

4.3.1 Body weight at birth

The mean birth weight of pups from the dams fed the 18% normal protein diet was 6.00g (SD ± 0.68) and the mean birth weight of pups from the dams fed the 9% protein restricted diet was 5.26g (SD ± 0.64). The pups from dams fed the low protein diet weighed on average 13% lighter at birth $p < 0.001$. Maternal weight at delivery was not recorded. This is discussed further in section 4.4.

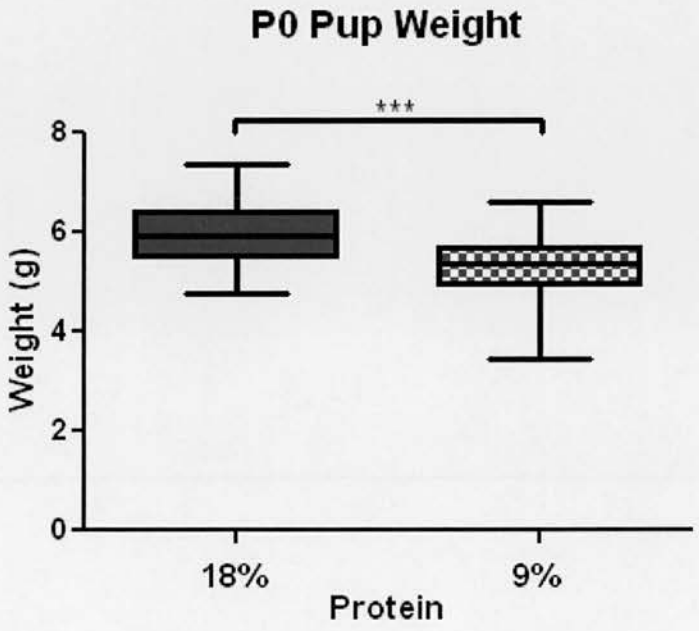

Figure 21 - Birth weight of pups

Birth weight in grams on y axis, dam dietary protein on x axis.
Box and whiskers plot, bars = minimum to maximum value, n=60 per group.
Unpaired t test comparing mean birth weight *** $p < 0.001$.

4.3.2 Body weights at P7

Accounting for diet only, pups fed on the normal 18% protein diet had a mean weight of 16.05g (SD ±1.52) and those fed on the low protein diet had a mean weight of 10.51g (SD ± 1.22). The pups from dams fed the low protein diet weighed on average 35% lighter at P7 ($p<0.001$) demonstrating ongoing postnatal growth restriction as shown in Figure 22.

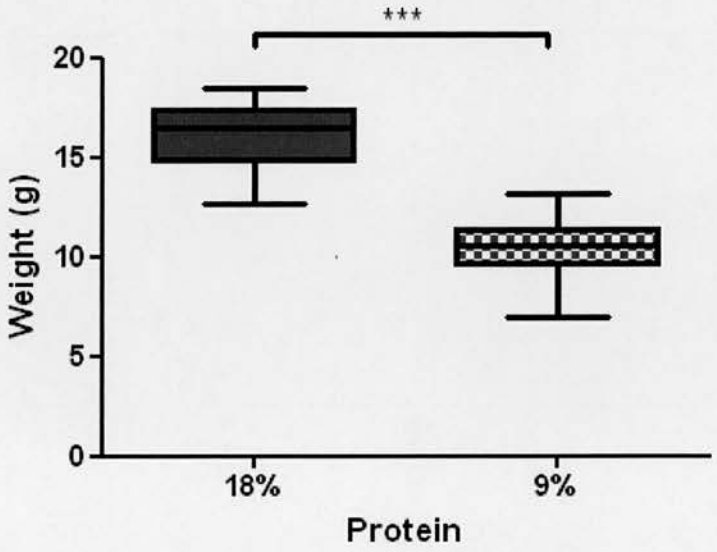

Figure 22 - P7 body weight based on diet group

Pup weight (grams) on y axis, dam dietary protein on x axis.
Box and whiskers plot, bars = minimum to maximum.
For the 18% plot n=60 (Both room air and oxygen groups fed 18% diet) and for the 9% plot, n=60 (Both room air and oxygen groups fed 9% diet).
Unpaired t test *** $p < 0.001$.

Pups from dams fed 18% protein can therefore be considered "normally grown" and pups from dams fed 9% protein are therefore "growth restricted".

The mean P7 weight of pups in the four experimental groups are given in Table 5 and summarised in Figure 23.

Table 5 – Body Weight at P7

Study Group	Body weight (g) (mean±SD)
RA 18%	16.58 ± 1.16
ΔO2 18%	16.42 ± 1.31
RA 9%	11.21 ± 0.82
ΔO2 9%	9.98 ± 1.2

There was a reduction in body weight in both growth restricted groups and a further reduction in P7 body weight in the growth restricted pups also exposed to fluctuating oxygen. There was no effect of oxygen on the normally grown pups.

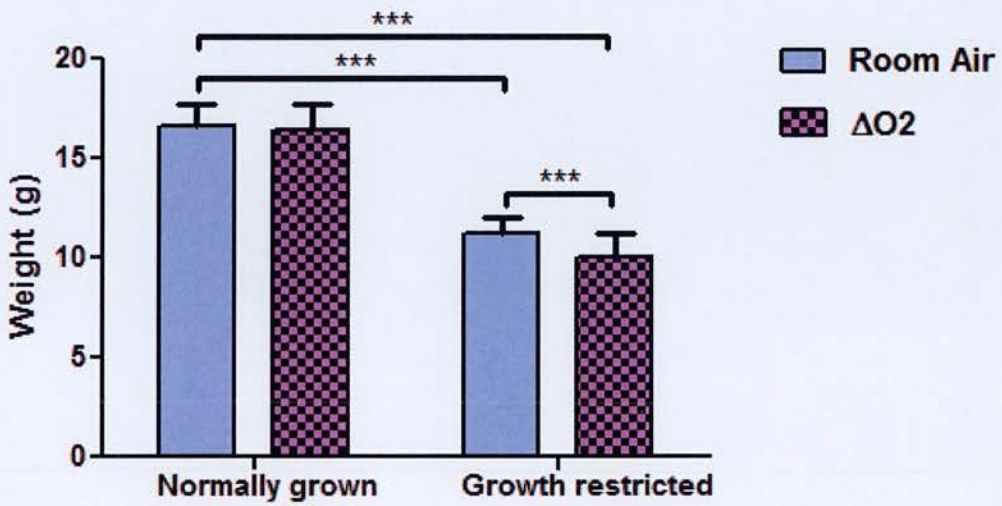

Figure 23 - Body Weight at 7 days

Pup weight (grams) on y axis, pup growth according to dam dietary protein on x axis.
Data shows mean ± standard deviation (SD), n=30 per group.
2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis confirms significant difference in weights between both normally grown groups, both growth restricted groups, and within growth restricted groups *** $p < 0.001$.

4.3.3 Brain weight at P7

Whole brain weight was measured as wet weight immediately following dissection from skull and submersion in PFA. Brains from growth restricted pups were smaller than normally grown pups; however there was no independent effect of oxygen on brain weight in normally grown or growth restricted pups.

Table 6– Brain weight at P7

Study Group	Brain weight (g) (mean±SD)
RA 18%	0.64 ± 0.03
ΔO2 18%	0.64 ± 0.03
RA 9%	0.58 ± 0.04
ΔO2 9%	0.55 ± 0.04

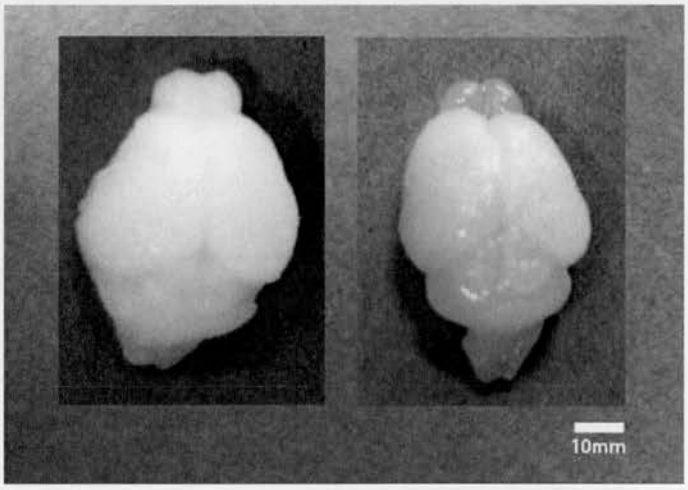

Figure 24 - Brain from normally grown and growth restricted rat pup

Brain from normally grown pup (left) is larger than brain from growth restricted pup (right).

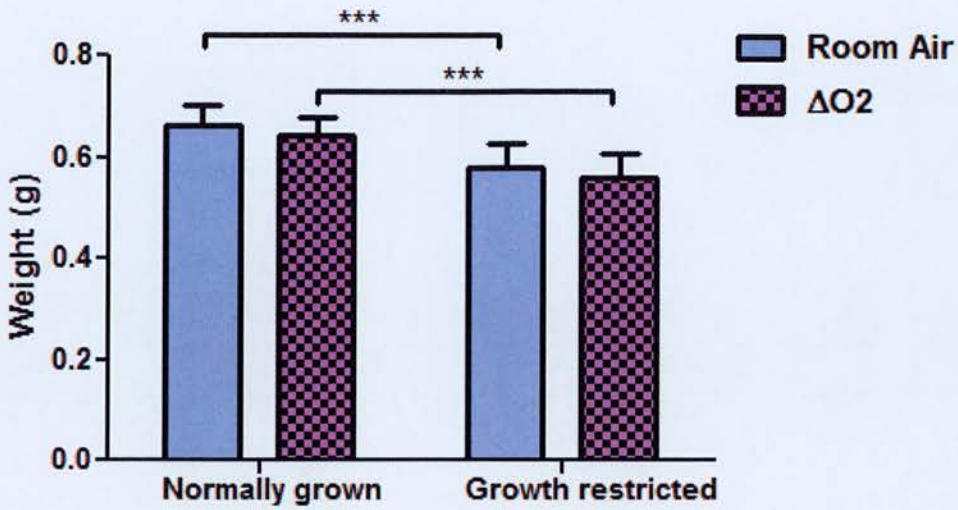

Figure 25 - P7 brain weight

Brain weight is reduced in both growth restricted groups in comparison to the normally grown groups with no additional effect of oxygen.
Pup weight (grams) on y axis, pup growth according to dam dietary protein on x axis.
Data shows mean ± standard deviation (SD), n=30 per group.
2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis to compare groups, *** $p < 0.001$.

When brain weight is considered as a percentage of body weight (see Figure 26), the growth restricted pups demonstrate a greater preservation of brain weight relative to body weight. The dual group exposed to both growth restriction and oxygen preserve brain weight to a greater extent in view of the reduced P7 total body weight seen in this group.

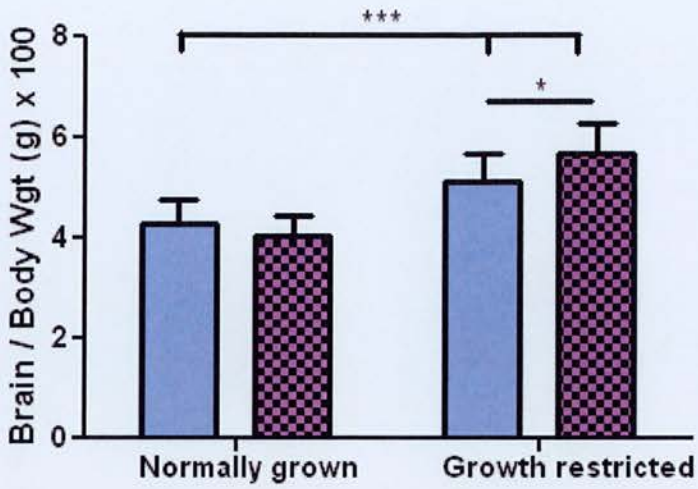

Figure 26 - Brain as a percentage of body weight

Growth restricted groups demonstrate relative preservation of brain growth and are therefore asymmetrically growth restricted.
Pup weight (grams) on y axis, pup growth according to dam dietary protein on x axis.
Data shows mean ± standard deviation (SD), n=30 per group.
2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis to compare groups, * $p < 0.05$, *** $p < 0.001$.

4.4 Discussion

It has been shown that a 9% isocaloric low protein diet fed to dams from E15 until P7 caused prenatal and postnatal growth restriction in the offspring with an additive effect of postnatal oxygen. Brain weight was reduced ($p < 0.001$) in growth restricted pups irrespective of postnatal oxygen exposure. As a percentage of body weight, both growth restricted groups preserved their brain weight relative to their body weight and this was most evident in those exposed to fluctuating hyperoxia. There was no independent effect of oxygen on actual brain weight at P7.

Several models have been described to induce growth restriction in rodent offspring and assess effects of intrauterine growth restriction and fetal programming, including various nutrition manipulation models, mid-gestation surgical procedures e.g. uterine artery ligation, and models administering growth prohibiting substances e.g. glucocorticoids (Snoeck et al. 1990; Woodall et al. 1996; Holemans et al. 1998; Holemans et al. 2003). We used a protein restriction model from day 15 of gestation as this has been previously successfully shown to induce fetal growth restriction in rodent pups with a greater effect on truncal growth relative to brain growth (Dhaliwal et al. 2011; Langley-Evans et al. 1994). This mirrors the clinical situation where asymmetrical growth restriction in preterm infants is most common. Although the commonest cause of human IUGR is placental insufficiency due to placental vascular disease, maternal under nutrition in rodent models results in reduced substrate delivery to the fetoplacental unit, decreased uteroplacental flow and reduction in total

milk volume during lactation, all of which hamper fetal and neonatal growth of the pups (Langley-Evans et al. 1998; Holemans et al. 2003; Gao et al. 2012). Preterm infants have a high metabolic rate and catabolise high amounts of protein in the initial postnatal period. Preterm infant nutrition within the intensive care unit tends to be high in carbohydrate and fat and low in protein (Vasu et al. 2007). One of the consequences of this is that postnatal growth failure of preterm infants is common (Cooke et al. 2004) and therefore the experimental model demonstrating pre- and post-natal growth restriction reflects a number of features of the clinical situation.

A weakness of the current study is that maternal weight was not documented at conception, delivery or in the postnatal period. Maternal weight has an important influence on pup weight and a protein restricted diet has been shown to reduce maternal body and brain weight (Bennis-Taleb et al. 1999). In all experiments, 2nd time mothers were used to provide consistency across experimental groups.

Low protein diet in animal pregnancy has previously been shown to cause lower body and brain weights in pups (Snoeck et al. 1990; Bennis-Taleb et al. 1999). To determine if antenatal growth restriction alone is sufficient to cause a reduction in brain weight, cross-fostering experiments where pups from a 9% dam are reared until P7 by an 18% dam could be performed using our model. Evidence from the literature suggests there may be a graded effect of protein restriction.

One study demonstrated that if the low protein diet was continued after weaning, adult rats similarly had lower brain weights. If pups exposed to low protein in utero were then fed by dams on a normal diet and the normal diet continued after weaning, brain weight in adulthood was not reduced (Bennis-Taleb et al. 1999). A previous study however, showed that if a 6% low protein diet was used in pregnancy, and a normal 20% diet continued after birth, brain weight of pups remained reduced at P21 (Resnick et al. 1982). This suggests there is a fetal programming element to the effect of growth restriction on the developing brain.

In keeping with this, protein restriction during gestation was found to reduce cerebral cortex vascularisation and this did not recover when a normal diet was introduced after birth (Bennis-Taleb et al. 1999). This effect on vascularisation seems specific to the brain as similar experiments examining the pancreas and duodenum showed recovery of vasculature when a normal diet was restored after birth (Iglesias-Barreira et al. 1996). A previous study reported that offspring of rats fed a low protein diet during gestation, had lower numbers of dendrites as well as lower sensory cortico-cortical and thalamo-cortical evoked potentials (Resnick et al. 1982). Taken together, these studies suggest there may be implications for cerebral function in pups of dams when low protein diet is given ante- and postnatally.

The mechanism underlying the 'brain-sparing' effect in response to protein restriction has been suggested as a consequence of increased carotid blood flow (Hawkins et al. 1999).

This would appear contradictory to the study above (Bennis-Taleb et al. 1999), that also reported forebrain weight recovered after the normal diet was reinstated despite the ongoing effects seen in cortical vasculature and conjectured that blood perfusion to the brain may have been normal despite the reduced vascular density (Bennis-Taleb et al. 1999). A more recent study has shown that low protein in gestation causes a reduction in brain oxygen consumption in utero at E21 (Gallagher et al. 2005). This suggests a reduction in cerebral metabolism as a further method by which brain-sparing may be mediated. Adaptive changes by a fetus to an altered intrauterine environment may improve immediate chances of survival despite detrimental effects in later life. The 'Predictive Adaptive Response' theory suggests that although these changes may have no immediate benefit, the adaptations allow the fetus to develop a phenotype based on the predicted postnatal environment (Gluckman et al. 2004).

These findings are supported in the human situation by imaging studies in preterm SGA infants showing reduced intracranial volume (Tolsa et al. 2004) and behavioural observations of poorer neurodevelopmental outcome than their appropriately grown peers (Yanney et al. 2004).

Previous work employing the clinically derived oxygen profile used in this study induced vascular changes in the rat pup retina comparable to those seen in retinopathy of prematurity (Cunningham et al. 2000; McColm et al. 2000b; McColm

et al. 2004; Dhaliwal et al. 2011). Within the brain, increased cell death, astrocytosis and reduced expression of myelin basic protein have been reported (Sedowofia et al. 2008). Reactive oxygen species are formed as a natural by-product of oxygen metabolism and can damage all components of cells including protein, DNA and enzyme systems (Maltepe et al. 2009). Growth restricted and preterm infants have lower total antioxidant capacity (Toy et al. 2009). In this model, the pups exposed to growth restriction and then oxygen fluctuation had a lower body weight and conserved brain weight in comparison to growth restriction alone. The additional reduction in body weight caused by oxygen in this group may represent reduced antioxidant capacity and a reduction in growth factors key to somatic growth as discussed further in Chapter 7.

The following Chapters will now examine the effects of ante- and postnatal growth restriction and the clinically relevant oxygen fluctuations on cortical development, neuronal subtypes and on brain growth in the animal model.

Chapter 5:

Growth Restriction and Fluctuating Hyperoxia on Cerebral Cortex Lamination in the Developing Brain

5.1 Introduction

In humans, cerebral cortical volume increases nearly four-fold between 29 and 35 weeks gestation and this is a critical period for neuronal migration and cortical growth. Infants born during this time period have decreased cortical grey and white matter at term equivalent which persists in to adolescence (Inder et al. 1999; Nosarti et al. 2002). The cerebral cortex is the source of cognition and therefore damage incurred will potentially result in altered cognitive function. As described previously, the rat cerebral cortex is smooth whilst the human cortex is highly convoluted and supports a greater surface area. During development the rat cerebral cortex follows a similar sequence to that of the human cortex (Sidman et al. 1973). In both species, the cortex is composed radially of six layers (Figure 27), each layer characterised by distinct cell types. Migration of neurons from the proliferating zones (ventricular zone, VZ and subventricular zone, SVZ) to the deep layers of the cerebral cortex is largely complete by E18 in the rat and 24 weeks gestation in the human, although maturational events including synaptogenesis continue into the third trimester (Kostovic et al. 2006). From 24 weeks, the superficial layers continue to expand radially as they receive migrating neurons throughout the third trimester and maturational changes continue into infancy. Broadly, the deep layers contain neurons that project extra-cortically to structures such as the thalamus and spinal cord, whilst the superficial layers contain neurons that project locally within the cortex and are involved in intracortical circuitry (Hill et al. 2005). Layer IV is significant as it receives the vast majority of afferent neurons projecting from the thalamus.

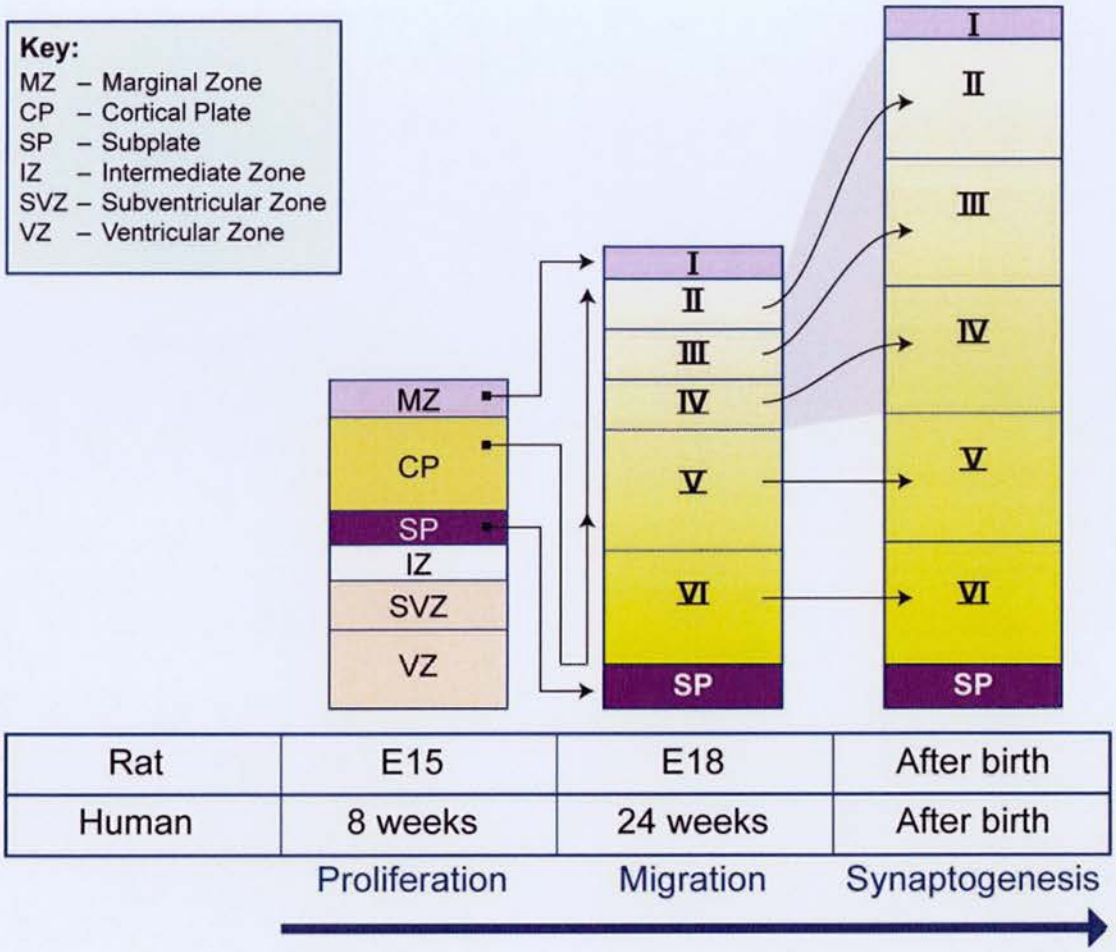

Figure 27 - Schematic Illustration of cortical development in rats and humans

The cerebral cortex, developing from the cortical plate (CP) is shown in yellow. By 24 weeks gestation in the human fetus, and embryonic day 18 in the rat (E18), deep layers VI and V have mainly formed. The superficial layers IV-II undergo growth and maturational changes such as differentiation, dendritic branching and synaptogenesis during the latter weeks of gestation and continuing after birth.

As discussed in Chapter 1, periventricular leukomalacia (PVL) diagnosed by ultrasound or MRI imaging, has long been associated with preterm brain injury and impaired neurodevelopmental outcome. Volumetric MRI imaging studies have demonstrated that preterm infants with PVL have a reduced cortical grey matter at term in comparison to infants without PVL (Inder et al. 1999; Thompson et al. 2007; Nagasunder et al. 2012).

In particular, the posterior periventricular white matter underlying the motor cortex is frequently associated with diffuse and cystic white matter change (Volpe 2009b). Previous work has shown that in the context of fluctuating hyperoxia, there is disruption of white matter myelination in the region of the internal capsule (Sedowofia et al. 2008; Pilley 2010) underlying the motor cortex. Furthermore, in studies of growth restriction, preterm infants with a higher risk of cerebral palsy score less well on motor and memory skills testing in childhood (Yanney et al. 2004). For these reasons, the motor cortex was selected as an initial region of interest in an exploratory study to determine the effects of growth restriction and fluctuating oxygen.

The corpus callosum is the largest white motor tract in the mammalian brain and is key to interhemispheric communication of motor and sensory signals. Although the basic structure of the corpus callosum is established by 20 weeks gestation, it continues to grow in size, receiving more axonal fibres during the third trimester (Malinger et al. 1993) with myelination occurring in the postnatal period in both humans and rodents. Preterm birth during the third trimester may therefore disturb normal corpus callosum development through disruption of normal axonal trajectory processes or myelination of nerve fibres. Recent studies have shown that preterm infants have a reduced cross sectional area of corpus callosum in comparison to term infants (Thompson et al. 2010).

Fractional anisotropy within the corpus callosum has also been linked to intelligence and coordination scores (Counsell et al. 2008) and corpus callosum deficits have been linked to delayed motor functioning (Rademaker et al. 2004).

It was hypothesised that pre- and postnatal growth restriction and postnatal exposure to oxygen will result in reduced thickness of the late born superficial cortical layers and reduced thickness of the corpus callosum.

5.2 Methods

Brains were removed from the animals in each study group as described in Chapter 2. Pups were perfused with fixative, the brains soaked in fixative, cryoprotected and then snap-frozen in 2-methylbutane (section 2.6.2). Coronal cryosections at 20 micron thickness were obtained (section 3.6.1) and comparable sections caudal to the level of the corpus callosum decussation were matched for each group. Sections were hydrated, stained with cresyl violet and imaged on a bright field microscope as described in sections 3.7.1.

5.3 Results

5.3.1 Thickness of Motor Cortex (M1) Laminae

1. Total Cortical Thickness (Layers I-VI)

In comparison to control, cortical thickness was preserved in the growth restricted pups. This was despite the decrease in brain weight observed in the growth restriction study group (section 4.3.3). In contrast, despite preservation of brain weight (section 4.3.3) postnatal fluctuating hyperoxia caused cortical thinning, particularly in the context of growth restriction (Figures 28, 29, Table 7). In Figure 28 below, each data point represents the average of 3 measurements made for each pup and each colour represents a different litter within each group. This graph illustrates similar variability within the litters and no skewing outliers.

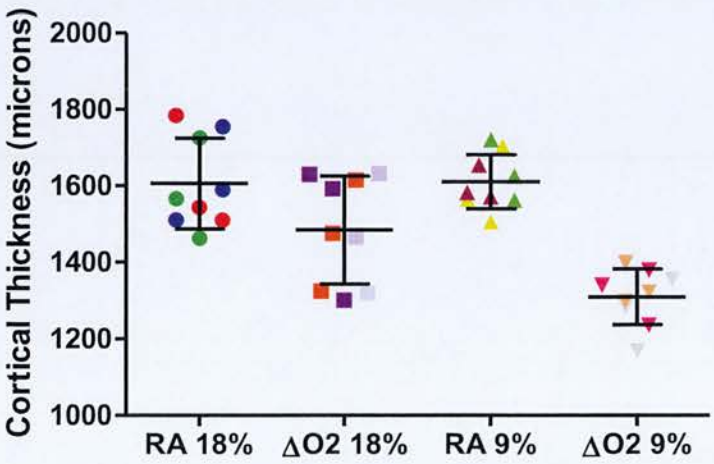

Figure 28 - Mean cortical thickness ±SD for each study group

Cortical Thickness in microns measured at the centre of primary motor cortex, against study group on x axis. Each data-point represents an average of 3 counts per pup. Each different colour represents a different litter, confirming no major outliers between the litters in each study group.
The values for each group are normally distributed by D'Agostino and Pearson omnibus normality test. Data shows mean ± standard deviation (SD), n=9 per group.
Figure 28 shows spread of data. Statistical analysis not shown - see Figure 29.

The following table summarises the mean cortical thickness and standard deviation for each group.

Table 7 – Total Cortical Thickness (microns, μm) per group

Study Group	Total Cortex (VI-I) Thickness (mean±SD)
RA 18% (Control)	1606 ± 118.2
ΔO2 18% (ΔO2 only)	1484 ± 120.1
RA 9% (GR only)	1610 ± 71.22
ΔO2 9% (Dual)	1310 ± 72.56

Statistical analysis by 2-way analysis of variance (2-way ANOVA) confirmed variation between study groups and is illustrated in Figure 29. Bonferroni post-tests defined a significant effect of fluctuating hyperoxia (ΔO2) on cortical thickness (1606μm ± 118.2 *versus* 1484μm ± 120.1), $p<0.05$. There was no effect of growth restriction (GR) alone. The dual group demonstrated particularly significant cortical thinning in comparison to both the ΔO2 group (1484μm ± 120.1 *versus* 1310μm ± 72.56), $p<0.01$, and the GR group (1610μm ± 71.22), $p < 0.001$.

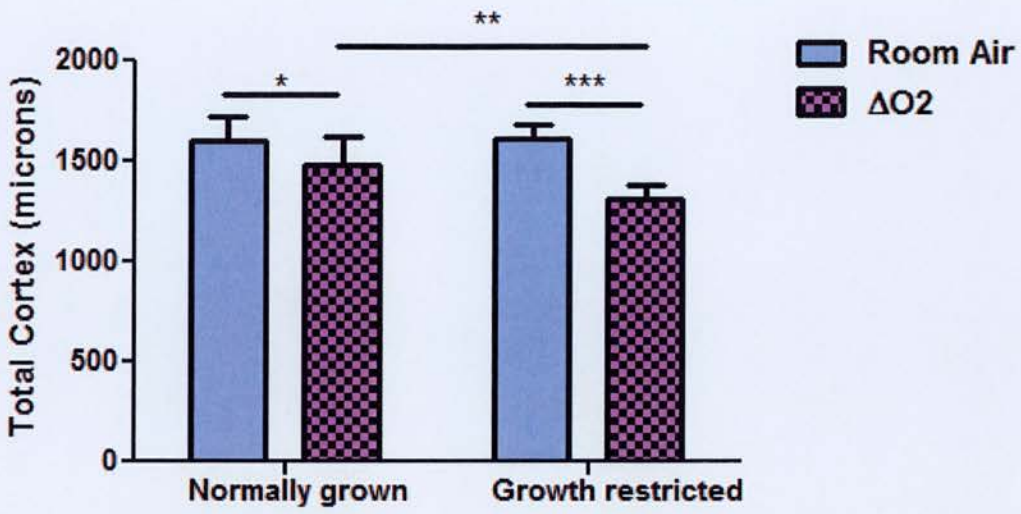

Figure 29 - Comparison of Total Cortical Thickness (layers I-VI) between study groups

Total cortical thickness, layers I-VI (microns) against study group on x axis.
Data shows mean ± standard deviation (SD), n=9 per group.
2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis to compare groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2. Deep Layer (V & VI) Thickness

When thickness of the early born deep cortical layers was considered alone, there was relative conservation of deep layer thickness in the ΔO2 only and GR only groups. Mild thinning was seen in the dual group. The raw data is presented as mean ± standard deviation in Table 8.

Table 8 – Deep cortical layer thickness (microns)

Study Group	Deep layer (VI & V) Thickness (mean±SD)
RA 18% (Control)	988.8 ± 107.7
ΔO2 18% (ΔO2 only)	970.3 ± 88.75
RA 9% (GR only)	1059 ± 48.78
ΔO2 9% (Dual)	895.9 ± 81.31

Statistical analysis by 2-way ANOVA and Bonferroni post-test demonstrates significant cortical thinning in the dual study group in comparison to the GR group, $p<0.01$. This may not represent a true difference as the 'GR only' group had a higher mean and lower SD than the rest of the groups.

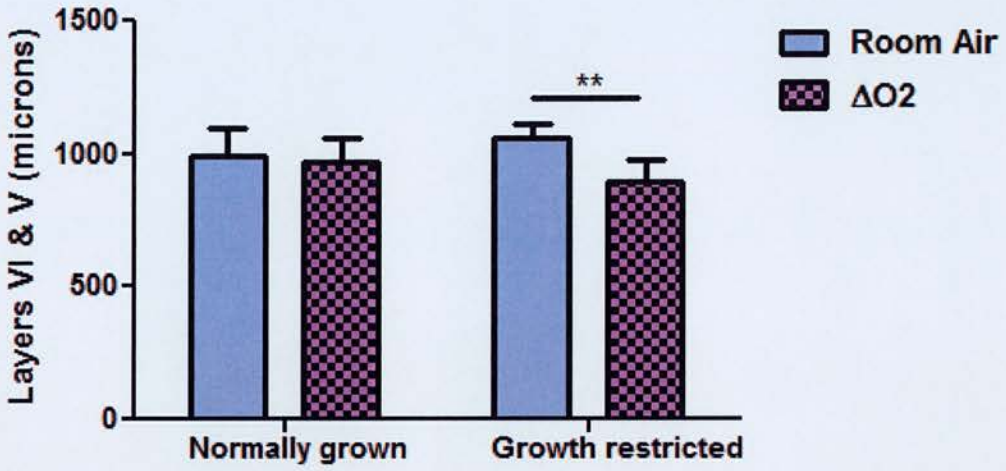

Figure 30 - Deep cortical layer (V +VI) thickness

Deep layer cortical thickness, layers V+VI (microns) against study group on x axis.
Data shows mean ± standard deviation (SD), n=9 per group.
2-way ANOVA confirms variation present in dataset $p<0.05$. Bonferroni post-test analysis to compare groups, $**p<0.01$.
There was no effect of ΔO2 or GR alone. Reduced thickness in dual group in comparison to GR group only.

3. Superficial Layer (II-IV) Thickness

The late born superficial layers were adversely affected by hyperoxia in both normally grown and growth restricted pups as shown by the mean values and SD in Table 9.

Table 9 – Superficial cortical layer thickness (microns)

Study Group	Superficial layers (IV-II) Thickness (mean±SD)
RA 18% (Control)	476.1 ± 33.61
ΔO2 18% (ΔO2 only)	401.1 ± 61.6
RA 9% (GR only)	428 ± 53.52
ΔO2 9% (Dual)	328.5 ± 26.04

Figure 31 shows 2 cresyl violet stained images from the control group (A) and the dual group (B), with total diameter marker and horizontal markers to define layer boundaries. Roman numerals indicate the relevant layer of cortex. Superficial layers II-IV are thinner in B in comparison to A.

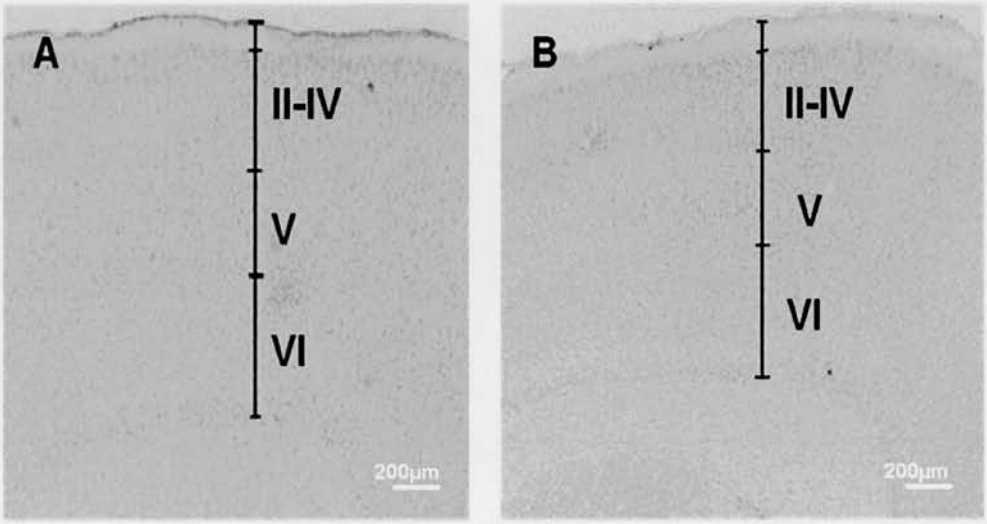

Figure 31 - Cresyl violet stained section from Control versus Dual group

A – Control group: Cortical diameter in at centre of motor cortex.
B – Dual group: Cortical diameter in at centre of motor cortex.
Thickness of superficial layers (II-IV) and to a lesser extent, deep layers (V &VI), were reduced in the dual group.

Statistical analysis by 2-way ANOVA confirmed variation between the groups and Bonferroni post-tests indicated significant differences in both groups exposed to fluctuating oxygen. Superficial cortical thinning was observed in the 'ΔO2 only' group compared to control (401.1±61.6 *versus* 476.1±33.61), $p<0.01$. The reduction observed in the 'GR only' group in comparison to control did not reach statistical significance (428±53.52 *versus* 476.1±33.61), $p>0.05$. Within the growth restricted groups, the dual group demonstrated the most significant thinning (328.5±26.04 *versus* 428±53.52), $p < 0.001$.

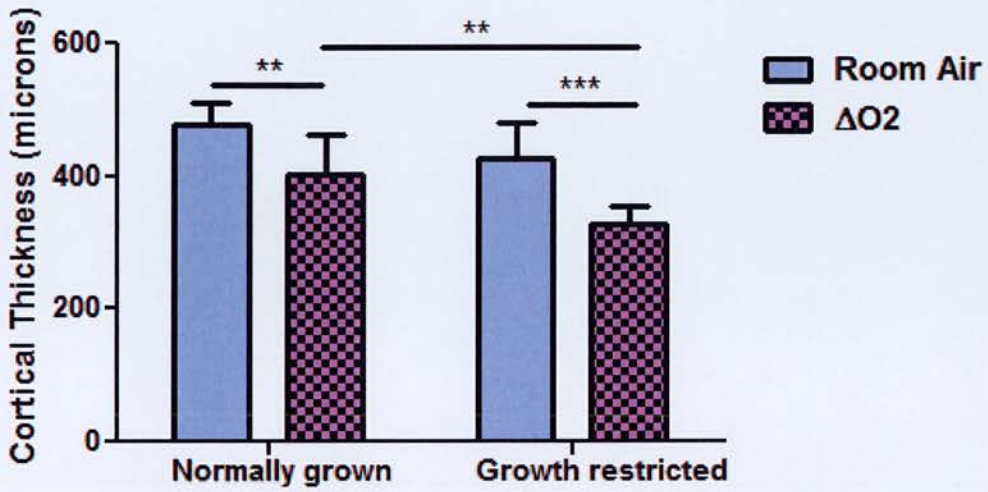

Figure 32 - Superficial cortical layer thickness

Superficial layer cortical thickness, layers II-IV (microns) against study group on x axis.
Data shows mean ± standard deviation (SD), n=9 per group.
2-way ANOVA confirms variation present in dataset $p<0.05$. Bonferroni post-test analysis to compare groups, **$p<0.01$, ***$p<0.001$.
There was no effect of growth restriction alone. Superficial cortical thinning was seen in the fluctuating oxygen group and in the dual group exposed to both growth restriction and fluctuating oxygen.

5.3.2 Superficial Cortical Cell Density

To set the scene for the effects of growth restriction and fluctuating hyperoxia noted on cell density within the superficial layers, the information in Figure 32 can be presented as a line graph and the order of the study groups changed to show the increasing effects of the environmental insults on cortical thickness as shown in Figure 33 below.

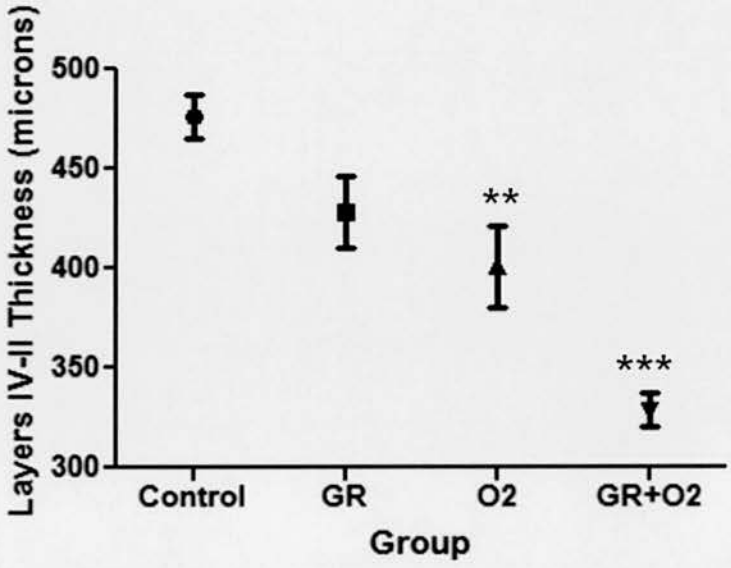

Figure 33 - Line graph of superficial layer thickness II-IV (microns)

Study group against superficial laminar thickness layers IV-II (microns).
GR = growth restriction only, O2 = oxygen only, GR + O2 = Dual.
Data shows mean ± standard deviation (SD), n=9 per group.
2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis to compare groups, * $p < 0.05$, $p<0.01$, ***$p<0.001$.
No significant difference between control group and GR only group.
Significant decrease in cortical thickness between control group and ΔO2 ($p<0.01$ **).
Significant decrease in cortical thickness between Control and dual group ($p<0.001$ ***).
Significant decrease in cortical thickness between fluctuating oxygen and dual group ($p<0.01$ **).

To determine the effect of superficial cortical thinning on cell number and cell density, total labelled nuclei within a 100μm diameter box placed in the centre of the motor cortex overlying the superficial cortical layers were counted and compared between groups (Chapter 3, Figure 18).

Figure 34 illustrates the number of nuclei counted within the 100μm diameter box overlying layers II-IV. This measurement is of limited value as it does not take account of brain volume differences between the groups, but illustrates that numbers of neurons within a set region are reduced in the fluctuating oxygen group in comparison to the other study groups. Of note, as described in Chapter 4, there was no difference in brain weight in the 'ΔO2 only' group in comparison to control.

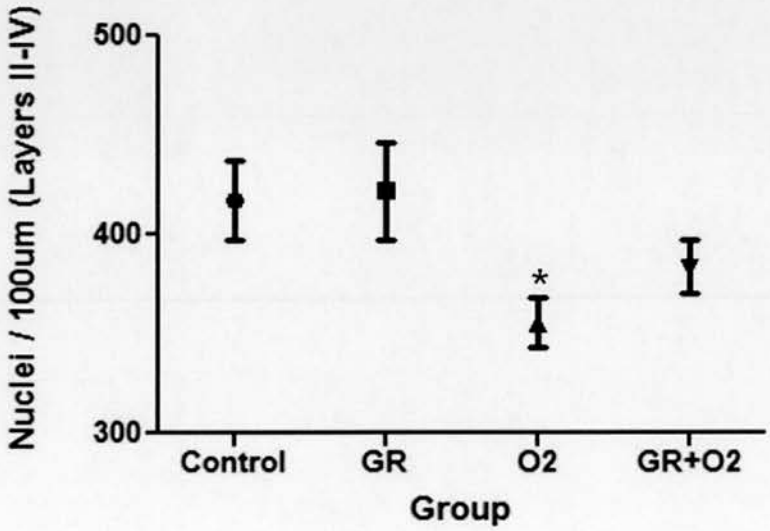

Figure 34 - Cell nuclei in 100μm diameter box in superficial layers II-IV

Nuclei number in superficial layers II-IV within a 100micron diameter box in M1 against study group. GR = growth restriction only, O2 = oxygen only, GR + O2 = Dual. Data shows mean ± standard deviation (SD), n=9 per group. 2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis to compare groups, * $p < 0.05$, $p<0.01$, ***$p<0.001$.
No significant difference in nuclei number between Control group and GR group ($p>0.05$).
Significantly reduced number of nuclei in the ΔO2 group in comparison to control ($p<0.05$ *).
No significant difference in the number of nuclei between the fluctuating oxygen group and the dual group.

A more comparable measure is to consider cell density differences between the groups, Figure 35. Cell density per square millimetre (mm^2) was calculated using the following equation: (nuclei / 100T) x1000, where nuclei = number of nuclei in 100um diameter box as presented in Figure 34 and T = cortical thickness as presented in Figure 33. 100T therefore represents the area of the box.

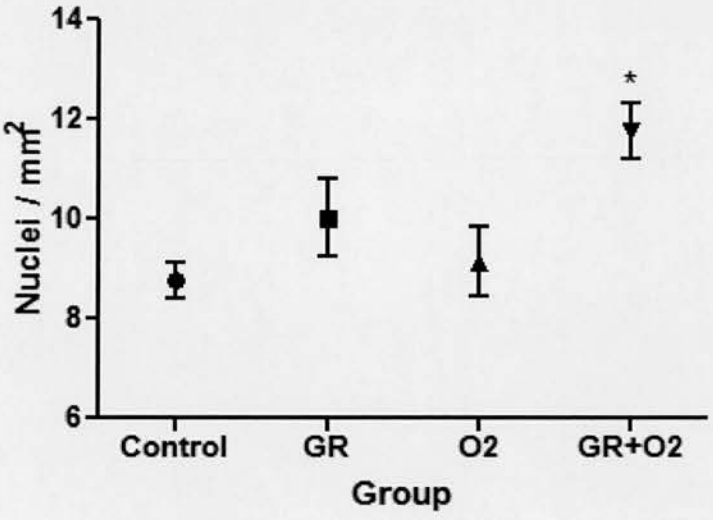

Figure 35 - Cell nuclei / mm^2 (Cell density)

Nuclei /mm^2 of brain against study group.
GR = growth restriction only, O2 = oxygen only, GR + O2 = Dual.
Data shows mean ± standard deviation (SD), n=9 per group. 2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis to compare groups, * $p < 0.05$
No significant difference between the control group and GR group ($p > 0.05$).
No difference between the control group and ΔO2 group ($p > 0.05$).
Significant increase in nuclei per mm^2 in the dual group ($p < 0.05$).

There was an increase in cell density observed in both growth restricted groups. The effect on the 'GR only' group did not reach statistical significance. There was no effect seen in the ΔO2 group, but the addition of fluctuating oxygen caused a significant increase in nuclei density in the dual group.

Table 10 - Summary of Effects of GR and ΔO2 on superficial laminae of cortex

	Layer IV-II Thickness (microns)	Layer IV-II Nuclei/ 100 micron diameter (M1)	Nuclei Density / mm2
Control (Mean ± SD)	476 ± 33.6	417 ± 59.8	8.76 ± 1.1
Growth Resticted (GR)	↔ (-10%)	↔ (+1%)	↔ (+12%)
Fluctuating Oxygen (O2)	↓ (-15%)	↓ (-15%)	↔ (+1%)
Dual (GR+O2)	↓↓ (-30%)	↔ (-8%)	↑↑ (+33%)
	Figure 33	Figure 34	Figure 35

First column represents superficial layer thickness in microns. The second column is the number of nuclei in the 100micron diameter box across the superficial layers. Third column is the density of nuclei within the 100micron box across the superficial layers

This table summarises the main directional changes in the three study groups relative to control. The mean raw values of the control group are given. The arrows represent the statistical findings relative to control. The (%) refers to the percentage change of study group mean relative to control mean. This table shows:

- In the GR group, a non-significant trend of reduced superficial layer thickness and an associated increase in cell density with no loss of nuclei.
- In the ΔO2 group, the significant loss in superficial layer thickness is accompanied by a similar proportional loss of cell number causing no observed effect on cell density.
- In the dual group, there is highly significant loss in superficial laminar thickness, a suggestion of a relatively modest loss of nuclei (not statistically significant) and a highly significant increase in cell density suggesting there has been loss of the intercellular matrix.

Although the following schematic diagram is an oversimplification, it is a composite pictorial representation of these results on superficial layer thickness, cell number and nuclei density, Figure 36.

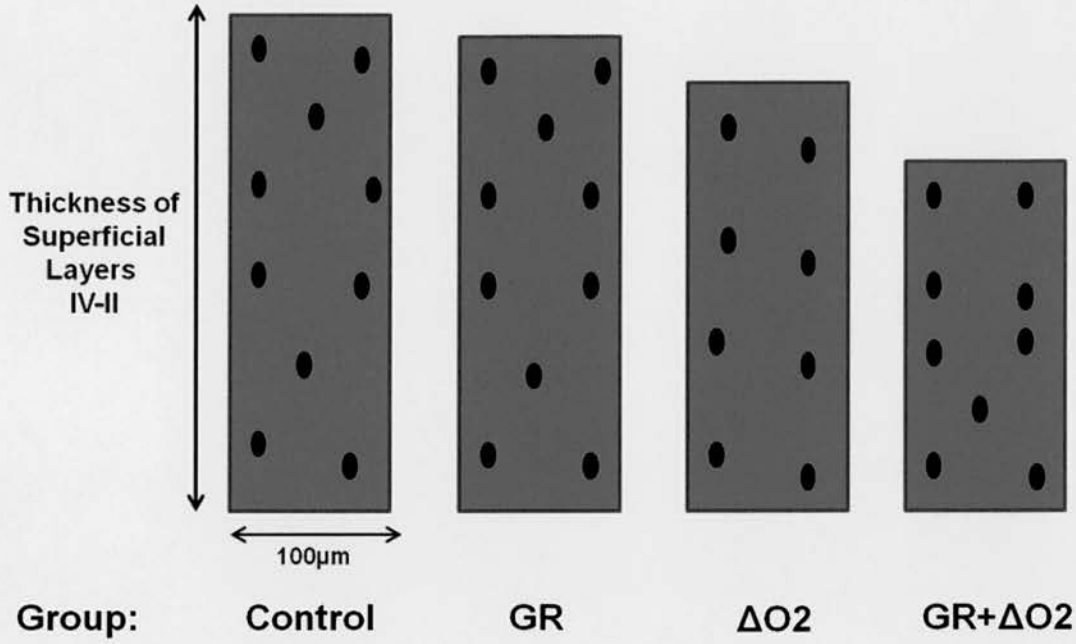

Figure 36 - Schematic of Superficial Layer thickness in each study group

In comparison to control:
GR group – slightly thinner cortex (not statistically significant), equal number of nuclei, slightly increased density (not statistically significant).
ΔO2 group – thinner cortex, less nuclei, same cortical density.
Dual group – significantly thinner cortex, slightly less nuclei (not statistically significant), significant increase in nuclei density.

5.3.3 Corpus Callosum Thickness

Corpus callosum thickness was measured on level matched sections immediately caudal to the decussation on 3 sections per pup in each of the groups. Statistical analysis by 2-way ANOVA did not find any significant variation in corpus callosal thickness between any of the study groups.

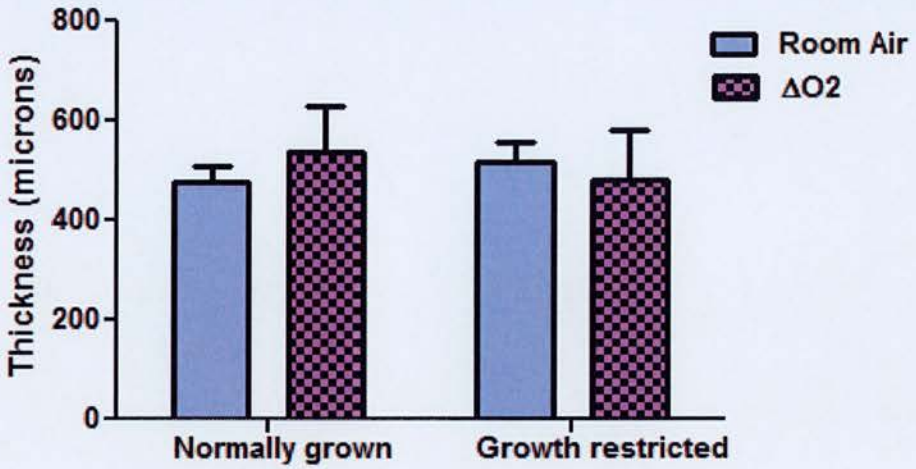

Figure 37 - Corpus Callosal Thickness immediately caudal to decussation

Corpus callosum thickness (microns) against study group on x axis.
Data shows mean ± standard deviation (SD), n=9 per group.
No variation identified by 2-way ANOVA $p>0.05$.

5.4 Discussion

5.4.1 Summary of results

This work shows that using a clinically relevant oxygen profile, postnatal oxygen adversely affects cortical growth, particularly of the late born superficial laminae, resulting in a thinned cortex with a maintained density and therefore an overall reduction in neuronal number. In the context of growth restriction, fluctuating oxygen caused cortical thinning, an increase in nuclear density and a non-significant reduction in neuron number. Growth restriction alone was associated with slight cortical thinning and a non-significant increase in nuclei density. There was no observed effect of growth restriction or oxygen on corpus callosal thickness.

5.4.2 Brain volume and the Preterm Infant

Previous work has suggested the premature infants are more likely to have a reduced brain volume at term (Thompson et al. 2007). Thompson et al. used MRI imaging to demonstrate specific reductions in the sensorimotor, premotor and orbitofrontal cortical grey matter in preterm infants at term. Within childhood, even those born at later gestations between 30-34 weeks without serious neonatal morbidity or cerebral pathology show global grey matter volume loss (Soria-Pastor et al. 2009) and this correlates with a reduced intellectual score at age 8-10 years. This volume loss is also noted in adolescence and one study documented an 8.8% reduction in grey matter volume on MRI in comparison to term-born controls (Nagy et al. 2009). Another study reported an 11.8% reduction in grey matter volume in adolescence

(Nosarti et al. 2002). The increasing volume of the developing brain is secondary to increases in white matter volume (Zhang et al. 2000), cortical surface area (Rakic 1995), deep grey matter nuclei and the thickness of the cerebral cortex. Volume loss, therefore, may be a consequence of reductions in white matter, cortical surface area, deep nuclei or cortical thickness which may reflect loss of cells or connectivity networks.

5.4.3 Cortical Thickness and the Preterm Infant

As described in Chapter 1, specific areas of the cerebral cortex are specialised in particular functions and therefore adverse effects on cortical thickness may interfere with the inherent specialist functions for each cortical area. Cortical thickness has been explored in ex-preterm adolescents using T1 weighted 3D MRI which demonstrated thinning of the cortex in multiple cortical areas including temporal, parietal, frontal and occipital lobes with large areas around the central sulcus, including the motor cortex, being affected (Martinussen et al. 2005; Nagy et al. 2009). The thinning around the central sulcus was most pronounced in adolescents who were born at a lower gestational age and birth weight (Nagy et al. 2011). Although, as previously discussed, the links between cerebral volume loss and poorer cognitive outcome are well established in preterm infants, there are few specific studies linking *cortical thinning* after preterm birth to poor cognitive outcome (Peterson et al. 2000; Tolsa et al. 2004). One study reported a positive correlation between thinning of the entorhinal cortex in adolescents born preterm and lower performance in cognitive and executive function testing (Skranes et al. 2011).

The entorhinal cortex, located in the medial temporal lobe is the gateway between the hippocampus and the neocortex and is involved primarily in long-term memory (Squire et al. 2004). Reduced density of pyramidal neurons in specific cortical layers leading to altered cortical architecture has been reported in the cerebral cortex overlying areas of periventricular leukomalacia (Andiman et al. 2010). This work underlines the concept that white matter necrosis must have destructive consequences on the developing cortical neurons and their axons. Recent MRI work using tractography has shown that thalamocortical connectivity is reduced following preterm birth. In particular connectivity between the thalamus and the supplementary and pre-motor areas is significantly reduced in preterm infants at term-corrected (Ball et al. 2012b). Reduced connectivity of afferent fibres in layer IV would be in keeping with thinner superficial layers. The supplementary and pre-motor areas are crucial for planning and performing movements (Picard et al. 1996). As previously discussed, preterm infants may develop particular problems with executive functioning and poor motor coordination (Anderson et al. 2004; Saiegal et al. 2003; Marlow et al. 2005). Recent work in Alzheimers disease using T1-weighted MRI have used cortical thinning at discrete regions within the cortex to predict progression of disease from mild cognitive impairment to Alzheimer's disease (Eskildsen et al. 2012).

Cortical thinning postnatally is in fact a physiological event within the first 1-2 years of life as a consequence of synaptic proliferation and pruning (Huttenlocher 1994) and ongoing myelination of small axons at the inner cortical border so that grey

matter becomes thinner as the white matter in this area expands (Sowell et al. 2001). Studies examining cortical thickness in ex-preterm infants in early childhood at age 18-22 months and at age 3-4 years therefore relate the cortical thickening and reduced surface area found in these infants to a delay in normal cortical maturation secondary to prematurity (Phillips et al. 2011). Within animal work, cortical thickness studies have found an increased cortical thickness in a mouse model of Huntington's disease (Lerch et al. 2008) and have been used to study the effects of intermittent ethanol exposure of cortical thickness in rats (Lee et al. 2011).

As far as can be determined, there have been no pathological studies of cortical thickness measurements at birth in preterm infants. In addition, the majority of the published work on cortical thickness has been carried out using MRI imaging as opposed to neuropathology and this has generally been in adolescents. Challenges in MRI measurements of cortical thickness include accurate removal of the skull overlying the cortical area of interest and modelling the cortical surface in regions where opposing sulcal surfaces may touch (Giedd et al. 2010). A major strength of this current work is therefore the use of tissue samples from a lissencephalic species.

This work is novel as there is no published literature as far as can be determined at present, relating specific neonatal morbidities and events, such as growth restriction and postnatal oxygen therapy, to cerebral cortical thinning.

5.4.4 The Effect of Growth Restriction and Oxygen on Brain Volume, Cortical Thickness and the Corpus Callosum

Bronchopulmonary dysplasia and supplemental oxygen are independent predictors of poor brain growth and neurodevelopmental outcome in the preterm infant (Short et al. 2003; Boardman et al. 2007). Studies have shown that intrauterine growth restriction is associated with a posterior reduction in brain volume while bronchopulmonary dysplasia is associated with a global reduction in brain volume (Thompson et al. 2007). The mechanisms underpinning such associations are not well-established. Experimental models designed to examine the relationship between oxygen and the brain traditionally employ high concentrations of oxygen (Nemoto et al. 1987; Sirinyan et al. 2006; Ratner et al. 2007). These models have offered important insights into the adverse effects of oxygen but such levels are seldom experienced by neonates in contemporary clinical settings where oxygen saturations are targeted to a normoxic range. Despite such targets, preterm infants with ventilation perfusion mismatch and immature respiratory drive frequently deviate outside the target range into hypoxic and hyperoxic levels (Quine et al. 2009). Little is known about the effects of such milder oxygen fluctuations on the brain.

Previous work employing the clinically derived oxygen profile used in this study induced vascular changes in the rat pup retina comparable to those seen in retinopathy of prematurity (Cunningham et al. 2000; McColm et al. 2000; McColm et al. 2004; Dhaliwal et al. 2011) and within the brain, increased cell death, astrocytosis and reduced expression of myelin basic protein (Sedowofia et al. 2008).

This work has shown that clinically relevant levels of oxygen cause cortical thinning in 7 day old rat pups with a preservation of cell density. This may be explained by a loss in neuron number secondary to increased cell death (Felderhoff-Mueser et al. 2004; Gerstner et al. 2008), free radical oxygen damage (Vento et al. 2003; Maltepe et al. 2009), or increased gliosis (Loeliger et al. 2006). In the context of growth restriction, the fluctuating oxygen may have precipitated a loss of neuronal processes, dendrites, synapses and interneurons from the inter-cellular space, hence the observed increase in nuclei density. Further work examining caspase activity, markers for oxidative stress, cortical volume analysis and staining for synaptophysin and markers of interneurons would support clarification of the underlying mechanism accounting for the thinning and increased density.

Preterm growth restricted infants are at particular risk of developing both acute and chronic respiratory morbidities (Zaw et al. 2003). These infants are also at increased risk of neurological consequences demonstrated by reduced cortical grey matter on magnetic resonance imaging (MRI) at term (Tolsa et al. 2004) and poor neurodevelopmental outcome in childhood (Yanney et al. 2004).

Within the context of growth restriction, the reduction in brain weight was accompanied by a mild, non-significant decrease in cortical laminar thickness, and a corresponding mild, increase in cell density within the cortex. It is not possible to define from this study if there was reduced cortical volume, loss of specific intercellular components, loss of white matter or enlarged ventricles. Clearly however, within the context of fluctuating hyperoxia, growth restriction is a detrimental prerequisite to normal laminar growth in rodents. Previous work does report that growth restricted offspring of dams fed a low protein diet during gestation were found to have increased cortical apoptosis, reduced synapses and a reduced numbers of both cortical neurons and glia (Liu et al. 2011). This study also suggested some of these effects could be mitigated by administration of the essential amino acid taurine which inhibits apoptosis and promotes neuronal survival.

The superficial cortical layers in humans have a larger proportion of late-born neurons than other species (Hill et al. 2005). The precursor superficial neurons travel from the cell proliferating zones, through the subplate, and deep cortical layers to

take their place in the superficial layers. The high proportion of superficial cortical neurons in humans allows a more complex architecture of inter-neuronal connections to support vast neuronal communication, key to human cognitive ability.

It was found that cortical thinning was more profound in the late born superficial layers although deep layer thinning was seen in the dual group exposed to both growth restriction and oxygen. Proliferation of neuronal progenitors destined for the cerebral cortex is complete by E16 in rats and migration occurs from E16 until E19 (Bayer et al. 1991b). Deep layer V and VI migration is largely complete by E18 and 24 - 28 weeks gestation in humans (Bayer et al. 1991b; Kostovic et al. 2006). The low protein diet was introduced on E15. The deep layer thinning observed may be explained by a preconditioning effect of growth restriction upon neurons yet to complete migration from the cell proliferating regions.

Maternal compensation may explain why there was no or minimal effect of growth restriction on the deeper layers but a more significant effect seen in the late born superficial layers. There may also be a decrease in circulating growth factors such as IGF-1 as a consequence of growth restriction (Randhawa et al. 2005). Such growth factors have neurotrophic effects and are crucial to normal cerebral development (Popken et al. 2005) and are discussed in greater detail in Chapter 7. Given the larger proportion of superficial neurons in humans, it is possible that effects seen on cortical thickness in rodents are significantly amplified in humans.

The corpus callosum, the major inter-hemispheric channel that connects the majority of cortical areas, is smaller in children, adolescents and adults who were born preterm (Nosarti et al. 2004; Caldu et al. 2006). MRI work has demonstrated that particularly in the posterior corpus callosum, fractional anisotropy was lowered, diffusivity increased and connectivity reduced in preterm infants at term. In addition, the shape of the corpus callosum in the ex-preterm infants was found to be more circular as previously reported in states of pathology including Alzheimer's disease and schizophrenia (Tomaiuolo et al. 2007; Thompson et al. 2010). Increasing white matter maturation in the developing brain is associated with increasing fractional anisotropy and overall decreased diffusivity reflecting increasing fibre density and myelination (Huppi et al. 1998). The findings in the preterm infant brain at term suggest prematurity has an effect on the macrostructure and microstructure of the corpus callosum, where higher diffusivity levels represent ongoing immaturity of the white matter tracts. In particular, intraventricular haemorrhage and white matter injury have been associated with abnormalities in the integrity of corpus callosum (Thompson et al. 2012).

The thickness of the corpus callosum was measured at its midpoint between the hemispheres at 20-40 microns caudal to the initial rostral decussation. From the most recent literature, changes in corpus callosum have been documented as posterior and therefore more posterior measurements in the coronal sections may have identified differences in thickness between the study groups.

The literature reports reductions in cross-sectional area of the corpus callosum as opposed to thinning and therefore a weakness of this study's technique is the inability to comment on corpus callosum area as measurements were made in the coronal dimension only. Differences have been suggested by MRI studies (Thompson et al. 2010) in the sub regions of the corpus callosum as summarised in Figure 38. In this study, a single measurement was made in the genus of the rat corpus callosum as shown in Figure 39 and would therefore not detect regional variation.

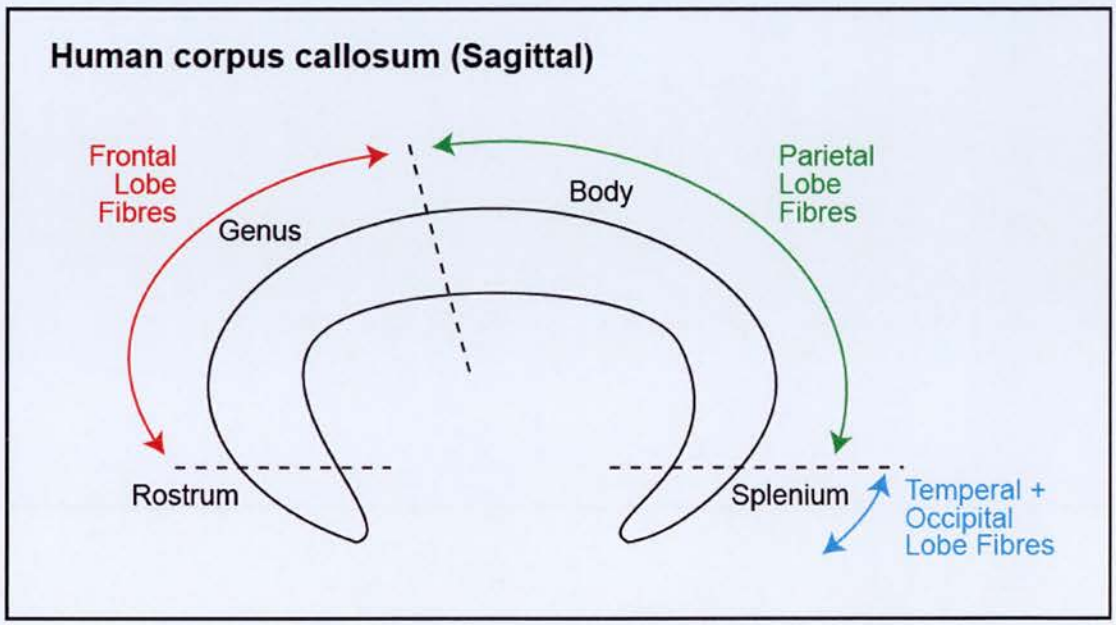

Figure 38 - Schematic sagittal view of human corpus callosum

MRI studies show that for term infants, anisotropy is highest in the splenium which is latest to develop, then in the genu which forms before the splenium and lowest in the body which is the first region of the corpus callosum to develop.

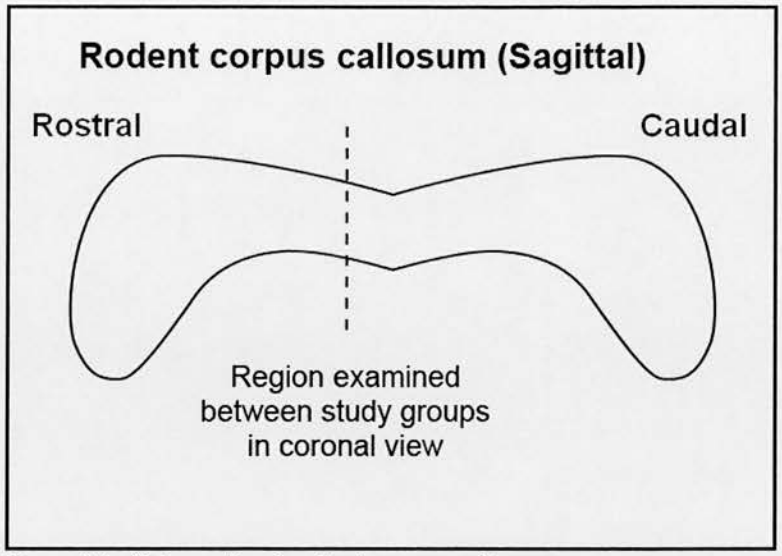

Figure 39 - Schematic of rodent corpus callosum
Measurements made on coronal section 20-40 microns caudal to corpus callosum decussation.

In this study, superficial thinning was observed in the motor cortex of the fluctuating oxygen groups with the most significant thinning observed in the dual group also exposed to growth restriction. A reduction in nuclei number was implied by the results in the ΔO2 group and therefore a corresponding reduction in associated white matter fibres may have been expected. In support of the negative findings however, a recent study examining perinatal correlates with corpus callosum structure did not find any association between corpus callosum area and presence of bronchopulmonary dysplasia or growth restriction at birth (Thompson et al. 2012). The disruption caused to brain development by premature birth causes alterations in corpus callosum integrity but to date the only perinatal variables found to be associated with this are IVH and white matter injury (Thompson et al. 2012), neither of which have been demonstrated in the study model. Furthermore, in keeping with previous literature, when infants without obvious white matter injury or IVH were studied, changes were only found in the posterior body (isthmus) and splenium of the corpus callosum (Hasegawa et al. 2011).

The explanation for this most likely lies in the developmental phases of the corpus callosum. The splenium is the last subregion to under its growth spurt which takes place in the third trimester and after birth (Volpe, 2001), enhancing it vulnerability to preterm delivery.

5.5 Summary

This work shows that a clinically relevant oxygen profile impairs cortical growth, particularly of the late born superficial laminae, resulting in a thinned cortex with maintained density and suggests an overall reduction in neuronal number. In the context of growth restriction, fluctuating oxygen caused cortical thinning, an increase in nuclear density and a nonsignificant reduction in neuron number. Growth restriction alone was associated with slight cortical thinning and a non-significant increase in nuclei density. There was no observed effect of growth restriction or oxygen on corpus callosal thickness but measurements made in more rostral sections may have shown callosal thinning.

Chapter 6:

Growth Restriction and Postnatal Oxygen Exposure on the Transcription Factor expression in the Cerebral Cortex of the Developing Brain

6.1 Introduction

Within the highly organised laminar structure of the cerebral cortex there are many different neuronal subtypes. Each layer or lamina is characterised by neuronal subtypes with distinct morphology, patterns of connectivity and gene expression. The formation of the laminae in the developing cortex is dependent on the precise proliferation and migration of specific cortical neuron subtypes. When precursor cells within the proliferating zones (ventricular zone (VZ) and subventricular zone (SVZ) as discussed in Chapter 1) commit to a definitive cellular fate, specific transcription factors are expressed within the neural cell and direct the cell to undergo key cellular events specific to the subtype function. Expression of these specific transcription factors allows particular cell subtypes to be identified and studied.

As outlined in Chapter 1, deep layers V and VI of the cortex generally contain large projection neurons which project axons to subcortical targets including the thalamus, striatum, pons, spinal cord and the contralateral cerebral hemisphere (Molnar et al. 2006). Layer IV is the major site to receive thalamic input and superficial layers II-IV are generally composed of smaller neurons that form more localised intra-cortical connections (Gilbert et al. 1979; Lewis et al. 2002). It is thought that upper layer neurons are a recent addition to evolutionary cerebral development, important to cognition and higher functioning in mammals and especially humans (Aboitiz et al. 2003) and that deep layer VI and V projection neurons serve similar functions to pyramidal neurons in a more primitive cortex.

The preterm infant brain may suffer both injury and interruption of normal development (Volpe JJ, 2009b). Damage to white matter structures, particularly the internal capsule and the finding of PVL, is associated with motor deficits, including cerebral palsy. It has been shown that axonal injury occurs in the white matter beyond the insult focus, therefore contributing to the white matter volume reduction (Haynes et al. 2008). This suggests that PVL-related brain injury causes disruption to particular types of neuron such as the corticospinal projection neurons originating from layer V of the motor cortex. Grade III / IV intraventricular haemorrhage and hydrocephalus also cause white matter damage and will impact on specific neural cell types (Hack et al. 2000). It is now recognised that damage to developing white matter, even in the form of diffuse noncystic PVL, does not occur in isolation, but has significant implications for the development of other brain areas including the overlying cortex, where complexity is reduced, and the thalamus (Nasgasunder et al. 2012). The thalamus feeds and receives crucial input to and from the cortex via white matter fibres such as the corticothalamic tracts originating in layer VI of the cortex (Volpe JJ, 2009b). Furthermore, in perinatal ischaemic stroke, hemiparesis is more likely if there is simultaneous damage to the posterior limb of the internal capsule, the basal ganglia and the motor cortex (Boardman et al. 2005); reinforcing the concept that white matter injury is linked to impaired grey matter function and together is associated with poorer neurological outcome. As discussed previously, cognitive deficits are now recognised as highly prevalent in the ex-preterm population at school-age and occur both in the presence and absence of focal parenchymal white matter lesions (Volpe, 2009b).

Previous work in preterm infants has suggested a correlation between poor cortical growth in the perinatal period and increased cognitive deficit at ages 2 and 6 years (Rathbone et al. 2011), linking integrity of neuronal populations and their connectivity with cognition. The development of cortico-cortical connectivity in humans is most active during the later stages of gestation from 33 weeks and continues postnatally. This involves completion of migration, dendrite maturation and synaptogenesis (Kostovic et al. 2006).

In Chapter 1, the effect of preterm birth and perinatal factors such as growth restriction and oxygen on neurodevelopmental outcome, cognitive function, imaging and pathological correlates have previously been discussed (Ajayi-Obe et al. 2000; Vento et al. 2003; Inder et al. 2005). In an attempt to define whether particular subtypes are more vulnerable to damage, the effects of growth restriction and fluctuating oxygen on specific neuronal subtypes within the developing cortex are now examined.

Four transcription factors were selected as markers of the major neuronal subtypes in the motor cortex. Transcription factor Cut-like-1 (Cux-1) is selectively expressed in the pyramidal neurons of the neocortex superficial layer progenitors and projection neurons (Cubelos et al. 2010a; Li et al. 2010). Callosal interhemispheric projection neurons are specified by Special AT-rich sequence-binding protein 2 (Satb2) in superficial and deep layers of the cortex (Fame et al. 2010). Corticospinal neurons in

layer V express Chicken ovalbumin upstream promoter transcription factor-2 (Ctip2) (Shoemaker et al. 2010) and transcription factor T-box brain1 (Tbr1) promotes identity of corticothalamic neurons (McKenna et al. 2011). These are summarised below in Table 11 and Figure 40.

Table 11 – Neuronal subtype and corresponding marker transcription factor.

Neuronal Subtype	Transcription Factor
Superficial Projection neurons	Cux-1
Callosal neurons	Satb2
Corticospinal neurons	Ctip2
Corticothalamic neurons	Tbr1

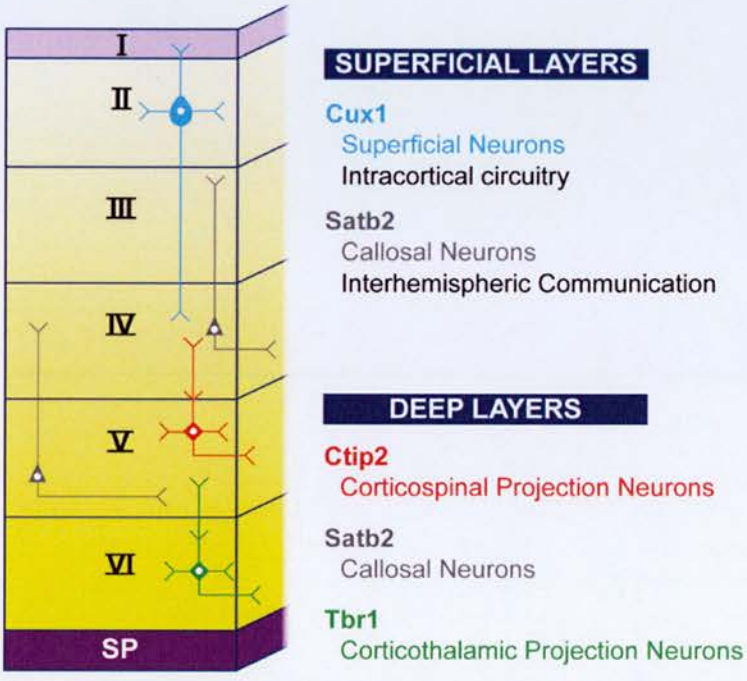

Figure 40 - Neuronal subtype markers and location in the cortex.

Each transcription factor is specific for a neuronal subtype within the discrete layers. Method established by Dr Georgala et al. in work examining embryological cortical development, see section 3.6.

The aim of this study is to determine the effect of growth restriction and postnatal fluctuating hyperoxia on specific neuronal subtypes in the motor cortex. Given the effects noted on laminar thickness in the superficial layers in Chapter 5, it was hypothesised that markers of superficial neurons, Cux1 and Satb2 would be more susceptible to these environmental conditions than the markers of deep layer neurons, Ctip2 and Tbr1. It was however considered important to measure deep layer markers to provide a reference and in view of the mild thinning observed in the dual group.

6.2 Methods

Rat brains were obtained from the animals in each study group as described in Chapter 2. Pups were perfused with fixative, the brain treated with fixative, cryoprotected and then snap-frozen in 2-methylbutane (section 2.6.2). Coronal cryosections at 20μm thickness were obtained as described in section 3.6.1. Comparable sections at the level of the motor cortex were selected and immunohistochemistry with primary antibodies, fluorescently labelled secondary antibodies and counterstain was performed (section 3.6.2). Imaging capture was achieved by confocal laser scanning microscope. Adobe photoshop was used to perform blinded total nuclei and transcription factor labelled nuclei counts within a 100μm diameter box placed in the centre of the motor cortex. Proportions of transcription factor labelled nuclei relative to total nuclei were compared between study groups.

During the immunohistochemistry step, there were initial challenges in optimising antigen retrieval. Fixation of the tissue to preserve morphology can lead to loss of immunoreactivity of many antigens. This is secondary to unpredictable cross-linking of proteins within the tissue (Key and Boenisch, 2006). 'Antigen retrieval' describes the process of restoring immunoreactivity without destroying epitopes (Shi et al. 1991). The most common technique used is applying heat near the boiling point of water in the presence of a citrate buffer at pH6.0 which protects protein from heat-induced denaturation (Cattoretti, 1994).

In this current study, microwave retrieval was initially attempted for 20 mins; 5 min intervals at 50% power. However despite good quality immunofluorescent staining, the brain sections became disrupted and large areas were lost from the slides. A steaming protocol was then trialled where slides were pre-immersed in citrate and placed in an enclosed steamer at 90°C for 15-25 mins (range of times attempted). The brains sections remained on the slides, but staining was poor. The microwave protocol was then reverted back to and optimal staining was achieved using the following settings: 1x 5 min 50% power, 1x 5min 30% power and 2x 5min 10% power.

6.3 Results

The specificity of staining for all the secondary antibodies was examined as shown in Figure 41. The microscope settings were unaltered between images. This confirms the secondary antibody used for the selected primary antibody was specific in all cases.

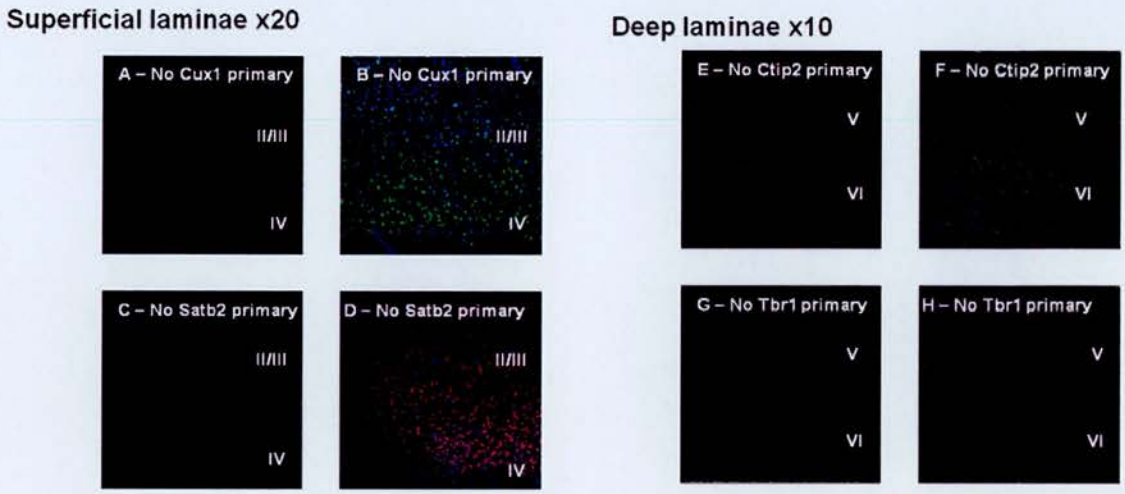

Figure 41 - Secondary only controls for transcription factor primary antibodies

A – Cux primary absent with 568nm secondary (red) – no nuclear staining.
B – Cux primary absent with 488nm secondary (green, Satb2) & Topro-3 nuclear counterstain (blue).
C – Satb2 primary absent with 488nm secondary – no nuclear staining.
D – Satb2 primary absent with 568nm secondary (red, Cux1) & Topro-3 nuclear counterstain (blue).
E – Ctip2 primary absent with 568nm secondary (red) – no nuclear staining.
F – Ctip2 primary absent 488nm secondary (green, Tbr1) & Topro-3 nuclear counterstain (blue).
G – Tbr1 primary absent with 488nm secondary – no nuclear staining.
H – Tbr1 primary absent with 568nm secondary (red, Ctip2) & Topro-3 nuclear counterstain (blue).

II/III, IV, V, VI correspond to cortical layers.

There was no misplaced staining, confirming that migration of neurons to the correct layer had taken place and that the ontological process of cortical lamination was not affected by environmental influences.

In particular, in view of the deep to superficial pattern of cortical development where superficial cells migrate through the deep layers, it was confirmed that there was no evidence of Cux1 staining in the deep layers in any of the study groups as shown in Figure 42 (Dual group). The bright red spots represent artefact and did not correspond to a nuclei.

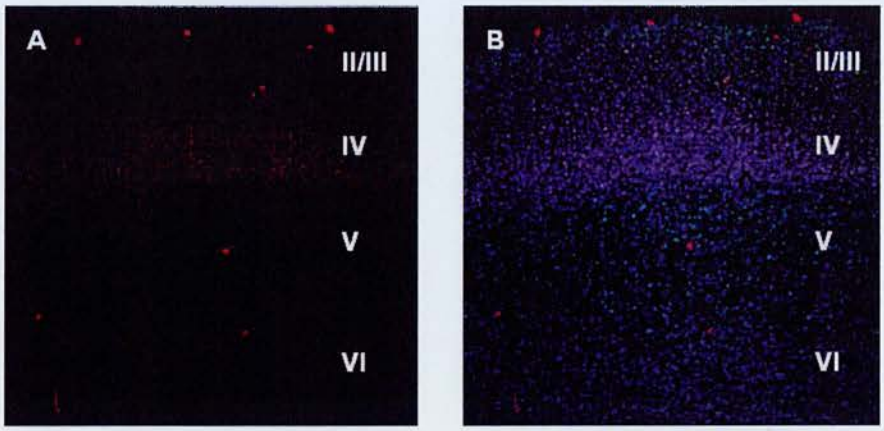

Figure 42 - Cux1 staining absent from deep cortical layers (x10 magnification).

A – Cux1 staining present in superficial layers II/III and IV. No Cux1 staining in deep layers V & VI.
B – Overlay of Cux1 staining, Satb2 staining (green) and Topro-3 nuclear stain (blue). Satb2 and Topro-3 staining present in all layers, including deep layers V and VI.
Bright red areas represent artefact.

6.3.1 Deep layer nuclei

Three transcription factors, Tbr1 expressed primarily in corticothalamic projection neurons in layer VI, Ctip2 expressed in corticospinal projection neurons in layer V and Satb2 expressed in layers V and VI were selected as markers of nuclei in the deep layers of the motor cortex. The proportion of neurons positively labelled with subtype transcription factor within the deep cortical layers was compared between each of the study groups. Table 12 shows the mean and SD in each study group for Ctip2 in layer V and Tbr1 in layer IV and demonstrates that the proportion of labelled neurons was not affected by exposure to GR or O2.

Table 12– Proportions of Ctip2 and Tbr1 in deep cortical layers

Study Group	Ctip2 / Layer V nuclei (mean±SD)	Tbr1 / Layer VI nuclei (mean±SD)
RA 18%	0.28 ± 0.04	0.50 ± 0.04
ΔO2 18%	0.27 ± 0.03	0.49 ± 0.04
RA 9%	0.27 ± 0.04	0.53 ± 0.06
ΔO2 9%	0.27 ± 0.03	0.50 ± 0.05

No differences were found in the proportion of Tbr1 or Ctip2 stained nuclei as shown in Figure 43.

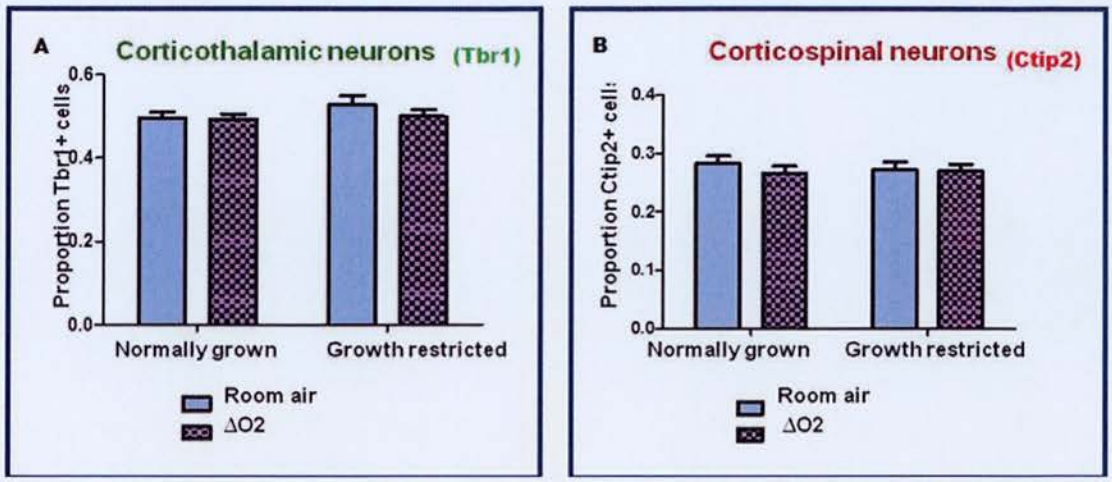

Figure 43 - Deep cortical layer labelled nuclei
Proportion of positively labelled nuclei against Study group (x axis). Data presented as mean ±SD. 2-way ANOVA demonstrates no significant variation in dataset $p > 0.05$.
Graphs A and B demonstrate no difference between study groups in the proportion of nuclei expressing Tbr1 (layer VI), or Ctip2 (layer V), in the rodent cerebral cortex.

6.3.2 Callosal Nuclei

Callosal neurons, identified by transcription factor Satb2 are present in all layers of the cortex. The proportion of neurons positively labelled relative to the total number of neurons within the superficial and deep layers respectively are shown in Table 13.

Table 13 - Proportions of Satb2 in deep and superficial cortical layers

Study Group	Satb2 / Layer V &VI nuclei (mean±SD)	Satb2 / Layer II-IV nuclei (mean±SD)
RA 18%	0.58 ± 0.01	0.67 ± 0.01
ΔO2 18%	0.59 ± 0.02	0.66 ± 0.01
RA 9%	0.58 ± 0.05	0.68 ± 0.02
ΔO2 9%	0.59 ± 0.03	0.66 ± 0.03

No differences were found in the proportion of Satb2 stained nuclei as shown in Figure 44.

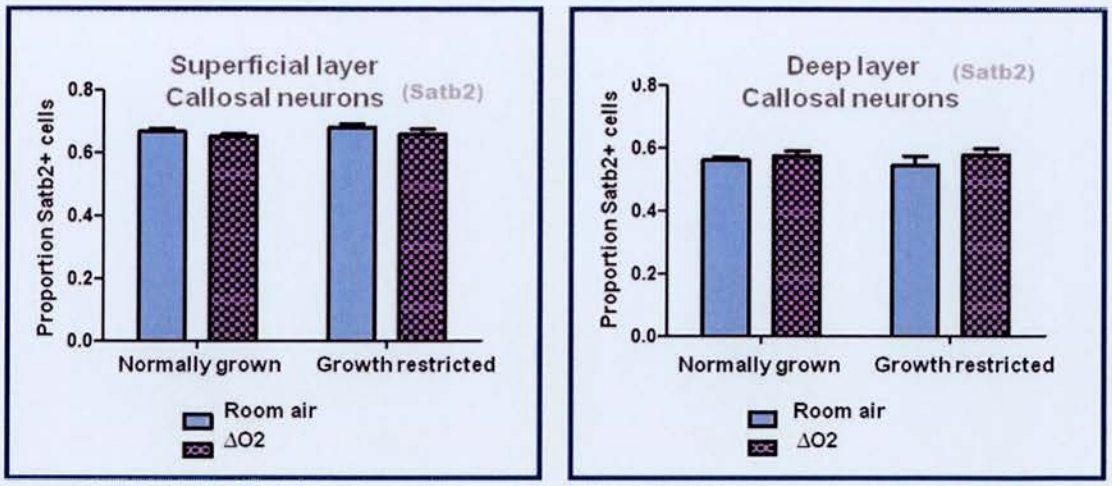

Figure 44 – Callosal nuclei in superficial and deep cortical layers

Proportion of positively labelled nuclei against Study group (x axis). Data presented as mean ±SD. 2-way ANOVA demonstrates no significant variation in dataset $p > 0.05$.
Graphs demonstrate no difference between study groups in the proportion of Satb2 positive nuclei in the rodent cerebral cortex.

6.3.3 Superficial layer nuclei

Significant differences were noted in the proportion of labelled Cux1 nuclei in superficial layers II-IV. Table 14 presents the proportion of positive nuclei relative to the total and the mean value and SD in each study group of Cux1 positive (Cux1+) nuclei and total nuclei in II-IV.

Table 14 – Cux1 positive nuclei in layers II-IV

Study Group	Cux1 / Total nuclei (mean±SD)	Cux1 nuclei (mean±SD)	Total II-IV nuclei (mean±SD)
RA 18%	0.52 ± 0.05	215 ± 32.22	417 ± 59.8
ΔO2 18%	0.46 ± 0.04	163.8 ± 18.27	355.6 ± 38.2
RA 9%	0.49 ± 0.03	206.2 ± 30.47	421.8 ± 74.08
ΔO2 9%	0.34 ± 0.03	129.9 ± 14.42	383.6 ± 41.25

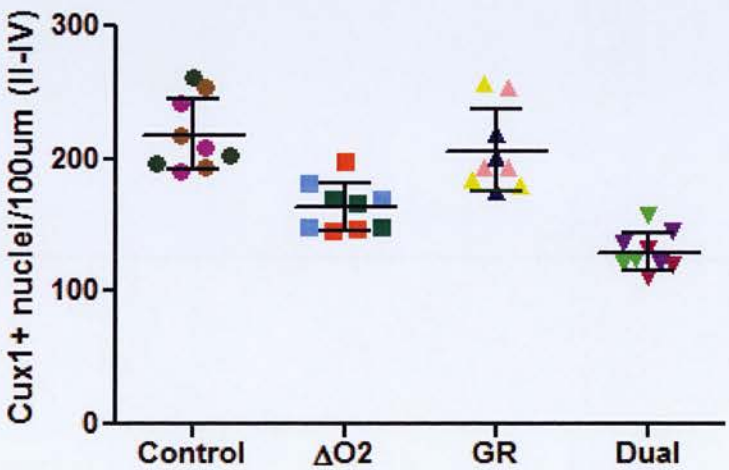

Figure 45 - Cux1+ nuclei in Layers II-IV

Cux1+ nuclei in 100μm diameter box overlying layers II-IV of motor cortex (M1) against study group on x axis. Each data-point represents an average of 3 counts per pup. Each different colour represents a different litter, confirming no major outliers between the litters in each study group.
The values for each group are normally distributed by D'Agostino and Pearson omnibus normality test. Data shows mean ± standard deviation (SD), n=9 per group.
Figure 45 shows spread of data. Statistical analysis not shown here – see Figure 46.

Statistical analysis by 2-way ANOVA suggested significant variation within the study groups which were then compared using Bonferroni post-tests. A significant difference was noted in the proportion of Cux1 expressing nuclei in the fluctuating oxygen group in comparison to control, $p < 0.01$ by 2-way ANOVA and a marked reduction in the proportion of Cux1 nuclei was observed in the dual study group exposed to both growth restriction and oxygen, $p < 0.001$. Within the fluctuating oxygen groups, growth restriction further impaired nuclear expression of Cux1 as demonstrated below ($p < 0.001$).

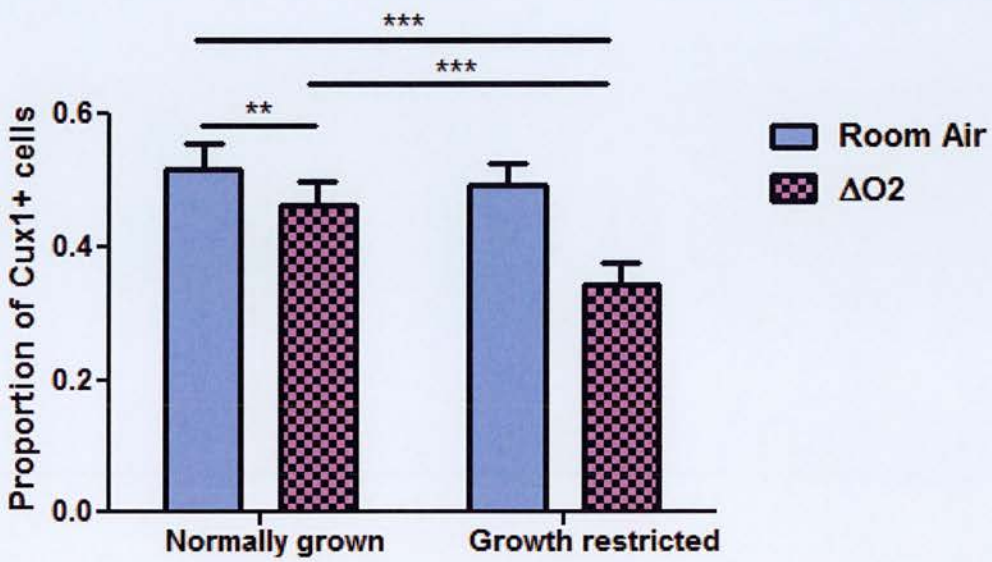

Figure 46 - Proportion of Cux1+ nuclei in layers II-IV.

Proportion of Cux1 positive nuclei against study group on x axis.
Data shows mean ± standard deviation (SD), n=9 per group.
2-way ANOVA confirms variation present in dataset $p < 0.05$. Bonferroni post-test analysis to compare groups, **$p<0.01$, ***$p<0.001$.
The proportion of Cux1 labelled nuclei was reduced in both groups exposed to fluctuating oxygen, and the greater effect seen in the dual group also exposed to growth restriction.

6.4 Discussion

6.4.1 Summary and context of findings

In this study, neuronal subtype populations in the cortical layers were examined and it was found that there was no observed effect of growth restriction or oxygen on the proportion of neurons expressing transcription factors marking deep layer neurons. Similarly, there was no effect of the environmental conditions on the proportion of Satb2 positive nuclei as a marker of callosal neurons in the superficial layers. However, there were significant effects observed in the proportion of superficial neurons expressing transcription factor Cux1:

- Fluctuating oxygen (ΔO2) caused a significant reduction in the proportion of Cux1 expressing neurons (Cux1+).
- The reduction in Cux1+ was most marked in the dual group where pups were also growth restricted.

In previous experiments using this rodent model it was shown that ΔO2 does not affect gross brain weight (Table 6 and Figure 25), but does cause superficial cortical thinning with a preservation of cell density (Figures 32, 33, 35, 36 and Tables 9 and 10). This implies there may be a loss of cells from cortical layers II-IV in this group. A reduction in the proportion of Cux1 nuclei in these superficial layers does not confirm or refute cell loss but does mean that expression of this transcription factor by neurons in the superficial cortex is affected.

In the dual group pre-exposed to growth restriction and then ΔO2, a reduction in gross brain weight was observed (Table 6 and Figures 24 and 25), significant cortical thinning but with a proportional increase in layer II-IV cell density and a suggestion (although not statistically significant) of cell loss from these layers (Figures 31, 32, 33, 35 and Tables 9 and 10). The marked reduction in the proportion of Cux1 expressing neurons similarly suggests disruption to superficial neurons within the superficial layers.

6.4.2 Aetiology of Reduced Cux1+

The reduction in the proportion of Cux1+ expressing neurons seen in the ΔO2 and dual study groups has not been established in this study, however two mechanisms are hypothesised:

A – **Reduced Cux-1 expression** by superficial neurons present in cortex, or,

B - **Loss of Cux-1 expressing neurons** through failed migration or cell death.

A – Reduced Cux-1 Expression

There are a number of markers in addition to Cux-1 which specify post-mitotic cells in layers II-IV, including transcription factors Cux-2 and LIM homeobox 2 (Lhx2). Cux-1 was selected for its specificity to superficial layer projection neurons.

Neurons expressing Cux-1 are negative for interneuron markers suggesting Cux1 specifically labels pyramidal projection neurons whereas Cux2+ neurons have also been shown to co-express interneuron markers (Nieto et al. 2004). Lhx2 is expressed throughout cerebral development and provides an example of how a single transcription factor can have differing roles at specific time points in development. In addition to specifying postmitotic superficial cortical neurons, Lhx2 is also involved in the embryonic onset of neurogenesis within the proliferating VZ and SVZ and also in establishing neocortical identity of all cortical layers (Monuki et al 2001). Despite Cux-1 therefore being the most specific transcription factor for superficial neurons, expression of one of the other markers in place of Cux-1 may indicate preserved function. There is evidence form Cux1 knockout and knockdown rodent studies that Cux-2 may compensate for Cux-1 (Nieto, 2004). Identification of proportions of nuclei positively expressing Cux-2 and / or Lhx2 would elucidate whether exposure to growth restriction and oxygen fluctuation affected (1) absolute numbers of superficial neurons and (2) preferential expression of key transcription factors by these neurons.

Identifying other cell types present in the superficial layers including interneurons and glial cells would also intimate whether there may be increased proportions of other post-mitotic cells. Animal work in a baboon model has previously linked respiratory support to the development of cortical gliosis and the persistence of radial glia within the cortex (Loeliger et al. 2006) and staining for glial cell types may therefore provide further information.

B - Loss of or failed migration of Cux1+ neurons

A reduction in Cux-1 expression particularly when combined with a reduction in other superficial layer transcription factors might be explained by a reduction in neurons within the superficial layers. This may occur through a process of cell death or through failure of neuronal migration during the later stages of cortical development. These hypotheses were not explored in this study, but measuring markers of cell death such as caspase-3 would provide evidence of cell death and could be supplemented with dual staining of neurons, astrocytes and microglia to fully characterise the cellular response. In the absence of cell death, the integrity of neuronal migration could be examined using Bromodeoxyuridine (BrdU) birthdate labelling to dams around the time of superficial neuronal migration (in rats E16-E18, Figure 4) and the laminar distribution of BrdU labelled cells examined at P7 along with laminar specific markers (Georgala et al. 2011). BrdU is incorporated into cells during S phase of the cell cycle, i.e. proliferating cells undergoing DNA replication. There was no evidence found of Cux-1 staining in the deep layers V or VI or in the subplate thereby excluding arrested migration of Cux-1 expressing cells (Figure 41). The identities of the individual progenitor populations that give rise to many projection neuron subtypes are largely unknown (Molyneaux et al. 2007). It is known that both Cux-1 and 2 are expressed by superficial layer progenitors in the proliferating SVZ but not the VZ from E13-E17 during the time of superficial neuron generation (Nieto et al. 2004). Therefore it is possible that the introduction of the low protein diet had an effect on the SVZ neurons that was not significantly seen in this model in the context of growth restriction alone but was with the additional

insult of postnatal oxygen fluctuation. There is evidence that pups of dams fed a low protein diet in pregnancy had lower numbers of dendrites and lower cortico-cortico evoked potentials (Resnick et al. 1982) which fits with reduced connectivity.

Although Cux-1 specifies post-mitotic superficial projection neurons, there are many other crucial transcription factors upstream of Cux1 in determining superficial neuron fate. In particular, Paired-box-6 (Pax-6) has multiple roles throughout development including establishing the identity of cortical progenitors and determining cortical arealisation (Muzio et al. 2002). In Pax6 mutants, deep-layer neurons are produced normally. The thickness of superficial cortical layers is however reduced and there is a global decrease in superficial layer neurons including a reduction in Cux-1 and Cux-2 expressing neurons (Nieto et al. 2004). In addition, it has been shown that overexpression of Pax-6 is equally damaging to cortical development and causes a reduced number of superficial neurons with no effect on laminar fate (Georgala et al. 2010). Recent work has shown that intermittent hypoxia (alternating 21% and 10% oxygen) increases the expression of Pax6 in neurons from the SVZ in P4 mice (Ross et al. 2012). It is therefore possible that the fluctuating hyperoxia / hypoxia profile caused disruption to Pax6 expression which in turn affects Cux-1 subtype identity and superficial layer thickness. There are no published reports documenting the effect of protein / nutrient restriction and subsequent growth restriction on gene expression of cortical transcription factors. As discussed in Chapter 5, one group reports that a low protein diet is associated with increased apoptosis, decreased synaptic density and fewer neurons (Liu et al. 2011).

In addition, as referenced above, offspring of dams fed a low protein diet have also been shown to have lower numbers of dendrites and lower cortico-cortico evoked potentials (Resnick et al. 1982). Although a statistically significant effect on cortical thickness in the 'GR only' group was not observed, there was a mild reduction in thickness and increased nuclear density, suggesting some loss of intercellular matrix. The additional insult of ΔO2 may further affect neuronal subtype integrity reducing Cux1 expression with further impact on the extracellular matrix (Cubelos et al. 2010b).

One of the main strengths of this study is the clarity of the effect on the proportional expression of Cux-1 on the background of no effect seen in the other transcription factors. This demonstrates the Cux-1 effect as convincing and is in keeping with the detrimental effects already observed on cortical thickness in the preceding Chapter. The superficial cortical layers are hugely expanded in humans in comparison to rodents and therefore effects seen in animal models hold crucial keys to understanding adverse effects on superficial layers in humans.

6.4.3 Consequences of reduced Cux1

Cux-1 is expressed in most tissues of the body (Nepveu, 2001) and expression and activity is regulated through the cell cycle. Cux-1 is involved in cell cycle progression and cell motility and has roles in the control of cell proliferation and differentiation (Sansregret et al. 2008). As a transcription factor, Cux1 acts as both a gene repressor and activator and it is not yet clear how this is determined (Sansregret et al. 2008). The gene was first studied in the Drosophila (Cut-like1) (Nepveu, 2001). The human homologue was initially found to be CCAAT-displacement protein (CDP) and is now known as Cux-1 in keeping with other mammals. Cux-1 knock-out studies in mice demonstrate a number of findings based on the effect caused by the knock-out on the protein. At the mildest end, a truncated protein causes wavy hair, curly whiskers and impaired lactation resulting in a high death rate in the litters (Tufarelli et al. 1998). The wavy hair was due to disrupted hair follicle morphogenesis. A further knock-out mouse model showed that homozygotes had a high death rate soon after birth, demonstrated failure to thrive secondary to muscle wasting, loss of body fat and thin bones and had curly whiskers and hair loss. The pups also had an increased risk of bacterial infection as a result of increased apoptosis of B cells in the bone marrow and T cells in the thymus (Sinclair et al. 2001). The third knock-out mouse model defined the cause of the high lethality observed in the other models as being a consequence of delayed lung development causing respiratory failure (Ellis et al. 2001). In humans, increased expression of Cux1 has been reported in tumours of breast and pancreatic origin with roles in tumour initiation and progression (Michl et al. 2005; Ripka et al. 2010) and Cux1 has

also been implicated in the aetiology of autosomal dominant polycystic kidney disease due to its role in maintaining microtubule dynamics (Alcalay et al. 2008).

It is only recently that Cux-1 and Cux-2, both related homeobox genes, have been identified as having key roles in cortical brain development (Cubelos et al. 2010b; Li et al. 2010). Expression of Cux transcription factors specifies superficial neuronal cell identity and Cux2 is specifically required for proliferation of superficial layer neurons in the SVZ (Cubelos et al. 2010b; Cubelos et al. 2008). Knock-out studies of Cux-1 and Cux-2 show that in the absence of either gene, there is reduced complexity of dendritic branching, simpler dendritic tree morphology, reduced dendritic spines, all elements required for interneuronal communication and the establishment of cortical circuitry (Cubelos et al. 2010a, Cubelos et al. 2010b). Given the high mortality associated with the Cux-1 knock-out, behavioural testing could only be observed in the Cux-2 knock-out mice who demonstrated working memory deficits (Cubelos et al. 2010b). An in-vitro experiment suggested contradictory findings where increased intracellular level of Cux1 reduced the dendritic complexity of cultured cortical pyramidal neurons (Li et al. 2010). This was an in vitro study in contrast to cortical neurons in vivo which function as part of a global cortical system. It is likely that the positive effects of Cux-1 on dendritic growth are likely to depend on the synaptic inputs to a given neuron (Hulea et al. 2012).

The reduced proportion of Cux-1 neurons in the superficial cortical layers in the ΔO2 and dual study groups of our model has implications for the dendritic integrity in the superficial cortical layers of these pups. Dendritic integrity is crucial in cortico-cortical circuitry and given that the superficial layers are generally considered to be the seat of higher cognitive functioning, deficits in such circuits will inevitably have consequences for function and behaviour.

6.4.4 Implications for humans

Cux genes are homeobox genes, widely conserved between species. Much less work has been done on Cux genes in humans, but it is known that Cux proteins are specific markers for the superficial layers in the human cortex, namely layers II and III and are expressed into adulthood (Arion et al. 2007). Human studies of nonsyndromic children with developmental delay have consistently reported dendritic abnormalities at post-mortem examination (Dierssen et al. 2006). This observation dates back to the 1970s, when researchers reported "dendritic spine dysgenesis" in children with nonspecific developmental delay to describe the appearance of long, thin, immature dendritic spines in contrast to short stubby dendritic spines found in children without learning difficulties (Purpura, 1974). In addition, other researchers reported reduced dendritic branching and complexity in children with developmental delay and seizures (Huttenlocher, 1974). More recent studies link the morphological shape of dendritic spines to memory function (Segal, 2005).

As far as can be ascertained, there are no specific studies in the literature examining dendritic morphology in preterm infants. Dendritic spines, as well as cortico-cortical connections, and synapses all develop during the neonatal period (Kostovic et al. 2007). There is however a substantial literature linking preterm birth to a reduction in cerebral cortical grey matter volume, and cortical thinning in adolescence affecting specific brain areas (Inder et al. 2005; Kapellou et al. 2006; Nagy et al. 2011; van Kooij et al. 2012). Loss of tissue may reflect either cell loss or loss of intercellular matrix containing intracellular connections. Specific areas of cortex affected by preterm birth include sensorimotor, premotor and orbitofrontal cortical grey matter (Thompson et al. 2007). In addition, recent MRI work indicates that reduced thalamic volume in preterm infants is a predictor for reduced cortical volume, reduced volume of both the frontal and temporal lobes and the hippocampus (Ball, 2012a). A subsequent study by the same research group used tractography MRI studies to demonstrate reduced thalamocortical connectivity specifically affecting the premotor and supplementary motor areas and the lateral frontal cortex (Ball, 2012b). Preterm infants with reductions in cortical and deep grey matter volume have an increased risk of poor neurodevelopmental outcome at 1 year (Inder et al. 2005) and a reduced intellectual score at age 8-10 years (Soria-Pastor et al. 2009). Such cognitive deficits may be a consequence of the premature interruption of cortical development and the effect of subsequent perinatal events on cortico-cortico superficial neuron connectivity and cortical circuitry involving other key cognitive structures such as the thalamus.

6.5 Summary

This Chapter has described that within the superficial cortical layers, postnatal fluctuating oxygen causes a reduction in the proportional expression of Cux1, a transcription factor key to the integrity of intracortical connectivity. In the context of pre-existing growth restriction, the reduction in Cux1 expression is more severe. Although the mechanism underlying the reduction have not been established in this work, inactivation of transcription factor expression by existing cells, failure of migration or cell death may be responsible. A reduction in Cux1 expression may lead to disrupted dendritic spine integrity, impaired neuronal circuitry and cognitive dysfunction.

Chapter 7:

The Effect of Growth Restriction & Postnatal Oxygen Exposure on Trophic Factors in the Developing Brain

7.1 Introduction

In this study, four key trophic factors for fetal cerebral growth were selected to investigate the effects of growth restriction and postnatal oxygen therapy on cerebral growth factor expression within the animal model. Trophic factors are substances provided in limited quantities by target cells to control the development and survival of specific groups of neurons in normal brain development. These factors regulate neuronal numbers by ensuring the survival of the correct number of neurons and allowing selective death of neurons not required (Bear et al. 2001). In Chapter 1, a description of the role of each of the trophic factors in brain development was given. In this section, a literature review of the specific effects of oxygen and growth restriction on each trophic factor is presented.

1 - Insulin-like Growth Factor I (IGF-I) is a peptide involved in neuronal progenitor proliferation, development and survival.

2 - Brain Derived Neurotrophic Factor (BDNF) is a neurotrophin, part of a family of intercellular messengers which promote survival and differentiation of specific cell subpopulations during neural development.

3 - Fibroblast growth factor (FGF) has significant trophic effects important to neurogenesis and also acts as an angiogenic factor.

4 - Vascular Endothelial growth factor (VEGF) is a signalling protein, key to vasculogenesis and angiogenesis.

Trophic factors involved in brain development work in concert with one another and therefore the effect on any individual growth factor has sequential effects on other trophic factors and therefore the developing neurons each factor regulates.

7.1.1 Insulin-like Growth Factor I (IGF-I)

IGF-I has pleiotrophic effects on brain development. It is produced by the fetal liver, locally by cortical and other CNS neurons and by endothelial cells of the cerebral vasculature. Previous work in a similar rodent model examining the effect of growth restriction and oxygen on early retinal blood vessel growth demonstrated delayed retinal vascularisation, particularly in pups exposed to fluctuating oxygen. This poor retinal vascularisation was associated with poor weight gain (Dhaliwal et al. 2011). Other studies have linked poor retinal vascularisation in ROP to low levels of circulating IGF-I, and that low levels of IGF-I are associated with more severe ROP (Hellstrom A, Low IGF-I suppresses VEGF-survival signalling, Prc Natl Acad Sci, 2001).

IGF-I - Relationship to Growth restriction & Oxygen

Studies of IGF-I mutations in humans are associated with intrauterine growth restriction, microcephaly and learning impairment (Walenkamp et al. 2005). The placenta produces a growth hormone which differs from mainstream Growth Hormone (GH) by 13 amino acids. This is only present in the maternal circulation but is reportedly low in IUGR pregnancies. The role of placental GH in relation to

regulation of the fetal IGF system is poorly understood (Westwood M, 2001). Infants who are small for gestational age (SGA) have lower levels of IGF-I in cord blood (Davidson et al. 2006). Such SGA infants also have reduced cerebral cortical volume and impaired cognition in comparison to appropriately grown preterm infants (Tolsa et al. 2004). Given the pleiotrophic roles IGF-I plays in the developing brain, it would seem plausible that lower levels in SGA infants may affect normal cerebral development with particular reference to the key role played in progenitor proliferation, development and survival.

In preterm infants, supplemental oxygen therapy has been demonstrated as an independent risk factor for impaired brain growth and loss of brain tissue volume at term (Boardman et al. 2007). Infants with bronchopulmonary dysplasia (BPD) have been shown to have lower concentrations of serum IGF-I (Lofqvist et al. 2012). From in vitro models, there is evidence that IGF-I is protective to cortical neurons exposed to hypoxia and re-oxygenation (Huang et al. 2012). IGF-I expression is reduced in the retinal vessels of rodent pups exposed to 75% oxygen who developed retinopathy of prematurity (Lofqvist et al. 2009). In hypoxic-ischaemic neonatal brain injury models, IGF-I mRNA expression is increased in regions where delayed neuronal and glial cell loss occur. The IGF-I was found to be produced by reactive microglia and astrocytes adjacent to surviving neurons encircling the area of ischaemia (Beilharz et al. 1998). Recent work has shown that exogenous IGF-I delivered to neonatal rats with an LPS-induced acute brain inflammatory response causes reduced neuronal cell loss (Pang et al. 2010). In rodent models of traumatic

brain injury, low IGF-I serum levels correlate with a poorer cognitive outcome (Ozdemir et al. 2012) and IGF-I administration after traumatic brain injury in rats is neuroprotective (Rubovitch et al. 2010).

It was hypothesised that growth restriction and oxygen fluctuation will have an adverse effect on IGF-I expression in the developing brain and on circulating levels within the serum.

7.1.2 Brain-Derived Neurotrophic Factor (BDNF)

BDNF has a neuroprotective role in the developing brain. Preterm infants have been found to have lower levels than term infants (Malamitsi-Puchner et al. 2004) and BDNF serum levels correlate with several antenatal and postnatal factors that influence neurodevelopmental outcome (Rao et al. 2010).

BDNF - Relationship to Growth Restriction & Oxygen

It has been previously argued that growth restriction does not have a specific effect on BDNF because of the brain-sparing effect (Malamitsi-Puchner A et al. 2004). However there is literature to support a link between nutrient intake and BDNF expression in the brain where dietary restriction is associated with increased levels of BDNF (Lee et al. 2002) and a high fat diet causes reduced levels of BDNF perhaps due to an increase in free radicals as a consequence of the high fat diet (Wu et al. 2004). Oxidative stress has also been previously reported to reduce BDNF levels (Wu et al. 2006). It was therefore hypothesized that fluctuating oxygen would have an adverse effect and growth restriction a positive effect on BDNF expression in the brain.

7.1.3 Fibroblast Growth Factor-2 (FGF2)

FGF-2 is highly expressed in both the developing and mature brain. FGF-2 is one of 22 members of the fibroblast growth factor family and has important roles in early cerebral patterning and as a trophic factor for neuronal and particularly glial progenitors in the SVZ (Tao et al. 1997; Kuhn et al. 1997). More recently, the role FGF-2 and other members of the fibroblast growth factor family play in synaptogenesis has become appreciated (Li A et al. 2002). FGF-2 also acts in concert with VEGF as a potent angiogenic factor (Kalghatgi et al. 2009). Within the developing and adult brain, FGF-2 promotes the development of learning and memory (Graham et al. 2010).

FGF2 - Relationship to Growth restriction & Oxygen

The protein restriction diet used in the experimental model is known to cause an increase in maternal glucocorticoids (Langley-Evans et al. 1994). Prenatal exposure to glucocorticoids has been shown to cause an acute upregulation in FGF-2 expression in specific brain regions (Molteni et al. 2001). Placental expression (Barut et al. 2010) and circulating FGF-2 levels from the umbilical vein (Wallner et al. 2007) are increased in SGA infants. Following ischaemic injury by unilateral ligation of the carotid artery, FGF-2 administered systemically prevents ischaemic related damage (Nozaki et al. 1993). This suggests that despite the abundant local expression of FGF-2 throughout the developing brain, circulating levels of FGF-2 are important for neuroprotection.

Following perinatal asphyxic episodes in rat pups, FGF-2 expression in the hippocampus was increased and persisted postnatally until P30. This was suggested to represent the activation of neuroprotective mechanisms (Morales et al. 2008). Modifications of oxygen tension have also been shown to increase FGF-2 mediated in vitro rat neuroblast cell proliferation and dopamine release (Jensen et al. 2011), low oxygen tension (3%) was the favoured environment for FGF-2 mediated neuronal proliferation and high oxygen tension (20%) more advantageous for FGF2 driven dopamine release, suggesting environmental oxygen affects FGF2 function.

It was hypothesised that FGF2 is up-regulated in response to the stress of growth restriction and fluctuating hyperoxia with mild hypoxia.

7.1.4 Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factor is a signalling protein necessary for normal blood vessel formation and development. Low levels of VEGF in the brain are not only associated with impaired vasculogenesis and angiogenesis (Ruhrberg C et al. 2003), but also impaired neuronal proliferation, survival and migration (Haigh et al. 2003), indicating a crucial role for VEGF in cerebral development. Within the brain of newborn rats, VEGF has been shown to guide the migration of neural progenitors in the subventricular zone of the cortex (Zhang et al. 2003).

VEGF - Relationship to Growth Restriction & Oxygen

One of the underlying abnormalities in the placentas associated with growth restricted infants is abnormal angiogenesis and inadequate terminal villi formation (Ahmed et al. 2000). An imbalance of angiogenic and anti-angiogenic factors including VEGF and VEGF receptors have been found in placentas from IUGR pregnancies (Jarvenpaa et al. 2007; Wallner et al. 2007) suggesting an intimate relationship between VEGF homeostasis and IUGR.

VEGF is up-regulated by hypoxic-inducible factor (HIF-1) and in studies of retinopathy of prematurity, much of the damage caused to the retina is by VEGF induced neovascularisation (Hellstrom et al. 2001). The new blood vessels are disorganised and weak and may cause bleeding within the retina. Hyperoxia is

inhibitory to VEGF function. Previous work has shown that fluctuating oxygen environments are more damaging to the retina than constant hyperoxia, considered due to the switching on and off of the hypoxic driven VEGF expression causing inefficient and chaotic neovascularisation (York et al. 2004; Penn et al. 1994). Within the neonatal lung, disrupted VEGF signalling impairs alveolar development and contributes to the development of BPD (Abman et al. 2010).

It was hypothesised that fluctuating hyperoxia and growth restriction downregulate VEGF expression in the developing brain which, in view of the neurotrophic effects, may have adverse effects on neuronal development.

7.2 Methods

Brain sections were obtained as described in section 2.4. Due to the fragility and small size of the P7 brains, dissection of discrete brain regions, e.g. hippocampus was not possible. The brains were therefore split into 4 sections:

Anterior Cerebrum – containing rostral cerebral cortex, partial dorsal hippocampus, rostral corpus callosum.

Posterior Cerebrum – containing caudal cerebral cortex, thalamus, hypothalamus, partial midbrain, majority of hippocampus and caudal corpus callosum.

Brainstem – containing partial midbrain, pons and medulla.

Cerebellum.

From each brain section, RNA was extracted, quantified and quality checked as per section 3.1. Reverse transcription to cDNA and then quantitative PCR was performed using validated primers and probes for rat for IGF-I, BDNF, FGF-2 and VEGF (TaqMan Gene Expression Assays, Life Technologies). Refer to sections 3.1 – 3.4 for further details. Significance analysis was performed on raw ΔCt values. Data is plotted as fold change $2^{-\Delta\Delta Ct}$ in comparison to control group. To quantify IGF-I protein in serum, a sandwich IGF-I ELISA was used, refer section 3.5.

7.3 Results

7.3.1 Insulin-like Growth Factor-1 (IGF-I)

Data was analysed by 2-way ANOVA which confirmed variation within the groups. Bonferroni post-tests confirmed a significant difference between the control group and each study group and between the growth restricted groups:

Table 15 – Serum IGF-I levels in 4 study groups

Comparison	mean ±SD	p value
Control & oxygen fluctuation group	224.7±8.4 *versus* 192.4±6.9	p = 0.011
Control & growth restricted group	224.7±8.4 *versus* 96.3±3.2	p < 0.0001
Control & dual group	224.7±8.4 *versus* 75.9 ±4.5	p < 0.0001
Growth restricted group & dual group	96.3±3.2 *versus* 75.9 ±4.5	p = 0.0004

At P7, there was a significant reduction in circulating IGF-I in the serum of both groups of growth restricted pups. Both groups of pups exposed to oxygen also have lower circulating IGF-I. The group of pups exposed to both growth restriction and fluctuating oxygen have the lowest level of serum IGF-I (ng/ml).

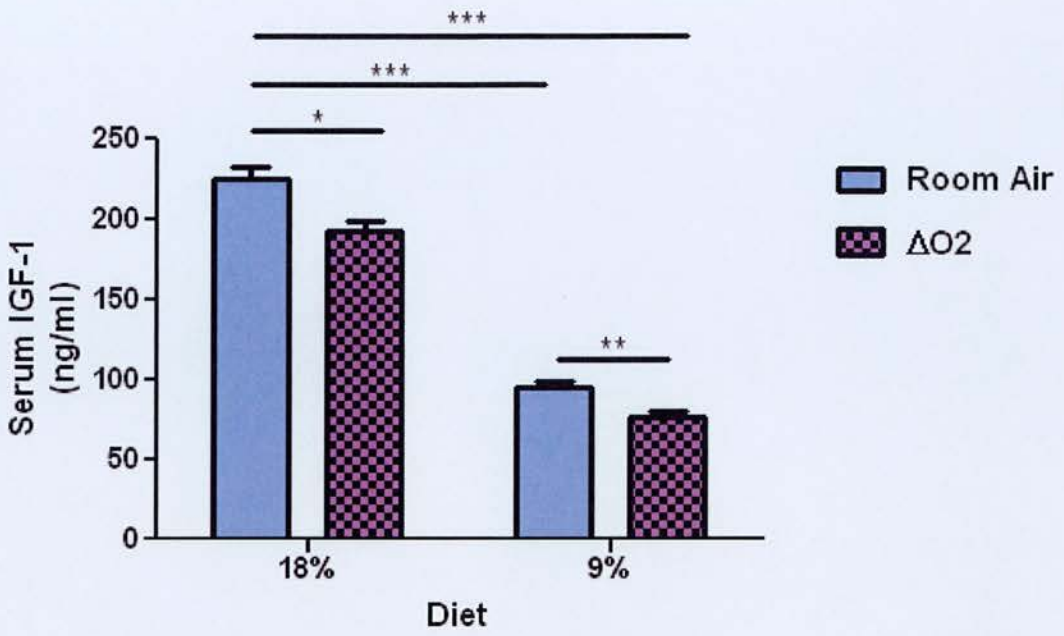

Figure 47 - P7 Serum IGF-I protein levels

Quantity of circulating serum IGF-I (ng/ml) at P7 in control group, oxygen fluctuation group, growth restriction group and dual group exposed to both growth restriction and oxygen fluctuation. n = 30 pups from 3 litters per group. Data from each group is normally distributed by D'Agostino and Pearson normality test. Data shown as mean ± SD. 2-way ANOVA confirmed significant variation between the datasets, $P<0.05$. There is a significant difference between the Control group and each study group by Bonferroni post-tests; $*p<0.05$, $***p<0.001$, $***p<0.001$ respectively. Within the growth restricted groups, there was a further reduction in serum protein in the pups exposed to oxygen $**p<0.01$.

Within the developing brain, there was no difference in IGF-I expression between study groups in any of the four brain regions examined.

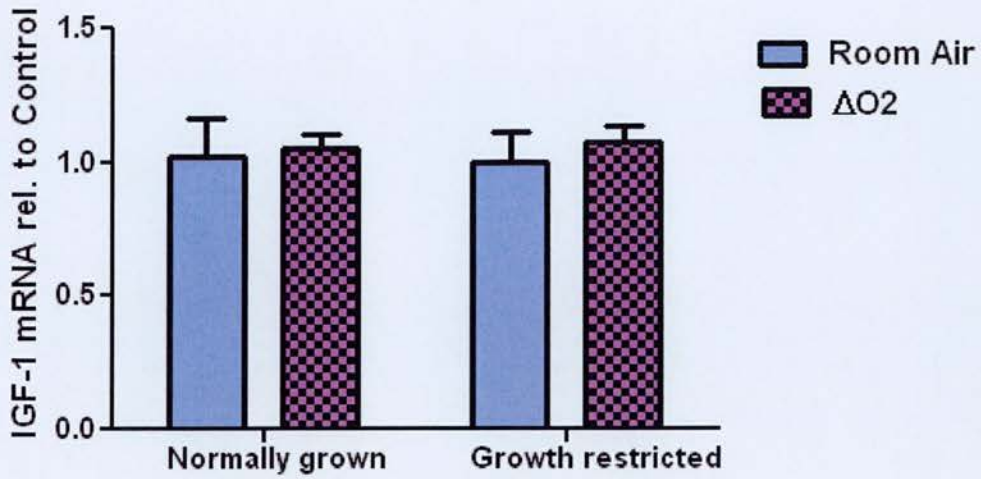

Figure 48 – Expression of IGF-I in brain sections

Expression of IGF-I mRNA relative to control group against study group on x axis.
Data shown as mean $2^{-\Delta\Delta Ct}$ ± SD, n=3 (9 pups from 3 litters) per group.
No variation was identified by 2-way ANOVA between the groups on any of the four brain sections.
Above data from posterior cerebrum section of the brain. Data from other brain sections not shown.

7.3.2 Brain Derived Neurotrophic Factor (BDNF)

There was no effect of growth restriction or fluctuating oxygen seen on the anterior, posterior cerebral section or in the cerebellum.

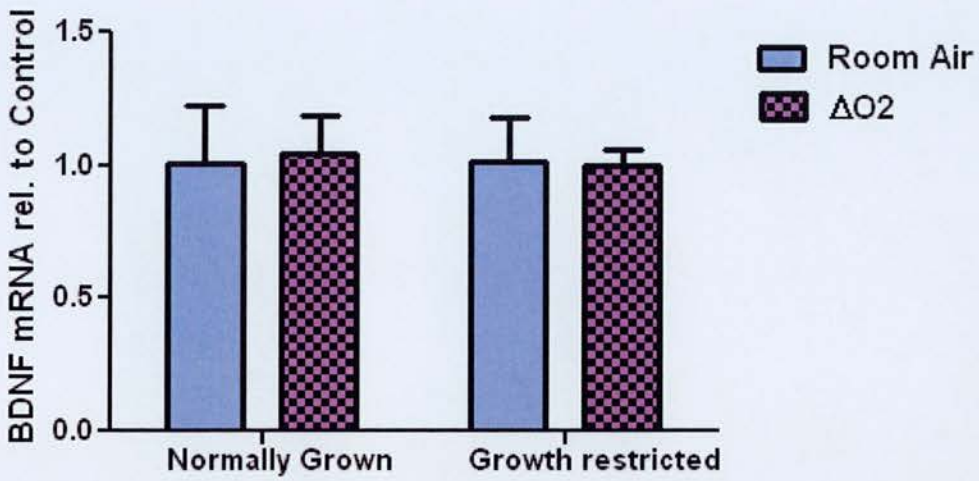

Figure 49 - Expression of BDNF in posterior cerebrum

Expression of BDNF mRNA relative to control group against study group on x axis.
Data shown as mean $2^{-\Delta\Delta Ct} \pm$ SD, n=3 (9 pups from 3 litters) per group.
No variation was identified between groups by 2-way ANOVA.
This data represents the posterior cerebrum section. Data from other brain regions not shown.

The BDNF expression results within the brainstem are summarised in Table 16 and shown graphically in Figure 50.

Table 16 – BDNF Expression in Brainstem

Data presented as mean $2^{-\Delta\Delta Ct}$ values ±Standard deviation

Group	BDNF Brainstem
RA 18%	1.13±0.13
ΔO2 18%	1.98±0.42
RA 9%	2.45±0.31
ΔO2 9%	3.04±0.7

Data was analysed by 2-way ANOVA and Bonferroni post-tests for multiple comparisons. There was an incrementally graded increase in the expression of BDNF across the groups with the highest expression seen in the dual group exposed to both growth restriction and oxygen fluctuation, $p<0.001$.

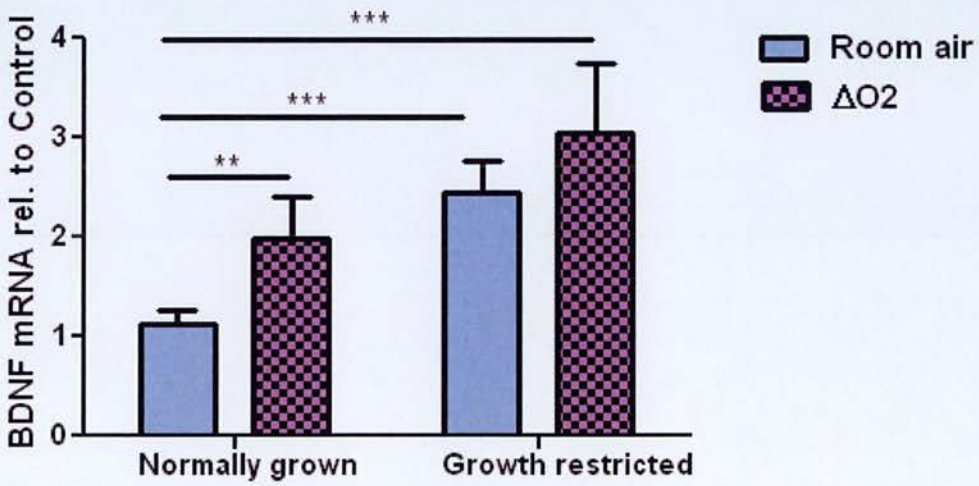

Figure 50 - BDNF Expression in brainstem

Expression of BDNF mRNA in brainstem: fold change relative to control group against study group on x axis. Data presented as mean $2^{-\Delta\Delta Ct}$ ±SD, n=3 (9 pups from 3 litters per group).
2-way ANOVA confirms variation present in dataset, $P<0.05$. Bonferroni post-test analysis identified increased BDNF expression in the oxygen only pups (**$p<0.01$), growth restricted only pups (***$p<0.001$) and in dual group pups (***$p<0.001$) in comparison to control.

7.3.3 Fibroblast Growth Factor–2 (FGF2)

Expression of FGF-2 was unchanged in all four regions of the brain across all four study groups.

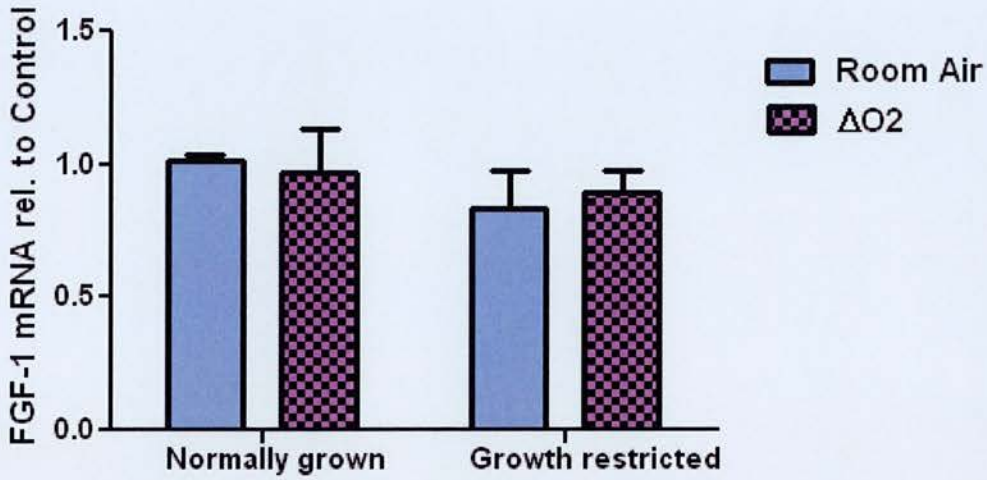

Figure 51 - Expression of FGF2 in the posterior cerebrum

Expression of FGF-2 mRNA at P7 relative to control group against study group.
Data presented as mean $2^{-\Delta\Delta Ct}$ ±SD, n=3 (9 pups from 3 litters per group).
2-way ANOVA did not identify variation between groups.
Above data represents the posterior cerebrum section. Data from other brain regions not shown.

7.3.4 Vascular Endothelial Growth Factor (VEGF)

Expression of VEGF is mildly reduced in the dual group exposed to growth restriction and oxygen in both the anterior and posterior cerebrum. There was no reduction seen in any of the groups in either the brainstem or cerebellum.

Table 17 – VEGF expression in Anterior and Posterior cerebrum sections

Mean±SD

Group	VEGF Anterior Cerebrum	VEGF Posterior Cerebrum
RA 18%	0.99±0.1	0.98±0.1
ΔO2 18%	0.98±0.1	1.0±0.05
RA 9%	1.06±0.08	1.1±0.07
ΔO2 9%	0.85±0.02	0.88±0.02

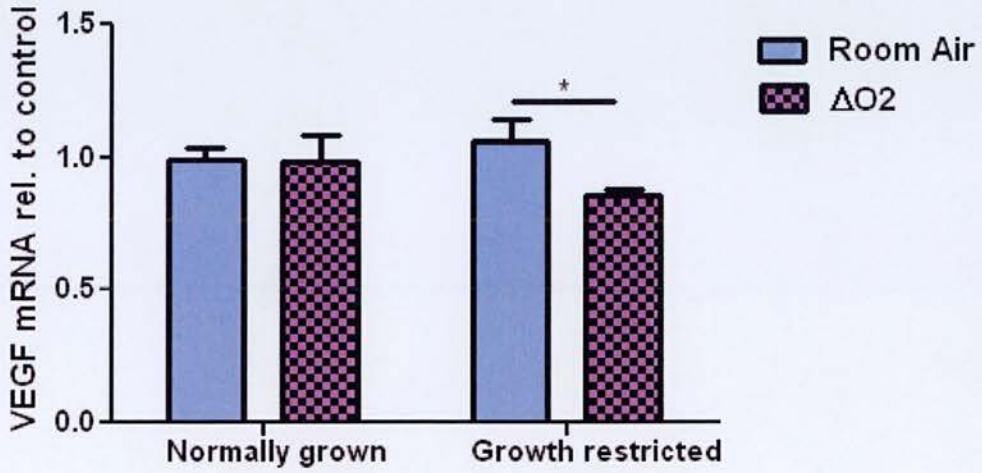

Figure 52 - Expression of VEGF in the anterior cerebrum

Expression of VEGF mRNA at P7 relative to control group against study group.
Data presented as mean $2^{-\Delta\Delta Ct}$ ±SD, n=3 (9 pups from 3 litters per group).
2-way ANOVA identified variation between datasets, Bonferroni post-test analysis identified reduced expression in dual group exposed to both growth restriction and oxygen in comparison to GP group, *p<0.05. Data from anterior cerebrum.

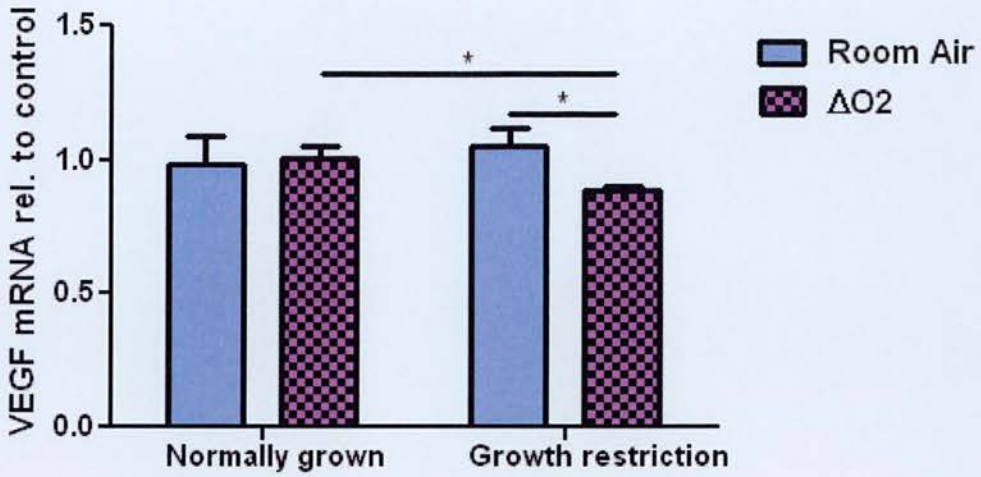

Figure 53 - Expression of VEGF in the posterior cerebrum

Expression of VEGF mRNA at P7 relative to control group against study group.
Data presented as mean $2^{-\Delta\Delta Ct}$ ±SD, n=3 (9 pups from 3 litters per group).
2-way ANOVA identified variation between datasets, Bonferroni post-test analysis identified reduced expression in dual group in comparison to ΔO2 group and GR group, *$p<0.05$. Data from posterior cerebrum.

In the rostral and caudal cerebrum, expression of VEGF in the dual group was slightly reduced in comparison to the other study groups, $p<0.05$.

7.4 Discussion

Proliferation and cell fate determination in the developing brain are regulated by complex interactions between a large number of growth factors as well as transcription factors and neurotransmitters. The most important growth factor in determining total body growth is IGF-I.

7.4.1 Insulin-Like Growth Factor - I

The reduction in circulating serum IGF-I that was observed in growth restricted pups is in keeping with the literature in both animal studies (Hellstrom et al. 2001) and with what is known about IGF-I levels in small for gestational age (SGA) infants (Davidson et al. 2006). This is not surprising given the effects which IGF-I has over the control of total body growth demonstrated by knock-out animal experiments, where birth weights of IGF-I knock-out rodents are 40% lower than wild type rodents (Liu JP et al. 1993). Similarly in humans, mutations of IGF-I cause severe intrauterine growth restriction (Walenkamp et al. 2005). Deficient levels of IGF-I can occur following preterm birth and this predisposes to retinal vessel loss and ROP (Zepeda-Romero et al. 2012). Animal studies of exogenous administration of IGF-I have shown positive effects on ROP prevention and particular benefit for total body growth (Hellgren et al. 2011). No beneficial effects on brain weight were observed and behaviour studies have not been reported. Preterm human studies have shown a beneficial effect of administering fresh-frozen plasma (FFP) containing IGF-I and IGFBP-3 from adult donors (Hansen-Pupp et al. 2009) and intravenous preparation

of IGF-I/IGFBP-3 (Lofqvist et al. 2009) on IGF-I and IGFBP-3 levels and is well tolerated. Any beneficial effect on growth or neurological outcome has not been determined.

All six IGF-binding proteins are present in plasma (Randhawa et al. 2005). IGF-I is bound to these proteins in plasma which limits the availability of IGF-I to the IGF-IR. The IGFBPs are under the control of a family of proteases that cleave the binding proteins into inactive fragments. These proteases thereby allow IGF peptides to exert their growth promoting effects (Collett-Solberg et al. 1996). The ELISA assay used in the determination of IGF-I within each study group was an IGFBP blocked assay so that *total* circulating IGF-I was measured as opposed to only that which is unbound in the circulation. The effect of growth restriction on total circulating IGF-I within the model can be commented on, acknowledging that some of this will be bound to plasma proteins. It has been previously reported that IGFBP1 is high in the cord blood of growth restricted babies, and that IGFBP1 is negatively correlated with birth weight, suggesting that IGF-I is less available in these infants (Ostlund et al. 1997). IGFBP3 is present in the highest quantity in plasma and circulates as part of a complex with IGF-I. IGFBP3 has been reported to be reduced by 50% in IUGR babies (Giudice et al. 1995), but it is not clear whether this is a primary effect on IGF-I or on IGFBP3. An effect on both as part of the 'IGF system' seems more likely given that IGFBP3 overexpression in transgenic mice is associated with a reduction in birth weight due to a reduction in circulating IGF-I (Modric et al. 2001). Determination of IGFBP levels in the model may have

provided more detail on this matter but the interaction between the IGF peptides and IGFBPs is constantly dynamic (Popken et al. 2005) and therefore the true unbound circulating fraction of IGF-I is difficult to ascertain reliably.

It has been shown that fluctuating oxygen has an effect on circulating IGF-I in the model with a reduction in serum IGF-I in both groups exposed to oxygen. Exogenous oxygen is associated with the development of BPD and a recent study has demonstrated that lower levels of IGF-I levels in preterm infants was associated with the subsequent development of BPD (Lofqvist et al. 2012). The P7 body weight of the oxygen only group was not reduced in the study, whereas the P7 body weight of the dual study group was significantly smaller than all other groups (refer Section 4.3), indicating that supplemental oxygen may affect total body growth. There is evidence that IGF-I correlates with the period of catch up growth following initial poor weight gain, or weight loss, often observed in growth restricted preterm infants (Hansen-Pupp et al. 2011). There is also some evidence from work in zebrafish that hypoxia increases IGFBP1 expression which is inhibitory to IGF-I action (Kajimura et al. 2005). The serum levels measured in the current study reflect total IGF-I as above, as opposed to unbound IGF-I. The oxygen profile used is hypoxic for 11% of the 7 days, normoxic for 4% and hyperoxic for 85%. Hyperoxia or the fluctuation between hyperoxia and hypoxia are the most likely determinants of the effect on IGF-I. The final component of the IGF system is the IGF-IR receptor, a transmembrane cell surface receptor. It is possible therefore that there is a direct effect of oxygen toxicity on IGF-I or its receptor. Levels of oxygen reactive species in the model were not measured but this may prove prudent towards explaining the

reduction in serum IGF-I seen in response to fluctuating oxygen. Preterm infants have reduced antioxidant defences and are therefore vulnerable to oxidative damage (Taglialatela et al. 1998; Kuster et al. 2011).

The conservation of IGF-I expression across all study groups in all brain areas was unexpected. IGF-I plays a significant role in the developing brain in the proliferation of neural progenitors (Popken et al. 2004), inhibition of apoptosis (D'Ercole et al. 2002) and neuron development (Niblock et al. 2000). However local production of IGF-I by neurons is considered more important to neuronal function than circulating IGF-I (Russo et al. 2005) and suggests a brain sparing effect on IGF-I expression within the brain, in the context of low circulating levels. It is also possible that endogenous IGFBPs were able to compensate for lower circulating levels by ensuring adequate concentrations within the brain.

These results do not yet explain why brain weight was reduced in the growth restricted pups. It is important to note that we have looked at IGF-I gene expression and not quantity of protein within the brain, the latter of which may have been significantly reduced during the period of restriction. In addition, the other components of the IGF system were not measured, namely the IGFBPs or the IGF-IR which are all expressed within the brain and regulate IGF-I activity and function (D'Ercole et al. 2002). Further work to quantify each of these components would complete the picture.

7.4.2 Brain-Derived Neurotrophic Factor

BDNF is a growth factor with key roles in neuroprotection. High levels generally correlate with a state of well being and health (Gomez-Pinilla et al. 2008) and suppressed levels are found in disease states such as depression and schizophrenia where these are associated with adverse effects on cognition (Autry et al. 2012). It is also reported that both diets high in saturated fats and oxidative stress have adverse effects of on BDNF levels and subsequently on cognition (Wu et al. 2004). Whereas dietary restriction of calories enhances BDNF levels in mice (Lee J et al. 2002) and calorie restriction has been shown to extend lifespan in animal models (Varady et al. 2008). Consumption by the dams in the current study was not measured because of inaccuracies of weighing of chow. It is therefore not possible to comment on the caloric intake of the dams, however the low protein diet was isocaloric. In contrast to the animal studies, adequate caloric intake is crucial for preterm infant weight gain and inadequate weight gain is associated with impaired cognitive development (Casey, 2008). Caloric intake by preterm infants in the neonatal period can be challenging because of low calorie intravenous infusions, low calorie content of breast milk and significantly increased energy requirements of preterm infants.

Increased expression of BDNF was found in the brainstem of the pups exposed to oxygen, growth restriction and most significantly, the dual study exposed to both growth restriction and oxygen. The effect of growth restriction is in keeping with the previous work as above, where dietary restriction, albeit protein as opposed to caloric restriction, up-regulates BDNF expression (Lee et al. 2002). These studies in adult

mice demonstrated no effect of BDNF on proliferation of neural cell precursors but significantly increased survival of newly generated cells and therefore enhanced neurogenesis. The exact mechanism is undefined, but it is suggested that dietary restriction induces a mild metabolic stress response which increases BDNF expression. The enhanced effect of fluctuating oxygen on brainstem expression of BDNF in the study model may reflect a further stress response. The brainstem is rich with BDNF (Katoh-Semba et al. 2003) and it is known that BDNF is required for the normal development of the central respiratory centres in the brainstem (Balkowrec et al. 1998). In a model of Rett Syndrome, methyl-CpG-binding protein 2 (*mecp2*) null mice and control mice were subjected to intermittent hypoxia and the effects on the respiratory centre in the brainstem examined. It was found that intermittent hypoxia fluctuated with normoxia and caused upregulation of *bdnf* in the control group, but not the *mecp2* null mice, as mecp2 is required for BDNF expression (Vermehren-Schmaedick et al. 2012). In the study oxygen profile, 11% of the 7 day period was spent in hypoxia. It is therefore possible that the upregulated BDNF expression in our model was secondary to the fluctuations between hyperoxia and hypoxia. The growth restricted pups exposed to oxygen had the highest levels of BDNF expression suggesting a synergistic effect of stress caused by oxygen fluctuation and growth restriction on BDNF gene expression. This may represent a neuroprotective response to protect the vital structures within the brainstem.

7.4.3 Fibroblast Growth Factor - 2

Prenatal exposure to glucocorticoids causes an upregulation of cerebral FGF-2 mRNA in the hippocampus of adult rats but no other discrete brain area (Molteni et al. 2001). Rats exposed to prenatal glucocorticoids and then an acute stress event shortly after birth show acute increases in FGF-2 in the hippocampus and prefrontal cortex but these are blunted responses in comparison to rats that were not exposed to prenatal glucocorticoids. This may represent a reduction in glucocorticoid receptors in these brain areas as has been previously proposed (Barbazanges et al. 1996) and suggests that prenatal exposure to stress may alter expression of trophic factors important to the structure and function of the developing brain. It has also been reported that in addition to neurotrophic effects, FGF2 has apoptotic effects at specific times in development (Yagami et al. 2010). When FGF2 is injected into the lateral ventricles of rat embryos at E20.5, total number and density of glial cells are increased with no effect on neurons (Vaccarino et al. 1999).

However in the study model no effect was seen on FGF-2 expression in any of the brain areas examined. This is most likely because the experimental design was not sufficiently sensitive to detect changes in gene expression within discrete structures of the brain. As described in section 2.6.3, the brains from the rat pups were divided into four sections; 'anterior cerebrum', 'posterior cerebrum', brainstem and cerebellum. The anterior and posterior sections contain parts of discrete structures, e.g. the hippocampus. In Chapter 5, adverse effects on laminar growth predominantly affected the superficial layers of the cerebral cortex.

Any changes in gene expression in the hippocampus or specific regions of the cortex would be masked by overall gene expression in the large section of brain. This also holds true for BDNF and IGF-I. The specific effects found on the expression of BDNF in the brainstem exemplify the advantages of studying distinct dissected brain areas. At P10, it is more likely that dissection of discrete brain areas would also be possible. Using micro-dissection techniques and handling the brain on ice-cold surfaces may also have improved the success of regional dissection (Chiu et al. 2007) and allowed further conclusions to be drawn on the effect of the environmental conditions studied on growth factor expression. The work by Lodygensky et al, 2008 also documented brain volume alterations in association with intrauterine growth restriction. In particular for preterm infants, IUGR is associated hippocampal volume loss (Lodygensky et al. 2008) and therefore regional dissection would have also allowed volumetric comparisons between discrete brain areas to assess the effect of protein restriction and oxygen fluctuation.

7.4.4 Vascular Endothelial Growth Factor

Expression of VEGF in the anterior and posterior cerebrum was reduced in the dual study group exposed to growth restriction and fluctuating oxygen. As described below, the absence effect observed between the other groups does not necessarily mean that growth restriction and oxygen did not have transient effects on VEGF expression and therefore function.

VEGF is sensitive to changes in oxygen environment. The underlying mechanism for the proliferative retinopathy in rats and preterm infants in association with fluctuating oxygen is thought to be the on / off switching of VEGF in response to hypoxia driven HIF-1 alpha, causing neovascularisation and weak vessels which are prone to leak (York et al. 2004; Penn et al. 1994). Within the brain, it is therefore likely there would be both up- and down-regulation of VEGF pathways during the 7 day period of oxygen fluctuation in the model. This suggests VEGF is a dynamic factor, intimately responsive to the environment. In addition, within the literature, there is ambiguity as to whether VEGF is up- or down-regulated in IUGR pregnancies. There is clearly an effect and disordered vasculogenesis within the placenta has been reported but this is poorly understood (Wallner et al. 2007; Jarvenpaa et al. 2007). The expression of VEGF within the brain at P7 is therefore unlikely to reflect the true profile of VEGF response in the model.

The reduced expression of VEGF in the anterior and posterior cerebral sections of the dual group may be explained by the increased vulnerability of this group as

demonstrated by increased levels of the neuroprotective trophic factor BDNF. In addition, there is evidence from human studies that growth restricted infants have reduced antioxidant reserve (Toy et al. 2009) and therefore cellular components within this group may be more vulnerable to damage. Within the brain, VEGF has roles in nerve growth and survival (Haigh et al. 2003) and therefore low levels may disrupt these roles.

7.4.5 Interaction between Trophic Factors in the Developing Brain

The existence of multiple trophic factors in individual brain areas suggests that neural development can be modulated by changes in any one of these factors. It is also likely that these growth factors interact in ways that have not yet been described and understood.

In-vitro experiments have confirmed that IGF-I and FGF-2 have additive growth promoting effects in maintaining survival of a larger number of neurons when added together (Torres-Aleman et al. 1990). Furthermore, in studies of mouse immature striatal cells, the presence of IGF-I allowed FGF and Epidermal Growth Factor (EGF) to induce proliferation of immature cells and development into mature cells, whereas when IGF-I was not present, neither EGF nor FGF were induced to cause cell proliferation or differentiation (Arsenijevic et al. 2001).

In knockout mouse studies and preterm studies of retinopathy of prematurity, adequate levels of IGF-I are crucial to VEGF-induced retinal vessel development via the induction of HIF-1α (Hellstrom et al 2001; Smith et al. 2005). Low levels of circulating IGF-I are associated with impaired VEGF signalling in the developing lung leading to impaired alveolar development and an increased risk of BPD, hence proposing a link between SGA infants and the likelihood of developing BPD (Lofqvist et al. 2012). Within the brain of newborn rats, VEGF has been shown to

guide the migration of neural progenitors in the subventricular zone of the cortex but can only achieve this is in the presence of FGF-2 (Zhang et al. 2003).

Within neuroblastoma tumour cells, BDNF increases VEGF expression by increasing promoter activity, causing increased angiogenesis and metastasis (Nakamura et al. 2006). Therefore despite the recognised neuroprotective roles of BDNF, increased expression is not without implications.

VEGF and FGF-2 are positive regulators of angiogenesis. Both are increased in the placenta of growth restricted infants in response to hypoxia and inadequate uteroplacental perfusion which impairs placenta function leading to a growth restricted fetus (Barut et al. 2010). Within the aging brain, protein levels of IGF-I, FGF-2 and VEGF in the hippocampus are all reduced by middle age in rats, reinforcing the importance each of these factors plays in maintaining neuronal function, the close link between neurogenesis and angiogenesis and emphasizing the effect reducing levels of one factor potentially has on the others (Shetty et al. 2005).

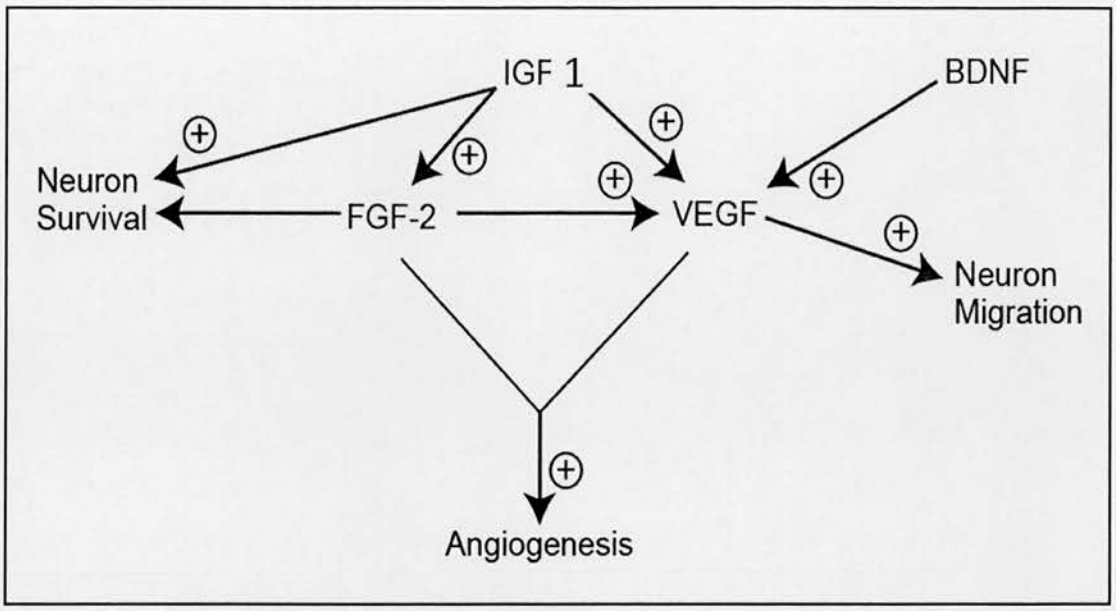

Figure 54 - Interaction of growth factors considered in this study

7.5 Summary

In this study the effects of the environmental influences of growth restriction and postnatal fluctuating oxygen on circulating levels of IGF-I protein and gene expression of IGF-I, BDNF, FGF-2 and VEGF in the developing brain in a rodent model were examined. An association was found between reduced circulating IGF-I and fluctuating oxygen. In the context of growth restriction, fluctuating oxygen was associated with a more significant reduction in IGF-I. Within the brain, gene expression of IGF-I and FGF-2 was largely conserved across groups and within the sections of the brain studied. This may reflect insensitivity in the methodology used as the design was not sufficiently sensitive to detect changes in discrete structures, for example, the hippocampus. An increase in BDNF expression in all study groups in comparison to control was detected within the brainstem. This is likely to reflect

increased metabolic stress caused by the set conditions and a neuroprotective response by BDNF in view of the key structures located in the brainstem. VEGF expression was reduced in the study group exposed to both growth restriction and oxygen fluctuation suggesting a synergistic effect of the environmental conditions. It is likely that even subtle changes in the expression of each of these trophic factors has subsequent effects on other factors that each interacts with, leading to a combined effect on the developing brain that may be amplified beyond the reduced role of a single trophic factor.

Chapter 8:

General Discussion

Intrauterine life lays the foundation for growth and wellbeing in infancy, through childhood and into adulthood. Both fetal brain and somatic growth are complex processes dependent on the genotype and epigenotype of the fetus, maternal health and availability of nutrients, growth factors and oxygen to the fetus. Intrauterine insults such as hypoxia or infection will have a negative impact on the in utero environment. Growth restriction is a common precipitant of preterm delivery and such infants often require respiratory support in the form of oxygen or ventilation. Growth restricted infants are at increased risk of poor neurodevelopmental outcome and the relative contributions of antenatal effects and postnatal insults on brain development, such as oxygen therapy are not clear.

In this thesis, the independent and combined effects of pre- and postnatal growth restriction and a clinically derived fluctuating oxygen profile on the developing brain of a rodent model is explored.

In each of the following sections, results are first summarised and placed in context of the most recent literature. Future directions arising from this work is then discussed.

8.1 Growth Restriction

8.1.1 The effects of growth restriction on body weight

In the experimental animal model, it was found that administering a 9% low protein diet to rat dams induced pre- and post-natal somatic growth restriction in the offspring. At birth, the growth restricted pups were 13% lighter than the control pups. The underlying mechanism to explain this somatic growth restriction is likely to be a combination of decreased substrate delivery to the fetoplacental unit, impaired uterine artery blood flow (Gao H et al. 2012), a reduction in maternal glucose, insulin and progesterone levels (Fernandez-Twinn et al. 2003) and fetal exposure to higher levels of maternal glucocorticoids (Langley-Evans et al. 1994). At postnatal day 7 in the animal model, the effect of postnatal growth restriction was marked with growth restricted pups being 35% lighter than control pups. This increase in restriction may be mediated by a reduction in total milk volume during lactation as has been reported in previous nutrition restriction models (Rosso et al. 1980) or perhaps a reduction in total milk protein.

8.1.2 The effects of growth restriction on brain weight

Brain weight was reduced in the growth restricted pups at P7. This is in contrast to the outcome achieved from 60% calorie restriction where despite a reduction in body weight, brain weight was not significantly different to the control pups (Agale et al. 2010). This may suggest a specific effect of postnatal protein restriction on brain growth and cross-fostering experiments would be required to determine if antenatal

growth restriction alone is sufficient to cause a reduction in brain weight. Evidence suggests there is a fetal programming element to the effect of low protein diet on the developing brain where more severe, prolonged protein restriction is associated with increased likelihood of impaired brain growth (Resnick et al. 1982).

As a percentage of body weight, both growth restricted study groups preserved brain weight relative to body weight. This mirrors the clinical situation where asymmetrical growth restriction in preterm infants is most common. Asymmetrical growth restriction has been renamed 'nutritional IUGR' by some authors (Cox et al. 2009) to reflect the failure of nutrient supply to the fetus toward the last trimester of pregnancy. In contrast, symmetrical IUGR reflects reduced growth potential and a smaller fetus with fewer cells, reflecting onset of growth restriction much earlier in the course of pregnancy where rate of growth is determined by cycles of cell division (Cox, 2009).

8.1.3 The effect of growth restriction on cortical thickness & neuronal subtypes

In the context of growth restriction alone, a statistically nonsignificant reduction in cortical thickness was seen with a corresponding statistically nonsignificant increase in cell density and no effect on neuron number. This may suggest a loss of stroma or neuronal connectivity. Previous work has shown that protein deprivation has adverse effects on dendritic branching complexity and synaptic spine number in the hippocampus (Diaz-Cintra et al. 1991) and in the cerebral cortex (Benitez-Bribiesca et al. 1999; Jones et al. 1981). The transcription factor Cux-1 is involved in directing superficial neurons to undergo maturational changes including dendritic branching and synaptogenesis. No reduction in the proportion of Cux1 expressing neurons in superficial neurons of the growth restricted group was detected. In addition, prenatal protein malnutrition in rats has been shown to negatively impact cerebral electrical activity (De Frias et al. 2010), again suggesting adverse effects of reduced protein on cortical circuitry.

Cerebral MRI scanning of preterm growth restricted infants at term shows reduced intracranial and cerebral cortical grey matter volume in comparison to term born controls (Tolsa et al. 2004). In particular grey matter is reduced in the frontal, parietal and temporal regions (Padilla et al. 2011). Infants who are born growth restricted are at increased risk of long-term cognitive impairments, and increased behavioural problems (O'Keeffe et al. 2003; Noeker 2005; Geva et al. 2006; Padilla

et al. 2011). These findings therefore suggest that preterm growth restricted infants have structural and functional brain differences in comparison to term born infants.

The growth restricted pups in this study did have a reduced brain weight compared to controls and a suggestion of loss of intercellular matrix despite normal proportions of Cux1 expressing neurons in the superficial layers. The study contained 9 pups from 3 litters per study group and therefore may not be sufficiently powered to detect small differences in cortical thickness or neuronal number. The reduced brain weight suggests there was loss of material from the brains in the growth restricted group which may include components involved in intercellular connections such as dendritic branching and synaptic spines (Georgieff et al. 2007). There is evidence from imaging studies to suggest growth restriction is also associated with reduced white matter volume which may be associated with enlarged ventricles and may also contribute to reduced brain weight (Padilla-Gomes NF et al. 2007; Padilla et al. 2011; Tolsa et al. 2004).

8.1.4 The effect of growth restriction on expression trophic factors in the brain

It was found that circulating IGF-I was significantly reduced in the growth restricted group at P7 providing an explanation for the 35% reduction in body weight. Within the brain, gene expression of IGF-I in each section was conserved across the study groups, suggesting preservation of brain IGF-I. Local production of IGF-I by neurons is considered more important to neuronal function than circulating IGF-I (Russo et al. 2004). These results suggest a brain-sparing effect on IGF-I expression within the brain, in the context of low circulating levels. It is also possible that endogenous IGFBPs were able to compensate for lower circulating levels by ensuring adequate concentrations within the brain. In addition, the methodology employed would not detect small changes in discrete structures such as the hippocampus.

No effect of growth restriction was observed on the gene expression of FGF-2 or VEGF within the brain. This may again be related to the methodology used as changes in FGF-2 expression may have been expected in the hippocampus where prenatal stress causes an increase in hippocampal and prefrontal cortex expression (Molteni et al. 2001). VEGF is a dynamic factor that has been shown to be both raised and reduced in the context of growth restriction (Jarvenpaa et al. 2007; Wallner et al. 2007).

Expression of BDNF was significantly increased within the brainstem section only and is likely to reflect the increased metabolic stress caused by the growth restriction and a neuroprotective response by BDNF in view of the key structures located in the brainstem. This is in keeping with the literature which supports a link between dietary restriction and increased levels of BDNF (Lee J et al. 2002).

8.2 Fluctuating Oxygen

8.2.1 The effect of ΔO2 on body & brain weight

There was no independent effect observed of the fluctuating oxygen profile on somatic or overall brain growth in the model, in keeping with previous researchers using the same oxygen profile (Sedowofia, 2007, Pilley E, 2010). This was despite a reduction in circulating IGF-I and preservation of IGF-I expression in the brain of the ΔO2 group. The fluctuating oxygen profile used has been previously shown to result in cell death, astrocytosis and reduced expression of myelin basic protein (Sedowofia et al. 2008). Therefore, although the weight did not change, other structural elements may have been altered.

8.2.2 The effect of ΔO2 on cortical thickness

The clinically relevant oxygen profile was shown to adversely affect cortical growth. This effect was on the superficial cortical layers II-IV, involved in intracortical circuitry, with no effect observed on the thickness of deep layers V and VI. There was a suggestion of a reduced number of nuclei in the context of preserved cell density in the ΔO2 group suggesting a direct effect of oxygen on neuron survival. This is in keeping with several previous studies and may be explained by increased cell death (Felderhoff-Mueser et al. 2004; Yis et al. 2008; Gerstner et al. 2008), or free radical oxygen damage (Vento et al. 2003; Vento et al. 2005; Maltepe et al. 2008).

8.2.3 The effect of ΔO2 on neuronal subtypes

Fluctuating oxygen caused a significant reduction in the proportion of Cux1 expressing cells. This means that in addition to a mild reduction in total nuclei in response to oxygen, oxygen reduces the proportion of neurons which express Cux1. There are no clear reports in the literature of direct effects of oxygen on Cux1 expression, however, a recent study has shown that intermittent hypoxia (alternating 21% and 10% oxygen) increases the expression of transcription factor paired-box-6 (Pax6) in the SVZ (Ross et al. 2012). Pax6 has key roles in fundamental brain patterning and cortical development. It crucial for normal superficial neuron migration, cortical integrity and Cux1 expression (Nieto et al. 2004; Georgala et al. 2010) and therefore disrupted levels secondary to oxygen fluctuation may impair superficial neuron migration and Cux1 function.

8.2.4 The effect of ΔO2 on trophic factors

As discussed in 8.2.1, circulating IGF-I was reduced following exposure to oxygen and brain expression levels of IGF-I was preserved across groups. There was no specific effect of fluctuating oxygen on FGF-2 and significantly, no effect on VEGF. Within the retina, fluctuating oxygen induces chaotic blood vessel development (York et al. 2004) due to the hyperoxia-/hypoxia-driven off and on switching of VEGF. Serum levels of VEGF or brain protein levels may have been more informative as gene expression may not reflect the rapid alternating levels of this growth factor. As in the case of growth restriction, BDNF was raised in the

brainstem in comparison to control, suggesting the brainstem has instituted a neuroprotective function in response to perceived stress caused by the oxygen.

8.3 Dual Exposure: growth restriction & fluctuating oxygen

8.3.1 The dual effect: body, brain weight & trophic factors

Within the context of growth restriction, body weight was further reduced by fluctuating oxygen. The significantly reduced circulating levels of IGF-I in this group may account for this. In addition, in humans, growth restricted infants have reduced antioxidant capacity in comparison to term born infants (Toy et al. 2009) and therefore cellular components in the dual group may be more susceptible to oxidative damage. In contrast, the absolute brain weight of the dual group was not reduced relative to the growth restricted only group; however brain weight relative to body weight was preserved to a greater extent. This was in the context of constant IGF-I and FGF-2 expression but significantly increased levels of BDNF in the brainstem. It is unlikely our method of measuring gene expression would detect small increases within discrete areas of the brain. Nonetheless, BDNF has roles in neuroprotection, cell proliferation and axonal outgrowth (Rao et al. 2009) and therefore may contribute to the preservation of brain weight within this model.

As a marker for the institution of neuroprotective mechanisms, the incremental expression of BDNF to three times the levels seen in the control group, suggests the combined effect of pre-/postnatal growth restriction and postnatal oxygen fluctuation enhances the vulnerability of the brain before the development of protective mechanisms.

The reduced expression of VEGF in the anterior and posterior cerebral sections of the dual group reinforces the increased vulnerability of this group to oxidative damage which may be explained by reduced antioxidant reserve (Toy et al. 2009). Within the brain, VEGF has roles in nerve growth and survival (Haigh et al. 2003) and in addition, animal studies have shown that low levels of VEGF are associated with increased apoptosis and adverse effects on neuron migration within the VZ and SVZ with subsequent effects on cortical thickness. The reduced VEGF may therefore be implicated in the cortical thinning observed in this group.

8.3.2 The dual effect: cortical thickness & neuronal subtypes

Within the context of growth restriction and oxygen fluctuation, severe cortical thinning occurred. This was more significant in the superficial cortical layers but also noted in the deep layers. Cell density was significantly increased in the dual study group, suggesting loss of intracellular matrix. As discussed above for growth restriction alone, previous work has shown that protein deprivation negatively impacts dendritic branching complexity and synaptic spine number in both the hippocampus and the cerebral cortex causing lower sensory cortico-cortical and thalamo-cortical evoked potentials (Resnick et al. 1982; Diaz-Cintra et al. 1991, Benitez-Bribiesca et al. 1999; Jones et al. 1981). The increase in cell density in the GR group did not reach statistical significance, however the increase in cell density in the dual group was highly significant, suggesting the added insult of postnatal oxygen fluctuation had a profound effect on extracellular space and perhaps structures such as dendritic branches and synaptic spines. As described above,

fluctuating oxygen alone was not associated with any changes in cell density in comparison to control; however there is evidence from animal models that hypoxia is associated with reduced density of dendrites in the cerebellum (Rees et al. 1999). The periods of hypoxia within the model in concert with the susceptibility induced by prenatal growth restriction may have been sufficient to impair the development of intercellular processes and connections.

In keeping with this, the significantly reduced proportion of Cux1 in the dual group is consistent with disrupted dendritic branching, reduced dendritic spines and interrupted circuitry in the developing cortex of the model (Cubelos et al. 2010a; Cubelos et al. 2010b).

There are suggestions from the roles of Cux1 elsewhere in the body that this transcription factor acts to protect cells from cell death. In the bone marrow and thymus, reduced levels of Cux1 have been shown to increase apoptosis (Sansregret et al. 2008) and increased levels of Cux1 within pancreatic tumours protect against apoptosis (Ripka et al. 2010a). In addition, treatment of cells with IGF-I has been shown to increase Cux1 expression within those cells. It is therefore possible that low levels of circulating IGF-I are implicit in the reduced expression of Cux1 in the cerebral cortex of the dual group. This reduced expression may increase the vulnerability of cerebral cortex cells to cell death which this work suggests occurs in

response to fluctuating oxygen in the context of both normal growth and growth restriction.

There was a statistically non-significant reduced neuron number in the dual group coexisting with a thinned cortex and increased cell density. As discussed above, low VEGF expression in the developing rodent brain causes reduced neuronal proliferation, increased apoptosis and aberrant neuronal migration from the VZ and SVZ reflected in a decreased cortical thickness (Haigh et al. 2003). Although no aberrant migration from the proliferating zones was noted in the model, it is possible that some superficial neurons fail to express Cux1 and therefore do not migrate from the cell proliferating zones. This would contribute to a loss in total cell number and proportion of positively expressing Cux1 neurons.

8.4 Future Directions

The results of the studies undertaken for this thesis have raised a number of questions and further work is required to address these.

8.4.1 The oxygen profile & markers of oxidative damage within the cortex

One of the major strengths of this work is the physiological oxygen profile used. Measuring markers of oxidative stress caused by such a physiological profile would not only unravel the mechanism underlying findings such as the reduction in circulating serum IGF-I (sections 7.4.1 and 7.5.1), but would contribute to general understanding of the impact such physiological treatments may have on developing body systems. It is widely appreciated that preterm infants have low antioxidant reserves and there is a need to validate biomarkers of oxidative stress so this can be monitored in the clinical situation (Perrone et al. 2012).

As described in Chapter 2, the profile is hyperoxic (above 21%) for 85% of the 7 days, hypoxic (below 21%) for 11% of the 7 days and the other 4% is spent at room air. To delineate the mechanistic role of the effect of hyperoxia or hypoxia in the model, fluctuating profiles which are either hyperoxic or hypoxic could be developed. This would allow findings such as which oxygen environment underpinned the upregulation of BDNF in the brainstem (section 7.5.2) and if

hypoxia was the underlying cause of increased cell density and reduced Cux1 expression in the dual group (section 8.3.2) to be clarified.

8.4.2 Growth Factor Systems – Ligands, receptors and binding proteins

A strength of the work on growth factors was the concurrent measurement of IGF-I in the serum and IGF-I cerebral expression, allowing the brain-sparing effect of gene expression to be appreciated fully. As discussed in section 7.5.1, there are many other components of the IGF system and measurement of each of these components would complete the picture of how the various elements interact within the physiological model.

8.4.3 Regional brain studies

To further define the effect of the environmental conditions on growth factors, detailed dissection of discrete brain areas is required. In particular, studies looking at growth factor expression in regions such as the hippocampus, a brain area affected by growth restriction (Jahnke et al. 2007) and implicated in volumetric and cognitive assessments of ex-preterm infants (Nosarti et al. 2002). In addition, regional studies of gene expression in the thalamus and other deep grey structures would provide further information because these are also associated with reduced volume in the

context of preterm birth and morbidities such as bronchopulmonary dysplasia (Jobe et al. 2010; Thompson et al. 2007).

The lamination study examined the motor cortex only. During the third trimester, areas undergoing rapid development and thus vulnerable to damage by preterm birth include the hippocampus, visual and auditory cortices (Georgieff et al. 2007). It would therefore be important to consider the effects of both growth restriction and oxygen on other areas of the cortex, for example, the visual cortex as visual impairment in preterm infants shows a higher prevalence than can be explained by focal retinal or white matter brain lesions (O'Connor et al. 2007) and in the somatosensory, premotor and lateral frontal cortical grey matter, all shown to be altered by preterm birth (Thompson et al. 2007; Ball et al. 2012b)

Despite thinning of the posterior corpus callosum being an observation reported in preterm infants (Thompson et al. 2012), corpus callosal thinning was not observed in response to the environmental conditions in the model of fullterm rodent pups. This may have been a consequence of the sections measured being too rostral and so to test this hypothesis, matched caudal sections could be stained and measured using the same technique as in section 3.7.1.

In Chapter 5, the thickness of laminae II – VI was studied as these layers contain the vast majority of postnatal cortical neurons. The subplate is the most prominent transient layer in human development (Kostovic et al. 2006) and is the site where the majority of afferent neurons, particularly from the thalamus, temporarily reside to establish synapses and participate in cellular interactions that are crucial for subsequent cortical development and connectivity. The subplate synaptic compartment is undergoing significant developmental maturation at the time of preterm birth (Volpe et al. 2009b). The subplate is much smaller in rodents (Bayer and Altman, 1991); however an assessment of thickness would indicate effects in the rodent model which would be extrapolated to be more profound in the larger subplate region of the human.

As outlined in Chapter 1, layer I is largely a non-cellular layer, generated early in development and initially called the marginal zone, see Figure 2. The major cell type in layer I are the Cajal-Retzius cells which produce Reelin and have been shown in knockout rodent studies to be crucial for normal migration and lamination in both the cerebral cortex and hippocampus (Frotscher et al. 1998; Dixit et al. 2011). It would be hypothesized that this layer is unaffected by the environmental conditions due to early formation in cortical development. However changes in this layer would have significant implications for cortical neuron migration. Attempts to quantify thickness of this layer suggested similar effects to the superficial layers; however variation in thickness along the length precluded robust statistical conclusions.

8.4.4 Markers of cell death

As discussed previously, increased cell death may be a significant consequence of oxygen fluctuation and may underpin the consequences of reduced circulating IGF-I, reduced cortical thickness with preserved cell density and decreased proportion of Cux1+ neurons within the cortex in response to fluctuating oxygen. Studies examining cell death markers such as caspase-3 and TUNEL would allow further definition of the exact effect of the fluctuating oxygen profile in the cerebral cortex of the model. Caspase-3 has been previously shown to be increased in the white matter of day 14 postnatal rats in response to the same oxygen profile (Sedowofia et al. 2008).

8.4.5 Other cerebral cell types

The Topro-3 stain is a non-specific nuclear stain. Other cell types in the cortex such as microglia, oligodendrocytes and astrocytes will therefore have been stained with Topro-3 and included in the nuclear counts. Specific temporal markers to identify these cell types could be used, for example anti-brain lipid binding protein (BLBP) is a marker for young astrocytes (Ma et al. 2012). The two major categories of cortical neurons are (1) projection neurons that are glutamatergic and extend axons to distant intracortical, subcortical and subcerebral targets and (2) interneurons that are GABA-nergic and make local connections (Molyneaux et al. 2007). In this study only projection neurons have been considered and clearly interneurons are fundamental to cortico-cortical communication. Immunohistochemical staining of this subtype of

neuron would provide further information on the integrity of neuronal circuits, particularly studied at later time points when significant numbers within the cortex can be reliably determined. In addition, there is considerable afferent input to the cortex which makes a significant contribution to the cerebral neural networks and connectivity. The importance of thalamocortical connectivity in preterm development has been previously discussed. Thalamic axons carry the majority of sensory input to the cortex and almost all areas of the cortex receive some form of thalamic input (Molnar et al. 2003). Thalamic neurons are one of the first afferent axon types to reach the subplate of the cerebral cortex where crucial connections are made before migration to predominantly layer IV (Kostovic et al. 2006). Thalamocortical fibres in fixed rodent brains could be studied using well established carbocyanine dye methods (Carme Auladell et al. 2000).

8.4.6 Cortical Migration

As previously discussed, the effect of oxygen on cortical thickness, neuron number and Cux1 expression may be related to impaired neuronal migration. Dual pulse labelling with BrdU and EdU labelling would allow superficial cortical neurons to be marked and their trajectory through the cortex studied by immunohistochemistry at defined postnatal ages (Georgala et al. 2010). As discussed in Chapter 1, radial glial cells act as progenitors for all neuronal cell types. Recent evidence suggests that radial glia in mice have distinct fate potential from early in gestation including glia which are destined for only superficial layer identity (Franco et al. 2012). Further

study by histological staining of the radial glia would allow a greater understanding of migration within the model.

8.4.7 Developmental Time Points

As discussed in Chapter 1, the rat cortex is most comparable to a human newborn cortex at P10 and therefore studying the cortex at this postnatal age and beyond would provide further information on the ongoing effects caused by the environmental conditions. At P10, it is more likely that dissection of discrete brain areas would also be possible. Using micro-dissection techniques and handling the brain on ice-cold surfaces may also improve the success of regional dissection (Chiu et al. 2007).

8.4.8 Magnetic Resonance Imaging

The use of MR imaging to correlate histopathological findings in the rodent model with imaging findings would allow inferences to be made regarding rarely available neuropathology in preterm infants and with spectroscopy may give insight into the metabolic environment of the brain.

8.4.9 Behaviour Testing

Placing structural findings in context of behaviour studies is crucial to interpreting the significance of results on functional outcome and to determine if there are long-term consequences to cortical thinning, Cux1 reduction or growth factor disruption. In preterm infants, growth restriction is associated with cognitive impairments, poorer memory, increased behavioural problems and reduced motor scores (O'Keeffe et al. 2003; Noeker 2005; Eshel et al. 2006; Leitner et al. 2007). Neurobehavioral experiments could therefore be performed at six weeks postnatal age when the rodent pups are fully grown and tests of cognition, exploratory behaviour and memory performed.

8.4.10 Development of Cortical Systems

Recent imaging studies support the concept that preterm birth has specific effects on developing neural systems in the immature brain, for example the thalamocortical system where volume reduction in the thalamus is shown to be linked to volume loss in the cortex and hippocampus (Ball et al. 2012a). In infants with the classic preterm brain pathology of PVL, thalamic volume is reduced in correlation with PVL severity and it is suggested that the reduced the thalamic volume accounts for the cognitive deficits demonstrated by children with PVL because of the extensive interconnections between the cerebral cortex and the thalamus (Nagasunder et al. 2011). In keeping with Volpe's concept of the 'Encephalopathy of Prematurity', it is now widely recognised that focal and diffuse white matter injury in preterm infants is

associated with adverse effects on other developing brain areas including the thalamus, basal ganglia, brainstem, cerebellum and cerebral cortex (Volpe 2009a).

The major strengths of this thesis are findings of specific effects on neural systems within the developing brain. Future work could include an assessment of deep grey matter, and a comparison of the sizes of other key cerebral regions between groups. The effect of preterm birth on the developing neural systems is the focus of current neonatal research in the literature. The findings of markedly reduced cortical thickness at birth as a consequence of specific neonatal morbidities and events, such as growth restriction and postnatal oxygen therapy, is novel and warrants further investigation as to whether this is a regional effect on the motor cortex or a global effect with significant implications for cortico-cortico connectivity and function. The reduced proportions of Cux1 positive superficial neurons is an original finding with no previous reports in the literature of reduced superficial neuron subtype specificity in association of common perinatal environmental insults. Dendritic integrity is crucial in cortico-cortico circuitry and given the superficial layers are generally considered to be the seat of higher cognitive functioning; deficits in such circuits will inevitable have consequences for function and behaviour.

By achieving a more complete understanding of how the brain responds to injury and interruptions to normal development, perinatologists can be guided to deliver optimal care and avoid the development of neurological deficits by preterm survivors. There

are opportunities for therapeutic interventions, such as antioxidants or the administration of exogenous growth factors, but clearly experimental work would be required in these areas before clinical studies could be performed.

8.5 Conclusion

In conclusion, the work of thesis indicates that common perinatal events such as growth restriction and oxygen therapy, have significant effects on the specific systems underlying cortico-cortical communication and this may have implications for neuronal development and cognitive processing. Future work is required to delineate long term consequences of these effects and to understand the underlying mechanisms before specific therapies or modification of practice can be undertaken in an attempt to protect normal neural development in the context of prematurity.

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