Jennifer Tatar Olsen

Brain Development after Postnatal Growth Restriction and Intermittent Hyperoxia Hypoxia in the Neonatal Rat

A Longitudinal MRI Study

Master's Thesis in Neuroscience

Trondheim, June 2016

Supervisor: Marius Widerøe
Co-supervisor: Tora Sund Morken

Norweigan University of Science and Technology
Faculty of Medicine
Department of Circulation and Medical Imaging

Abstract

Children born prematurely are commonly exposed to perinatal events such as infections, apneutic attacks and poor lung performance. These conditions put the children at risk of a reduced oxygen uptake and tissue hypoxia. Furthermore, overcompensations in treatment of hypoxia with supplementary oxygen often introduce too high levels of oxygen in the body, leading to tissue hyperoxia. The resultant periods of intermittent hyperoxia hypoxia (IHH) in preterms during critical periods of growth and development have been suggested to cause damage to white and grey matter in the brain, and to impair neurodevelopment. Body weight (BW) has also been suggested to play a role in the effects of IHH on brain development.

In this study we wanted to investigate the combined effect of IHH and postnatal weight gain on grey matter volume development in the brain. The purpose of this study was to increase the understanding of how a clinically relevant oxygen paradigm affects long term brain development, and possibly contribute towards improving the quality of the supplemental oxygen therapy that is provided to preterm born children.

Newborn rats were exposed to chronic levels of 50% O_2 interrupted every three hours by three consecutive five-minute episodes of 12% O_2, each eleven minutes apart, for the first two postnatal weeks. Postnatal growth restriction was obtained by litter culling, where litters consisted of either 8, 12 or 16 animals. T_2-weighted magnetic resonance images were acquired at postnatal day (P) 15 and P28, together with BW. Volumes of various grey matter structures were measured.

Animals that had been exposed to IHH were found to have higher BWs and brain volumes compared to RA controls, suggesting that IHH had a positive effect on postnatal weight gain and volume increase. The study was unable to determine if the increase in brain volume resulted from positive growth or pathological processes such as edema. However, the general promotion of IHH on postnatal growth led to the conclusion that exposure to IHH most likely increased BW, and that the increase in BW again led to the increase in brain volume.

Acknowledgements

I first and foremost would like to honour my supervisor **Marius Widerøe**, who's immense knowledge in the field of MR, and profound experience in the world of research, not only makes him a person of proud stature in the MR Core Facility at NTNU, but also an amazingly caring, supportive and enthusiastic supervisor. Thank you for your kindness and your critiques, and for being such a reliable guide throughout this whole *adventure* that this master thesis was to me.

I thank **Tora Sund Morken** for her support and interest in my work, and for trusting me with highly important assignments. But most of all I thank her for the possibility to present my research to an accomplished audience, it was truly an invaluable learning experience.

I thank **Tina Bugge Pedersen** for her brilliant organizational expertise, for her help with the laboratory preparations, and for her witty humor, which always puts a smile on the face of everyone within a hearing distance.

I also salute my co-master student, **Julia Anna Adrian**, whom I have had a pleasure to cherish this experience with. Despite our non-compete practice, you made me push myself towards always performing better, and you are the only person who knows exactly how much blood, sweat and tears that we put into this master. Thank you for your support and trustworthiness, and for your joyful company throughout our countless nights in the MR center.

Finally, I show gratitude to the most important person in my life, **Stian Aurdal Iversen**, for your unlimited love, for your overwhelming support, and for your excellent skills in zoning out during my endless talks about the master thesis. I treasure your patience, compassion and selflessness.

Table of contents

Symbols and Abbreviations

2D	2-dimentional
AHN	Anterior hypothalamic nucleus
B_0	External magnetic field
BBB	Blood brain barrier
BW	Body weight
^{13}C	Carbon-13
E	Embryological day
^{19}F	Fluorine-19
^{1}H	Proton
GR	Growth restriction
γ	Gyromagnetic ratio
IGF	Insulin-like growth factor
IHH	Intermittent hyperoxia hypoxia
IVC	Individually ventilated cages
MHz	Megahertz
MIPAV	Medical Image Processing Analysis and Visualization
MR	Magnetic resonance
MRI	Magnetic resonance imaging
NMV	Net magnetization vector
P	Postnatal day
ppm	Parts per million
PVL	Periventricular leukomalacia
RA	Room air
RARE	Rapid acquisition with relaxation enhancement
RF	Radiofrequency
SGA	Small for gestational age
SN	Substantia nigra
T	Tesla
T_1	Longitudinal relaxation
T_2	Transverse relaxation

TE	Echo time
TR	Repetition time
VLBW	Very low birth weight
ω_0	Larmor frequency

1 Introduction

1.1 Pathologies related to preterm birth

Preterm birth is defined as birth before 37 weeks of gestation, and occurs in 11% of infants[1]. Major perinatal morbidities, such as infections[2], apneutic attacks[3], respiratory distress syndrome[4] and poor lung performance[5], are associated with preterm birth, and can through the impediment of oxygen uptake lead to a reduced brain oxygen delivery during critical periods of growth and development. Poor neurodevelopmental outcomes, including reduced grey and white matter volumes and alterations in cortical structures, have been tied to preterm birth[6-9], and appear to proportionally worsen with younger gestational ages at birth[10,11]. Also increases in the prevalence of cognitive[9,12], motor[13], behavioural[14,15] and psychiatric problems[16] have been found in prematurely born children, of which some have been correlated to changes in cortical structures.

The increase in survival rate among preterm born children[17] is attributed to an improvement in the quality of treatment methods that aim to minimize the neonatal morbidities. However, the use of supplemental oxygen in treatment of reduced oxygen uptake in preterms is a challenge, as too high oxygen levels lead to more pathology, and too low levels increase mortality[18]. There are also no solid guidelines for the treatment dose and duration[19]. Throughout the early postnatal development, the preterm neonate therefore experiences continuous fluctuations in blood oxygen levels, as periods of low blood oxygen levels due to respiratory complications are followed by periods of high blood oxygen levels due to the oxygen supplement, before the blood oxygen levels normalize and the cycle repeats again[5]. Such large variations in blood oxygen concentrations due to suboptimal oxygen treatment therapies transfer to long lasting detrimental effects on the growth and development of various tissues in preterms.

1.2 Effect of hyperoxia and hypoxia on brain development

Hyperoxia occurs when tissue is exposed to higher than physiological levels of oxygen concentrations, and can lead to an increased amount of reactive oxygen species that have a destructive effect on biological tissue[20]. Hypoxia occurs when tissue is deprived of oxygen, and leads to reduced energy production at the cell level and eventually cell death[21]. The brain is especially vulnerable to hypoxic events due to its high energy requirement, large oxygen consumption and small energy reserves[21]. Limitations in the brains oxygen availability can

therefore lead to severe pathologies. For instance, patients with chronic obstructive pulmonary disease that frequently experience hypoxia were found to have reduced grey matter volumes in cortex, thalamus, caudate nucleus, putamen, parahippocampus and amygdala[22]. On the contrary, exposure to two and ten hours of 12% O_2 in healthy young males was found to increase grey matter and total brain volume, but simultaneously decrease white matter volumes[23]. Studies investigating the effect of hyperoxia in neonates have also found that it increases the risk of several neonatal morbidities, including lung complications and retinopathy of prematurity[24].

The effect of hyperoxia and hypoxia on the brain has also been studied using animals. Research in animals allows for a more controlled and tailored variation in the oxygen exposure, where established models of different exposure paradigms allows for comparison of results across studies. The most frequently used oxygen exposure models utilize sustained levels of hyperoxia or hypoxia. Ramani et al.[25] exposed mice to chronic hyperoxia (85% O_2) and hypoxia (12% O_2) during the first two postnatal weeks, and found that both hyperoxia and hypoxia lead to reduced hippocampal volumes. Porzionato et al.[26] also found smaller hippocampal volumes in response to chronic 60% and 95% O_2 during the first two postnatal weeks in rats, but the volumes of the subventricular zone (SVZ) were increased. Felderhoff-Mueser et al.[27] found that exposure to 80% O_2 for 2 hours or more lead to cell death in the caudate nucleus, accumbens nucleus, cortex and white matter tracts in the forebrain in rats. On the other hand, hypoxic insults of 11% O_2 from postnatal day (P) 4-P8 in rats were found to increase the volumes of anterior cingulate cortex, with no change in volumes of the caudate-putamen, dentate gyrus, and SVZ[28]. Also exposure of rats to chronic levels of 9.5% O_2 from P3-P33 resulted in decreased cortical volumes, and subcortical white matter[29]. It therefore appears that sustained levels of hyperoxia and hypoxia both can cause increases and decreases in brain volume, depending on the experimental setup. It also appears that individual brain structures respond differently when exposed to the same levels of unphysiological oxygen concentrations.

To my knowledge there is only one study that has investigated the effect of IHH on brain development. Morken et al.[30] exposed rats to 50% O_2 interrupted by three consecutive two-minute episodes of 12% O_2 every sixth hours during the first two postnatal weeks, and found subtle reversible changes in white matter diffusivity, grey matter water content and general vasculature density. Interestingly, Morken et al.[30] also discovered that brain pathology was more severe in the smaller animals, implicating that restrictions in growth could be a reinforcing factor in the effect of IHH on brain development. Body weigh has also previously been found to be proportional to

brain weight[31,32] and brain volume[33] in various mammalian species. Unfortunately, the study included only three litters, of which two were exposed to IHH and one served as RA control, and a large degree of uncertainty is therefore tied to the results. A larger scale study with a more severe oxygen exposure would therefore be needed to make the results clearer.

1.3 Postnatal growth restriction

Postnatal growth restriction is defined as growth values <10th percentile of the predicted growth in body weight (BW), body length or head circumference, and it occurs in approximately 28% of preterm born children[34]. Common pathologies associated with impaired postnatal growth include higher risks of ischaemic heart disease, type II diabetes mellitus and higher blood pressure in term born children[35]. However, as the occurrence of growth restriction has been found to increase with decreasing birth weight and gestational age at birth[34], preterm born children are necessarily more susceptible to develop pathologies associated with reduced growth. One of the primary contributors towards reducing postnatal growth appears to be inadequate dietary intakes due to poor nutritional practices in hospitals[36,37]. Suboptimal early nutrition has been found to cause reduced motor[38] and cognitive abilities[39], and several studies have stressed the need for a better nutritional support and follow-up of weight gain in infants of high risk for growth restriction[34,35]. Other factors that also have been found to relate to growth restriction include sex[40], genes[41], the occurrence of other illnesses[42], need for respiratory support[34], exposure to steroids during hospital course[43] and length of hospital stay[35].

Growth restriction is frequently employed in research to increase the variations in weight in research animals. There exist many different animal models for growth restriction that interfere with growth both *in utero* and *ex utero*. *In utero* models include interventions on the mother through nutritional limitations and pharmacological manipulations during gestation, and interventions on the foetus through placental nutrition limitations, genetic manipulations and infections[44,45]. *Ex utero* models include nutritional limitation in animals through litter culling[46]. While *in utero* models provide a good insight into diseases regarding infants born small for gestational age, *ex utero* models are more applicable to the type of growth experienced by most preterm infants[47].

1.4 Postnatal development of brain structures in rats

As rats are born premature in terms of brain development, a large part of the brain development occurs postnatally. Postnatal neurogenesis has for instance been found in a heterogeneity of grey matter structures located in both the telencephalon and in deeper parts of the brain. Telencephalon is situated in the rostral forebrain, and contains all higher order functions such as motor and sensory systems, speech and cognition[48]. Telencephalic structures include the cerebral cortex and olfactory bulb, in addition to several other subcortical structures such as the hippocampus, globus pallidus and corpus striatum[49] (Figure 1). Deeper grey mater structures in the literature commonly include thalamus, hypothalamus, and various small midbrain nuclei such as substantia nigra (Figure 1).

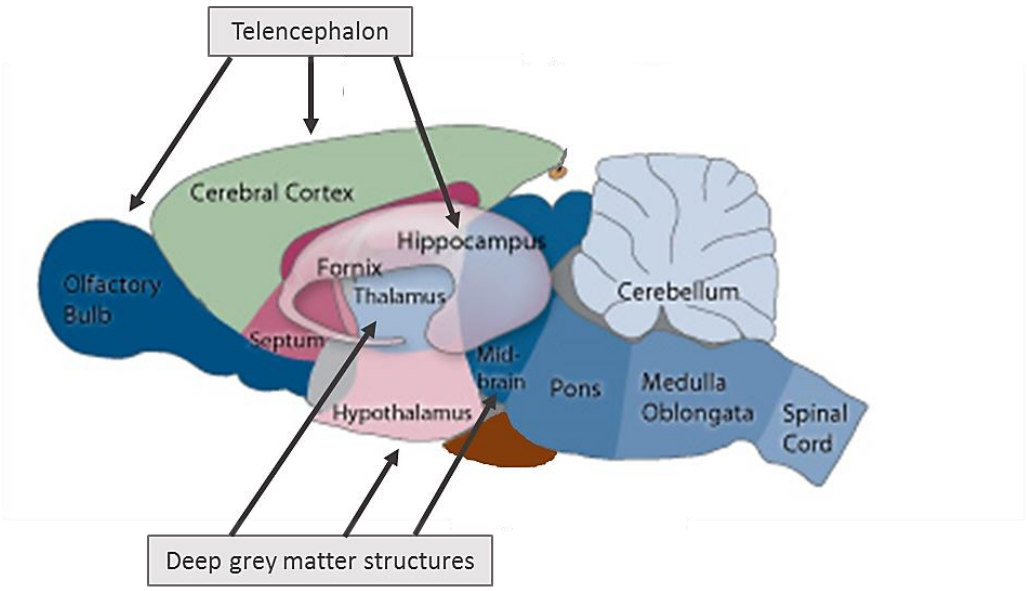

Figure 1 Midsagittal section of the rat brain illustrating the location of telencephalic and deep grey matter structures. Figure downloaded from: http://www.computescotland.com/extreme-reliability-of-design-and-the-agile-brain-5126.php, date: 22.05.2016. The original illustration has been edited.

In the telencephalon, granule cells have an exceptionally late time of origin, where approximately 85% of the cells develop during the first postnatal week, and where neurogenesis can continue until even the second and third postnatal week[50]. Furthermore, dentate granule neurons continue to be produced throughout adulthood, increasing the neuronal population by approximately 9000 new cells each day in young adult rats[51,52]. Also interneurons within the olfactory bulb are generated after birth[53]. The majority of interneurons develop during the first postnatal week, but neurogenesis is present beyond P20 within all interneuron populations[53]. As opposed to the granule cells in hippocampus, neurons in the olfactory bulb are continuously replaced throughout adult life, and do

not add to an increasing population[50]. Neocortical neurogenesis also occurs postnatally through the supply of cells from the SVZ, a late-appearing, secondary proliferate zone in the brain[54]. Studies disagree whether it is only glial cells, or also cortical neurons, that contribute to this postnatal growth[55], and other studies claim that neurons generated in adulthood are just temporary[56]. Adult neurogenesis has been discovered in the piriform cortex, temporal cortex, visual cortex and amygdala, although the extent and fate of these new neurons remain unclear at this point in time[57-59]. Finally, there is also a small degree of postnatal neurogenesis in the caudoputamen, where less than 10% of the neurons are generated between P0-P4[60]. Caudoputamen has however also been found to be supplied with neurons from the SVZ following tissue trauma, such as stoke, during the adult life[61].

The only two deep grey matter structures with a postnatal development are thalamus and hypothalamus. In addition to the prenatal generation of thalamic neurons from embryological day (E)14-E15[62], Mooney et al.[55] found that the ventrobasal nucleus of thalamus had a secondary in situ postnatal growth stage. During this second stage, the ventrobasal nucleus of thalamus increased 2.5-fold in neuronal numbers during the period from P3-P21, averaging with an estimated increase of 7500 neurons and glial cells per day. The thalamic volume was found to increase until P30. Hypothalamus is also mainly generated prenatally, with different nuclei developing between E12-E19[63]. Postnatal neurogenesis in hypothalamus in rats has however been found to occur during the first postnatal week, it makes up 5-7% of the total amount of labelled cells and it occurs in only a few hypothalamic nuclei[64].

1.5 Principles of magnetic resonance imaging

Magnetic resonance imaging (MRI) is a technique that utilizes atomic nuclei with an uneven mass number to form images. Atoms consisting of uneven mass numbers, such as for instance ^{1}H, ^{13}C and ^{19}F, have an unequal amount of protons and neutrons in their nucleus. This state causes individual nuclei to spin around their own axis, forming a small magnetic moment. When describing MRI, the magnetic moments are regarded as magnetization vectors[65].

In the absence of external magnetic fields, the magnetic vectors are randomly oriented in all directions (Figure 2A). But, when exposing the magnetic resonance (MR)-active nuclei to a static magnetic field (B_0), the magnetic vectors align themselves parallel or anti-parallel to B_0 (Figure 2B). The sum of all magnetic vectors from one specific type of MR-active nuclei form a net

magnetization vector (NMV) for that nuclei type, and provide the sample signal that is measured using MRI. As ^{1}H is the most abundant nuclei type in the body, it has a large NMV, and is therefore most commonly used in MRI[65].

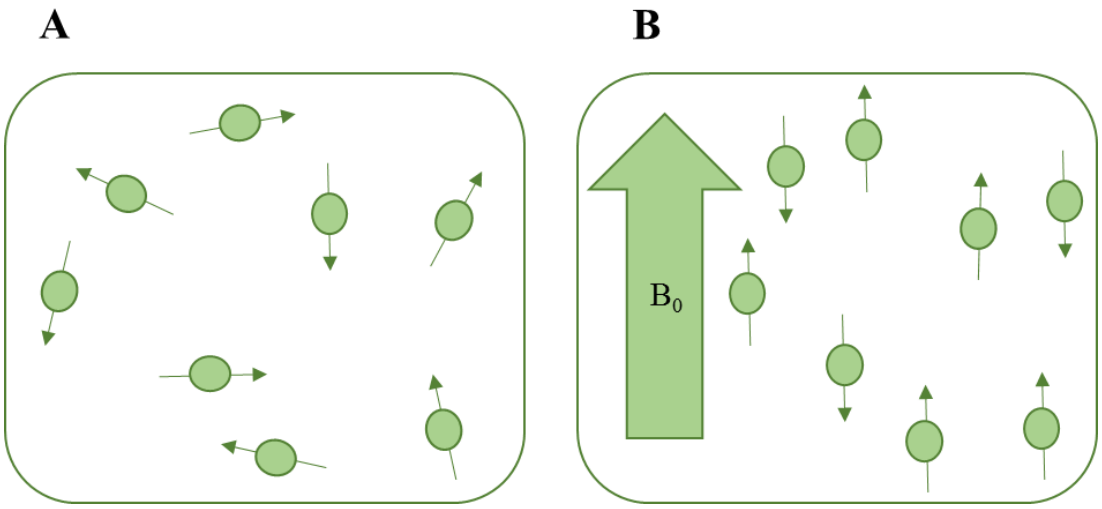

Figure 2 Alignment of MR-active nuclei. A) Random alignment with no external magnetic field. B) Parallel or anti-parallel alignment with external magnetic field (B_0).

The exposure of the sample to the external magnetic field (B_0) also causes the NMV to start precessing around B_0. The frequency of precession is called Larmor frequency, and is given by:

$$\omega_0 = \gamma \times B_0 \qquad \text{Eq. 1.1}$$

where y is the gyromagnetic ratio and B_0 the external magnetic field strength. The gyromagnetic ratio is denoted by the properties of the nuclei, and vary with different types of MR active nuclei. E.g. ^{1}H has a gyromagnetic ratio of 42.58 MHz/T. The Larmor frequency is therefore determined by both the properties of the nuclei and the strength of the applied magnetic field. In essence, this means that different MR active nuclei will have their own distinct Larmor frequency when exposed to the same strength of B_0[65].

1.5.1 Signal acquisition

Signal acquisition is obtained when a radio frequency (RF) pulse at a specific Larmor frequency is applied perpendicularly to B_0. This causes the NMV to flip from the direction of B_0 into the perpendicular xy-plane at an angle corresponding to the magnitude and duration of the RF pulse. The NMV continues to precess around B_0 in the xy-plane, forming an oscillatory magnetic field

that can induce voltage in a coil located in the same plane. The MR signal is produced every time the magnetic field cuts across the coil, and hence the frequency of the MR signal is the same as the Larmor frequency[65].

The spatial location of a spin population is determined through the stepwise application of three magnetic gradients at an angle perpendicular to each another. The gradients alter the Larmor frequency of spins in a linear fashion according to the spins position along them. The slice selective gradient is applied perpendicular to the desired slice plane, and enables the selective excitation of nuclei within a band of Larmor frequencies. Only excited nuclei are able to resonate, and hence provide a MR signal that can be measured. The two remaining gradients encode signal along the two remaining axis, where the phase gradient induces a difference in phase along the first axis, and the frequency gradient induces a difference in frequency along the second axis. The information on signal position from the gradients is transformed through a process called Fourier Transformation to form a 2D pixel image, and each pixel is allocated a grey colour corresponding to the signal intensity at the spatial location it represents[65].

1.5.2 Signal decay

Once the RF pulse is switched off, the NMV gradually realigns itself with B_0, and there is a loss of signal in the xy-plane. This decay in signal is caused by two separate processes that both occur at the same time. T_1 recovery is when nuclei transfer energy to their surrounding lattice, and recover their magnetic moments in the longitudinal plane. T_2 decay is when the magnetic moments of neighbouring nuclei interfere with each other, and cause a loss of coherency in the NMV in the transverse plane. The chemical properties of the immediate environment in which the nuclei are located affect how fast the signal decays. In fat, the dense packing of large lipid molecules will cause a fast T_1 recovery and T_2 decay, while in water, the small and highly mobile molecules cause a slow T_1 recovery and T_2 decay[65].

1.5.3 T_1 and T_2 weighting

Through manipulation of scan parameters, one can weigh the image contrast to depend on differences in either the T_1 recovery time or the T_2 decay time of structures in a sample. The manipulation occurs through pulse sequences, which allows us to control the way the system applies RF pulses and gradients. There are many different types of pulse sequences available, but

the spin-echo pulse sequence is most commonly applied in MRI, as it provides good image quality and is versatile at use. Two of the parameters that need to be manipulated in order to obtain contrast weighting are the repetition time (TR), meaning the time period between subsequent applications of RF pulses, and the echo time (TE), meaning the time period from the application of the RF pulse to the time of signal readout. The TR controls the time available for T_1 recovery, and the TE controls the time available for T_2 decay. In T_1-weighted images, the TR is short and the TE is short. This maximizes the difference in T_1 times between structures, while at the same time minimizing signal arising from differences in T_2 decay times. In T_2-weighted images, both the TR and the TE are long. This maximizes the difference in T_2 times between structures, while at the same time minimizing the contribution of signal arising from differences in T_1 times[65].

2 Objectives

In this study we wanted to investigate the effect of IHH on brain development. Using a clinically relevant oxygen paradigm, the study would not only increase the knowledge and understanding of the influence that IHH has on the brain, but more importantly possibly contribute towards an improvement in the quality of the supplemental oxygen therapy that is provided to preterms. We also wanted to investigate the role of BW in the impact of IHH on brain development.

We hypothesized that IHH would have a negative effect on brain volumes, that animals with a lower BW would have smaller brain volumes, and that IHH and low BW together would have an additive negative effect on brain volumes.

3 Material and methods

3.1 The animal model

Animal handling

All experiments were conducted according to guidelines set by the Norwegian Ethics committee for Animal Research, and approved by the local authority on animal welfare (Forsøksdyrutvalget, permit number 8010).

Eight time-mated Sprague Dawley rats were obtained from Taconic Biosciences Inc. (Ejby, Denmark), and kept at the animal facility at the Norwegian University of Science and Technology in Trondheim. All animals and their offspring were housed in standardized individually ventilated cages (IVC) class 3 for rats fitted with environmental enrichments, and exposed to a 12:12 hours light:dark cycle. Animals had access to food pellets and water *ad libitum* at all times. After birth, all offspring were carefully health monitored once a day for the first 14 days using score sheets with predefined monitoring parameters and humane end points.

Animals were kept in the animal facility at all instances, except for during MRI acquisition. MRI was conducted in the MR Centre located approximately 300 meters away from the animal facility. Transportation of the animals occurred the same morning or the day before the MRI, utilizing an underground transportation corridor and secure transport cages to minimize unwanted external interferences. In the MR Centre animals were kept in a separate room maintaining the same environmental conditions as in the animal facility. To ensure oxygen availability, the tops of the IVC in all litters were removed during the stay in the MR Centre.

All animals were weighed at P0, P15 and P28. At P15, all animals were also equipped with a RF identification chip to enable individual tracking of BW and MR images. Chipping was not possible at P0 as the procedure would have caused the newborn animals too much harm.

Experimental groups

The experimental groups were divided according to oxygen exposure and litter size. Animals were allocated to either the IHH paradigm or RA, and naturally born litters were culled to form new litters consisting of 8, 12 or 16 animals (Figure 3). The culling was done to ensure that animals in larger litters received reduced food supply, inducing postnatal growth restriction. With variations

in the BW of animals both within and between different litter sizes, the effect of IHH on brain volume could be measured across a weight gradient. To reduce litter effects, offspring from different litters that were born within 12 hours from one another were mixed together.

Five litters were placed in the IHH paradigm, and three litters served as RA controls (Figure 3). Within twelve hours after delivery of the last animal, cages containing litters and dam were placed in specialized plexiglass A-chambers (Biospherix Ltd., Lacona, NY) used for inducing IHH (Figure 4). The chambers were large enough to fit two IVC at the same time, but the cage lids had to be removed for ventilator purposes. At P14, animals were removed from the A-chambers and placed in RA up until they were euthanized at P28. Animals kept in RA were at all times placed in closed IVC coupled to a cage holding rack located in the same room (Figure 5).

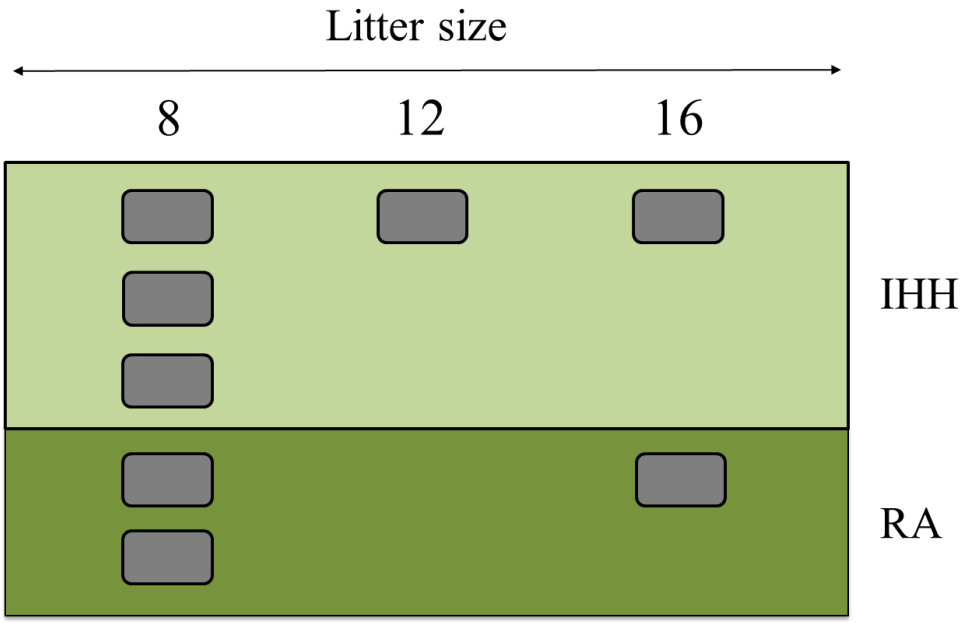

Figure 3 Experimental groups showing the division of litters according to litter size and oxygen exposure. Each grey box represents one litter. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air.

Figure 4 Specialized plexiglass A-chambers used for inducing IHH. The chambers were large enough to fit two IVC at the same time.

Figure 5 IVC rack with automated air supply from the integrated ventilator system in the animal facility.

All animals in the RA groups survived, but two animals from the IHH groups died during the experiment. The first was the highest weighing animal in the litter of 16 animals, which died before weight and MRI measures at P15 for unknown reasons. The second was an average weighing animal in a litter of 8 animals, which died in the MRI scanner at P15. Post-mortems were not performed. All data acquired before their deaths were utilized in the experiment.

The number of animals in the various subdivisions of experimental groups at all time points are listed in (Table 1).

Table 1 Number of animals in the various experimental subgroups at time points P0, P15 and P28. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, P0; postnatal day 0, P12; postnatal day 12, P16; postnatal day 16.

		Number of animals		
		P0	P15	P28
All animals		84	83	82
Litter sizes	8	40	40	39
	12	12	12	12
	16	32	31	31
Exposure groups	IHH	52	51	50
	RA	32	32	32
Exposure & Litter size	IHH, 8	24	24	23
	IHH, 12	12	12	12
	IHH, 16	16	15	15
	RA, 8	16	16	16
	RA, 16	16	16	16

The oxygen profile

An A84 Oxycycler (Biospherix Ltd., Lacona, NY) was used to program the IHH profiles. A computerized feedback system regulated the oxygen concentration within the chambers, using nitrogen from bottles to lower the oxygen concentration, and oxygen from the local air vault to increase the oxygen concentration. Sensors within the chambers monitored the levels of oxygen, CO_2 (mean = 1194 ppm, SD = 858), temperature (mean = 22.75°C, SD = 0.22) and relative humidity (mean = 48.22%, SD = 12.42).

A continuous high air oxygen concentration (50% O_2) was interrupted every three hours by three consecutive five-minute episodes of low air oxygen concentration (12% O_2), each eleven minutes apart (Figure 6). The ramp time from 50% O_2 to 12% O_2 was approximately eight minutes (Figure 6, A→B). Better ramping times were not possible to accomplish due to limited N_2 flow rate relative to the large size of the A-chambers. The ramping time from 12% O_2 to 50% O_2 was approximately three minutes (Figure 6, C→D). The total duration of one sequence of three episodes was approximately forty-eight minutes. The A-chambers were also opened daily for an average of under four minutes during the high air oxygen concentration for neonatal health check, food refill and change of water bottle.

Due to system failure and unreplaced nitrogen bottles, five sequences of low air oxygen concentration episodes were not induced on three separate occasions during the two week IHH profile period. This resulted in the oxygen concentration reaching only 21% O_2 instead of the designated 12% O_2 during the affected periods.

Animals exposed to RA were at all times supplied with regular air (21% O_2) from the integrated ventilator system in the animal facility that was coupled to the IVC rat racks. The air was maintained at a temperature of 20-21°C and a humidity of 55% ± 10%.

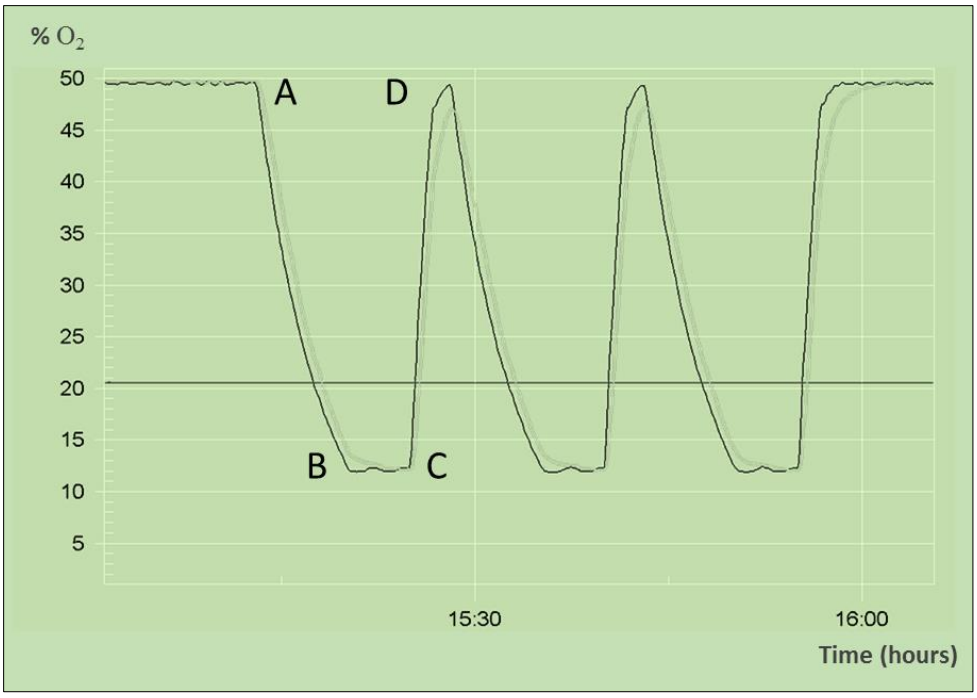

Figure 6 Screenshot of the oxygen profile showing three consecutive episodes of low air oxygen concentrations, each lasting five minutes (from B to C). The ramp time between the high and low air oxygen levels was eight minutes when going from 50% to 12% air oxygen levels (from A to B), and three minutes when going from 12% to 50% air oxygen levels (from C to D).

3.2 Magnetic resonance imaging

MRI was performed at P15 and P28 using a 7-Tesla magnet (Biospec 70/20 AS; Bruker Biospin MRI, Ettlingen, Germany) with water cooled gradients (BGA-12S, 660 mT/m). Animals were anesthetized (2% isoflurane with 30% O_2) and scanned longitudinally at both time points while laying prone in a dedicated water-heated bed (Bruker Biospin MRI). The head of every animal was fixated using nose-mask, polystyrene, tooth bar (P28 only) and ear fixators (P28 only). Temperature and respiration rate of each animal was monitored throughout the scanning procedure.

On P15 and P28 coronal T_2-weighted images were obtained using a turbo spin-echo (RARE) sequence with the following parameters: effective echo time = 70 ms; repetition time = 4000 ms; RARE-factor = 16; field of view= 20.48 x 15.36 mm^2; acquisition matrix = 256 x 192; 18 slices á 0.8 mm with 0.2 mm slice gaps; spatial resolution 80 x 80 x 800 µm. Number of averages was 8 giving a scan time of 6 minutes and 24 seconds.

3.3 Image analysis

Volume measurements of cerebrum, cortex, hippocampus, putamen, thalamus, hypothalamus, anterior hypothalamic nucleus (AHN) and substantia nigra were acquired by manually drawing the outlines of each brain structure in every image slice using the T_2 images and brain atlas'[66,67] for structure definitions (Figure 7). The drawing was performed on Wacom DTF-720 LCD tablet using MIPAV (Medical Image Processing Analysis and Visualization) software.

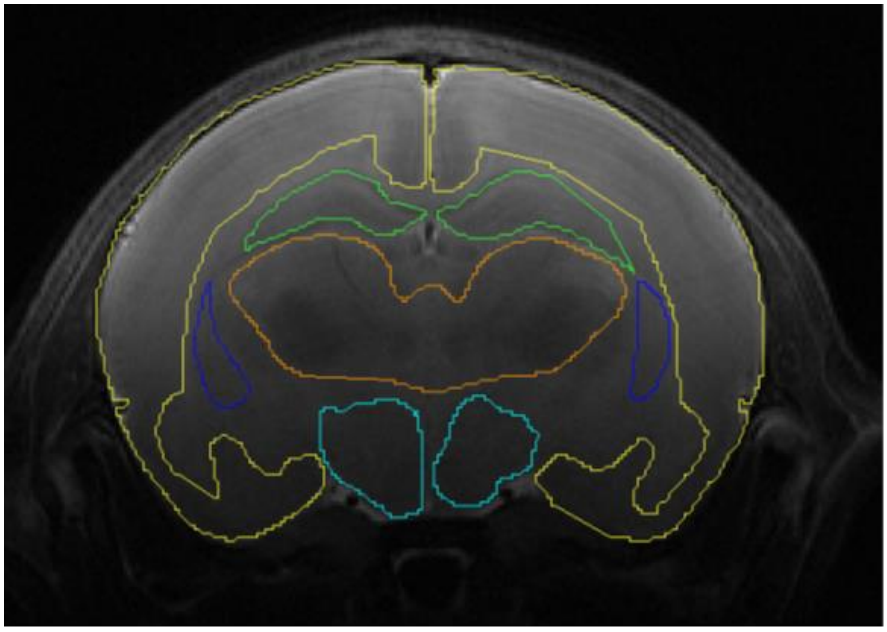

Figure 7 A T_2-weighted MRI image showing a coronal section of the rat brain with outlines of the hand-drawn brain structures. Different colours code for different structures. In this image: yellow = cerebral cortex, green = hippocampus, orange = thalamus, dark blue = putamen, light blue = hypothalamus.

Cerebral volume was defined as including all of brain tissue, with the exception of cerebellum and medial hindbrain structures. Superior colliculus, inferior colliculus and nucleus of lateral lemniscus was however also included for practical drawing purposes.

3.4 Statistical analysis

Version 23 of the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The level of significance was set to 0.05. Independent samples t-tests were used for comparison of means between two independent experimental groups, such as oxygen exposures and gender. Equal variances were not assumed. Paired samples t-tests were used for comparison of means between two time points. Pearson correlation was used to test the

correlation between two continuous variables, such as BW against brain volume. Spearman's correlation was used to test correlation between ordinal and continuous data, such as litter size against BW.

4 Results

4.1 Variations in BW between experimental groups

All animals had an increase in BW between P0 and P15, and between P15 and P28 (Figure 8A). The average BW varied inversely with litter size, where animals in smaller litters weighed more than animals in larger litters (Figure 8B). This variation was also present within the individual IHH and RA groups, at both time points (Figure 8D).

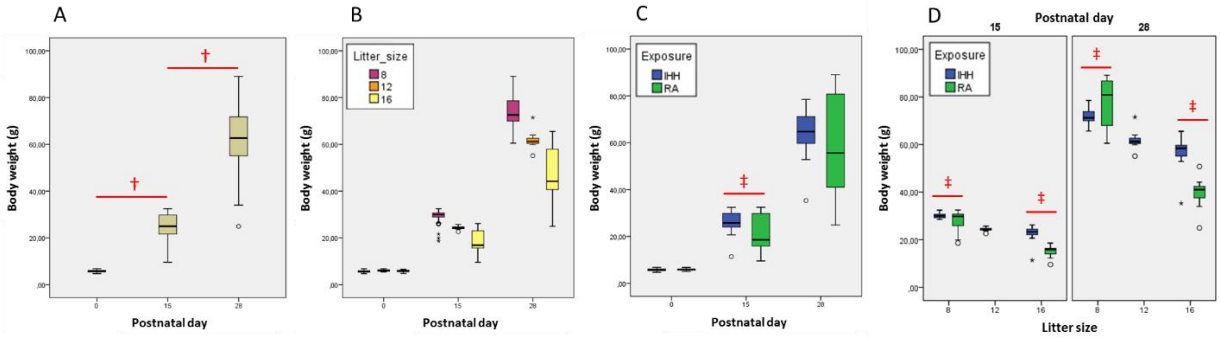

Figure 8 Box plots showing the BW distribution of animals at 0, 15 and 28 days after birth. A) BW distribution of all animals. B) BW distribution of animals according to litter size. C) BW distribution of animals according to exposure groups. D) BW distribution according to both litter size and exposure group. Circles and stars represent outliers and extreme outliers, respectively. †p-values < 0.001 between time points. ‡p-value < 0.05 between IHH and RA. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air.

Animals in the IHH group had a larger absolute and relative weight gain from P0-P15 compared with animals in the RA group (Figure 9A+B). The IHH group also had a higher average BW at P15 (Figure 8C), in both litter size 8 and 16 (Figure 8D). There were no group differences in absolute weight gain from P15-P28 (Figure 9C), but the RA group had a larger relative weight gain from P15-P28 (Figure 9D). The average BW of all IHH animals at P28 also tended to be higher than in all the RA animals, although the difference was not significant ($p = 0.128$) (Figure 8C). The RA_8 group did however have a higher average BW than the IHH_8 group, while the IHH_{16} group had a higher average BW than the RA_{16} group (Figure 8D).

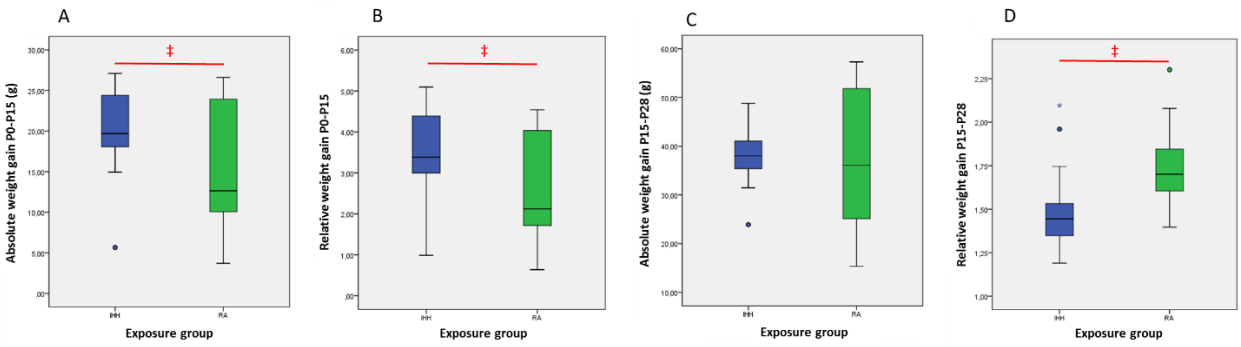

Figure 9 Box plots showing the absolute and relative weight gain of animals in relation to the exposure groups. A) Absolute weight gain from P0-P15. B) Relative weight gain from P0-P15. C) Absolute weight gain from P15-P28. D) Relative weight gain from P15-P28. Circles and stars represent outliers and extreme outliers, respectively. ‡p-value < 0.001 between IHH and RA. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, P0; postnatal day 0, P15; postnatal day 15, P28; postnatal day 28.

The difference in weight gain between the exposure groups was greater in litters of 16 animals compared with litters of 8 animals for both the absolute and relative weight from P0-P15 (Figure 10A+B). During this time period, the IHH group had higher weight gains compared with the RA group in all litter sizes. In the time period P15-P28, the difference in absolute weight gain between the exposure groups became more similar in litter size 8 and 16 (Figure 10C). However, the RA_8 group now had a larger absolute weigh gain than the IHH_8 group, while the IHH_{16} group maintained a larger absolute weight gain compared with RA_{16} group. The RA group also had larger relative weight gain from P15-P28 compared with the IHH group, in both litter size 8 and 16 (Figure 10D). The lower relative weight gain from P15-P28 in the IHH_8 group deviated from the trend where weight gain varied inversely with litter size, and led to a great difference between the exposure groups in litter size 8.

The difference in weight gain between IHH_8 and IHH_{16} was smaller than between RA_8 and RA_{16} for absolute and relative weight gain from P0-P15 and absolute weight gain from P15-P28 (Figure 10A+B+C). The difference in relative weight gain from P15-P28 between the litter sizes appeared to be approximately the same within the exposure groups, although weight gain varied inversely with litter size within the IHH group due to the small relative weight gain in IHH_8.

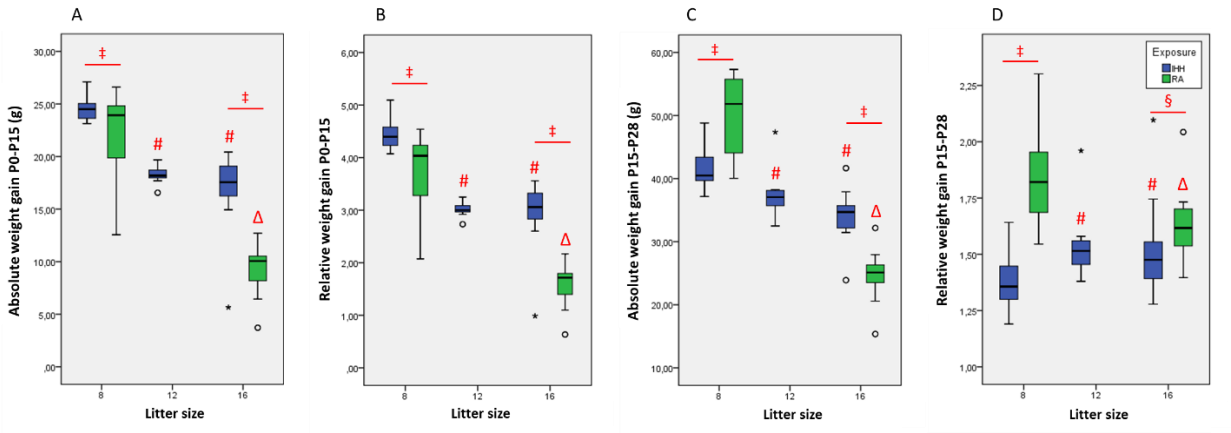

Figure 10 Box plots showing the absolute and relative weight gain in animals in relation to litter size and the exposure groups. A) Absolute weight gain from P0-P15. B) Relative weight gain from P0-P15. C) Absolute weight gain from P15-P28. D) Relative weight gain from P15-P28. Circles and stars represent outliers and extreme outliers, respectively. ‡p-value < 0.001 between IHH and RA. §p-value = 0.004 between IHH and RA. $^{\#}$p-value < 0.05 between IHH_8 and IHH_{12}, and IHH_8 and IHH_{16}. $^{\Delta}$p-value < 0.05 between RA_8 and RA_{16}. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, P0; postnatal day 0, P15; postnatal day 15, P28; postnatal day 28.

4.2 Variations in brain volumes between experimental groups

In general, brain volumes at P28 were larger than brain volumes at P15 in all structures, except in anterior hypothalamic nucleus (p = 0,373) and hypothalamus (Figure 11). The absolute increase in volume from P15 to P28 was smaller in AHN, hypothalamus and SN compared with the rest of the structures (Figure 11).

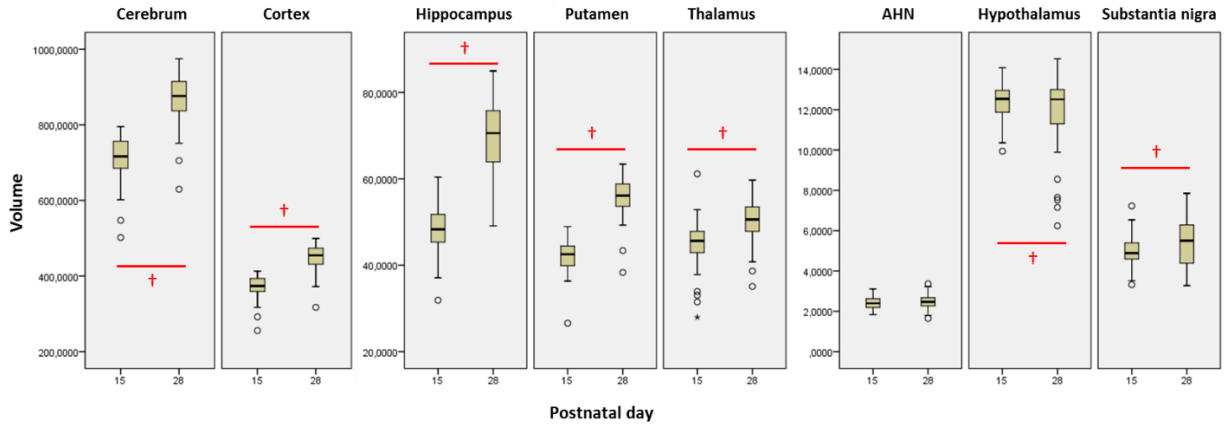

Figure 11 Box plots showing the volumes of cerebrum, cortex, hippocampus, putamen, thalamus, AHN, hypothalamus and substantia nigra at time points P15 and P28. Volumes are shown in units of mm^3. Circles and stars represent outliers and extreme outliers, respectively. †p-value < 0.05 between time points. Abbreviations: AHN; anterior hypothalamic nucleus.

4.2.1 The effect of IHH on brain volumes

Overall, brain volumes were larger in the IHH groups compared with the RA groups, with the exception of substantia nigra at P15, where the IHH group had smaller volumes than the RA group. (Figure 12). Also, the difference in volume between the exposure groups appeared to be dependent on the structure's location within the brain. Structures involving higher order brain functions in the telencephalon, such as the cerebrum, cortex and hippocampus, had more pronounced differences between the exposure groups, while deep grey matter structures, such as thalamus, AHN and hypothalamus had more subtle, if any, group differences (Figure 12).

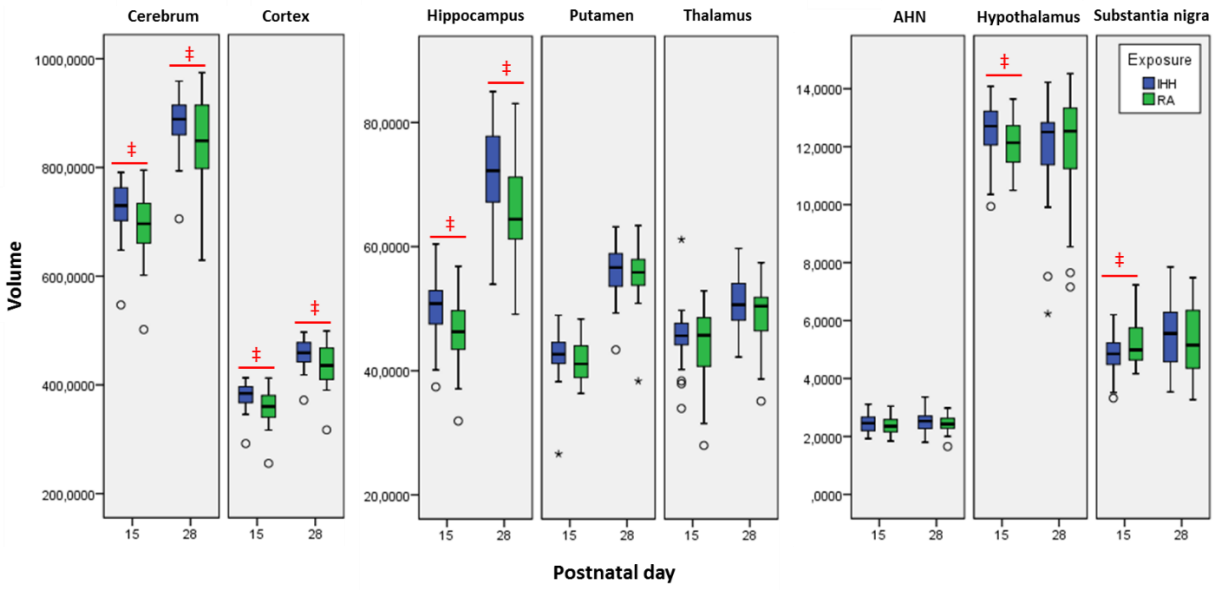

Figure 12 Box plots showing the volumes of cerebrum, cortex, hippocampus, putamen, thalamus, AHN, hypothalamus and substantia nigra at time points P15 and P28 in animals exposed to IHH and RA. Volumes are shown in units of mm^3. Circles and stars represent outliers and extreme outliers, respectively. ‡p-value < 0.05 between IHH and RA. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus.

When looking at the differences in brain volume between the exposure groups within same sized litters at P15, animals in the IHH group had larger volumes compared with animals in the RA group in the cerebrum, cortex and hippocampus in litter size 16, and in hippocampus, AHN and hypothalamus in litter size 8 (Figure 13). At P28, animals in the IHH group had larger volumes than animals in the RA group in only cerebrum and cortex in litter size 16 and hippocampus in litter size 8 (Figure 14).

The difference in brain volume between litter sizes within one exposure group also varied with different structures. At P15, the IHH_8 group had larger volumes than the IHH_{16} group in all structures except hippocampus ($p = 0.056$) and thalamus ($p = 0.055$), and the RA_8 group had larger volumes than the RA_{16} group in the cerebrum, cortex, hippocampus and putamen (Figure 13). At P28, the IHH_8 group had larger volumes than the IHH_{16} group in all structures except AHN ($p = 0.139$), and the RA_8 group had larger volumes than the RA_{16} group in all structures except thalamus ($p = 0.309$), AHN ($p = 0.887$) and hypothalamus ($p = 0.515$) (Figure 14).

The differences in brain volume between exposure groups were more pronounced at P15 compared with P28. Also the amount of significant differences in brain volume between exposure groups decreased from P15 to P28.

4.2.2 The effect of BW on brain volumes

There was a positive correlation between the BW at P15 and the volumes at P15 in all structures, except AHN and SN, for all animals (Table 2). There was also a positive correlation between the BW at P28 and volumes at P28 in all structures, except hypothalamus and AHN, for all animals (Table 3). Furthermore, the volumes at P28 were positively correlated with BW at P15 in all structures, except hypothalamus and AHN, for all animals (Table 3). Scatter plots show examples of the data point distribution for a selection of structures (Figure 15, Figure 16).

The absolute and relative weight gain from P0-P15 was correlated with both the volumes at P15 and the volumes at P28 for all structures, except AHN and SN (Table 4, Table 5). The absolute weight gain from P15-P28 was correlated with the volumes at P28 for all structures, except hypothalamus and AHN (Table 5). The relative weight gain from P15-P28 was only correlated with the volumes at P28 for hippocampus and AHN (Table 5). Scatter plots show examples of the data point distribution for a selection of structures (Figure 17, Figure 18, Figure 19).

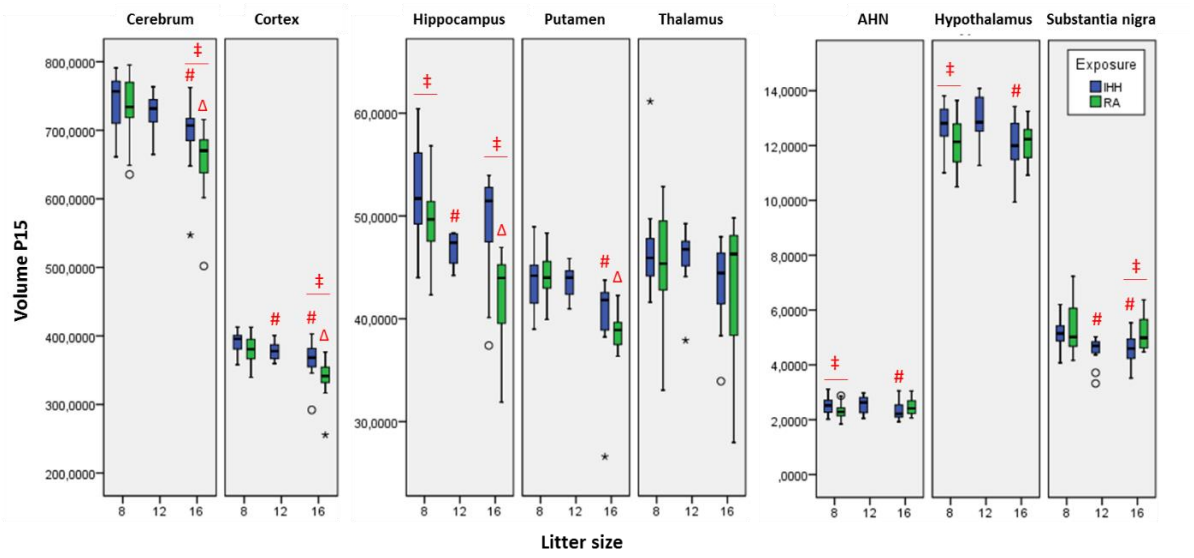

Figure 13 Box plots showing the volumes of cerebrum, cortex, hippocampus, putamen, thalamus, AHN, hypothalamus and substantia nigra at time point P15 for different litter sizes in animals exposed to IHH and RA. Volumes are shown in units of mm^3. Circles and stars represent outliers and extreme outliers, respectively. ‡p-value < 0.05 between IHH and RA. #p-value < 0.05 between IHH_8 and IHH_{12}, and IHH_8 and IHH_{16}. Δp-value < 0.001 between RA_8 and RA_{16}. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, P15; postnatal day 15.

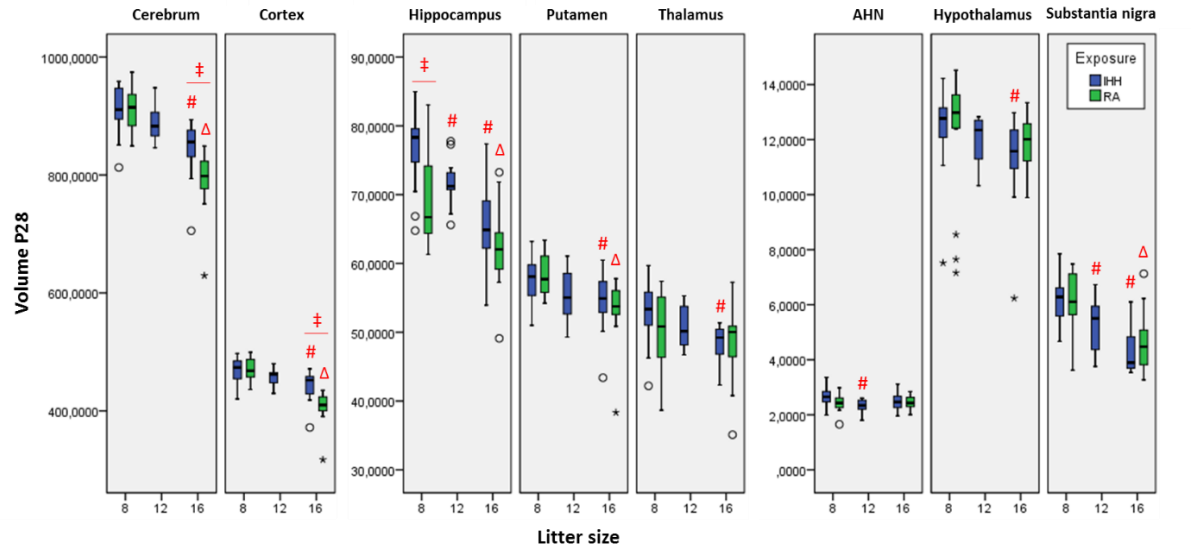

Figure 14 Box plots showing the volumes of cerebrum, cortex, hippocampus, putamen, thalamus, AHN, hypothalamus and substantia nigra at time point P28 for different litter sizes in animals exposed to IHH and RA. Volumes are shown in units of mm^3. Circles and stars represent outliers and extreme outliers, respectively. ‡p-value < 0.05 between IHH and RA. #p-value < 0.05 between IHH_8 and IHH_{12}, and IHH_8 and IHH_{16}. Δp-value < 0.001 between RA_8 and RA_{16}. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, P28; postnatal day 28.

Table 2 Pearson correlation between brain volumes at P15 and body weight for all animals, animals in the IHH group and animals in the RA group at P15. P-values in heavy print show statistical significance p < 0.05. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, SN; substantia nigra, P15; postnatal day 15.

		Body weight		
		All animals	IHH	RA
		P15	P15	P15
Cerebrum P15	r	0.689	0.611	0.692
	P-value	< 0.001	< 0.001	< 0.001
Cortex P15	r	0.765	0.717	0.735
	P-value	< 0.001	< 0.001	< 0.001
Hippocampus P15	r	0.668	0.558	0.662
	P-value	< 0.001	< 0.001	< 0.001
Putamen P15	r	0.653	0.530	0.821
	P-value	< 0.001	< 0.001	< 0.001
Thalamus P15	r	0.372	0.393	0.337
	P-value	0.001	0.004	0.059
Hypothalamus P15	r	0.268	0.411	-0.039
	P-value	0.014	0.003	0.834
AHN P15	r	0.037	0.285	-0.284
	P-value	0.740	0.042	0.115
SN P15	r	0.140	0.381	0.271
	P-value	0.206	0.006	0.134

Table 3 Pearson correlation between brain volumes at P28 and body weight for all animals, animals in the IHH group and animals in the RA group at both time points P15 and P28. P-values in heavy print show statistical significance p < 0.05. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, SN; substantia nigra, P15; postnatal day 15, P28; postnatal day 28.

		Body weight					
		All animals		IHH		RA	
		P15	P28	P15	P28	P15	P28
Cerebrum P28	r	0.728	0.742	0.681	0.652	0.720	0.785
	P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Cortex P28	r	0.706	0.719	0.590	0.555	0.721	0.780
	P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Hippocampus P28	r	0.644	0.561	0.727	0.704	0.471	0.528
	P-value	< 0.001	< 0.001	< 0.001	< 0.001	0.007	0.002
Putamen P28	r	0.473	0.505	0.492	0.468	0.535	0.582
	P-value	< 0.001	< 0.001	< 0.001	0.001	0.002	< 0.001
Thalamus P28	r	0.390	0.339	0.527	0.405	0.249	0.297
	P-value	< 0.001	0.002	< 0.001	0.004	0.170	0.098
Hypothalamus P28	r	0.194	0.175	0.495	0.529	0.018	0.021
	P-value	0.081	0.116	< 0.001	< 0.001	0.922	0.911
AHN P28	r	0.203	0.141	0.350	0.360	-0.013	-0.031
	P-value	0.067	0.206	0.013	0.01	0.944	0.867
SN P28	r	0.572	0.550	0.601	0.542	0.663	0.632
	P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

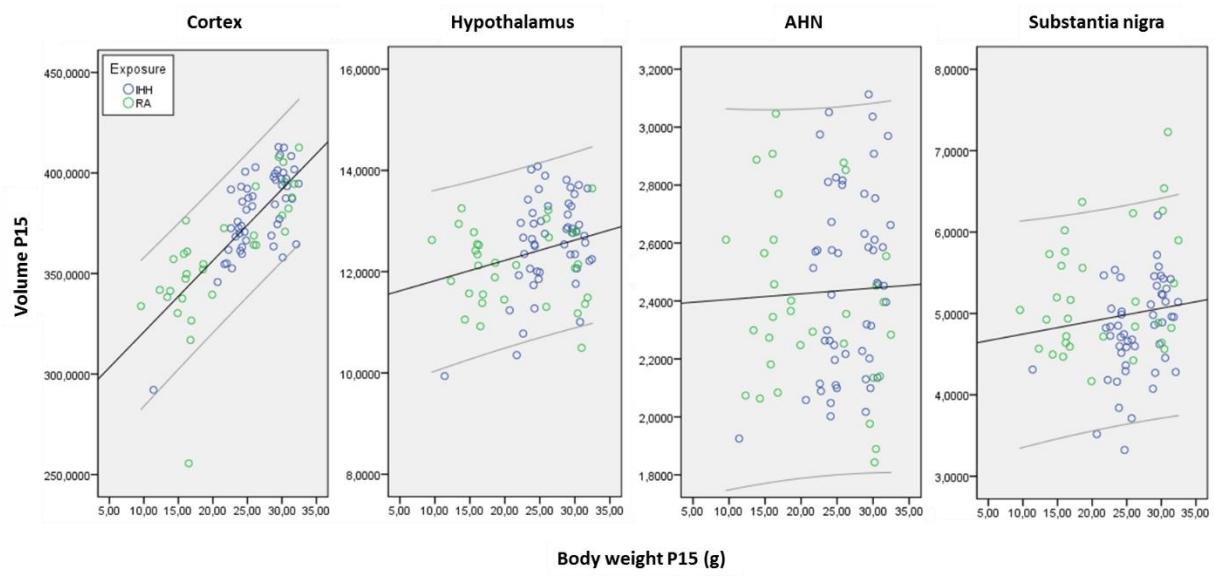

Figure 15 Scatter plots showing the correlation between BW at P15 and volumes of cortex, hypothalamus, AHN and substantia nigra at P15. Black lines represent the fittet linear regression lines for all subjects, grey lines represent the individual 95% confidence intervals. Volumes are shown in units of mm^3. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, P15; postnatal day 15.

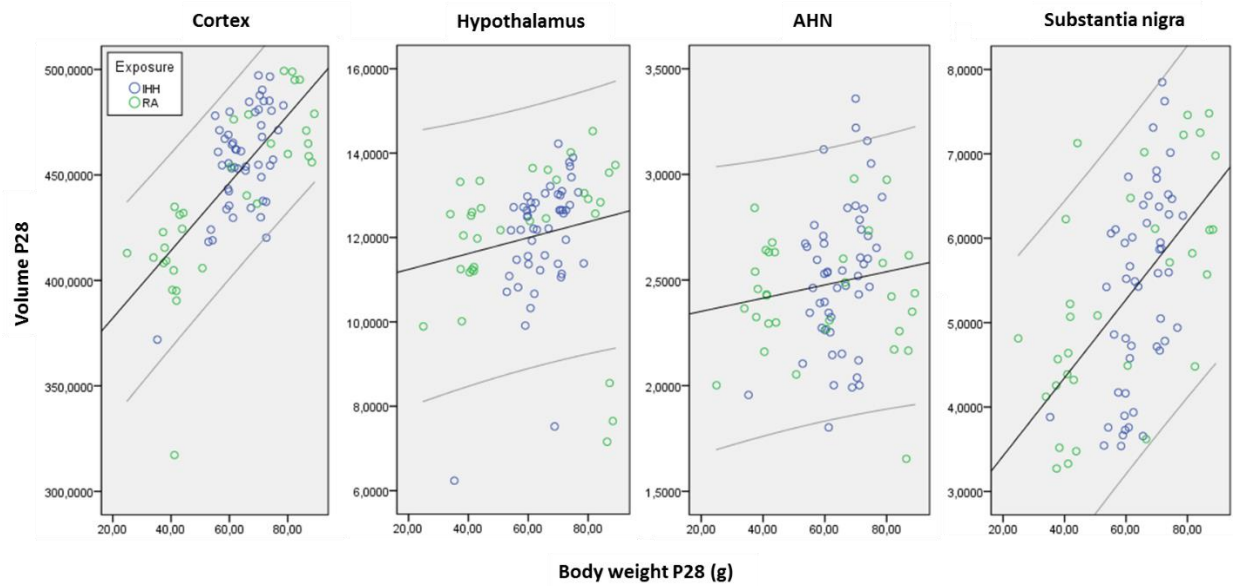

Figure 16 Scatter plots showing the correlation between BW at P28 and volumes of cortex, hypothalamus, AHN and substantia nigra P28. Black lines represent the fittet linear regression lines for all subjects, grey lines represent the individual 95% confidence intervals. Volumes are shown in units of mm3. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, P28; postnatal day 28.

Table 4 Pearson correlation between brain volumes at P15, and absolute and relative weight gain from P0-15. P-values in heavy print show statistical significance p < 0.05 between weight gain and volume. Abbreviations: AHN; anterior hypothalamic nucleus, SN; substantia nigra, P0; postnatal day 0, P15; postnatal day 15.

		Absolute weight gain P0-P15	*Relative weight gain P0-P15*
Cerebrum P15	r	0.682	0.649
	P-value	< 0.001	< 0.001
Cortex P15	r	0.762	0.739
	P-value	< 0.001	< 0.001
Hippocampus P15	r	0.670	0.668
	P-value	< 0.001	< 0.001
Putamen P15	r	0.642	0.597
	P-value	< 0.001	< 0.001
Thalamus P15	r	0.367	0.345
	P-value	0.001	0.001
Hypothalamus P15	r	0.266	0.258
	P-value	0.015	0.018
AHN P15	r	0.038	0.049
	P-value	0.732	0.658
SN P15	r	0.140	0.137
	P-value	0.205	0.215

Table 5 Pearson correlation between brain volumes at time P28, and absolute and relative weight gain from P0-15 and P15-P28. P-values in heavy print show statistical significance p < 0.05 between weight gain and volume. Abbreviations: AHN; anterior hypothalamic nucleus, SN; substantia nigra, P0; postnatal day 0, P15; postnatal day 15, P28; postnatal day 28.

		Absolute weight gain P0-P15	*Relative weight gain P0-P15*	*Absolute weight gain P15-P28*	*Relative weight gain P15-P28*
Cerebrum P28	r	0.721	0.688	0.775	-0.065
	P-value	< 0.001	< 0.001	< 0.001	0.562
Cortex P28	r	0.698	0.660	0.761	-0.060
	P-value	< 0.001	< 0.001	< 0.001	0.591
Hippocampus P28	r	0.646	0.656	0.504	-0.308
	P-value	< 0.001	< 0.001	< 0.001	0.005
Putamen P28	r	0.467	0.441	0.575	-0.006
	P-value	< 0.001	< 0.001	< 0.001	0.960
Thalamus P28	r	0.392	0.399	0.344	-0.153
	P-value	< 0.001	< 0.001	0.002	0.170
Hypothalamus P28	r	0.192	0.186	0.151	-0.063
	P-value	0.084	0.094	0.174	0.576
AHN P28	r	0.210	0.234	0.081	-0.237
	P-value	0.058	0.035	0.467	0.032
SN P28	r	0.569	0.558	0.502	-0.116
	P-value	< 0.001	< 0.001	< 0.001	0.300

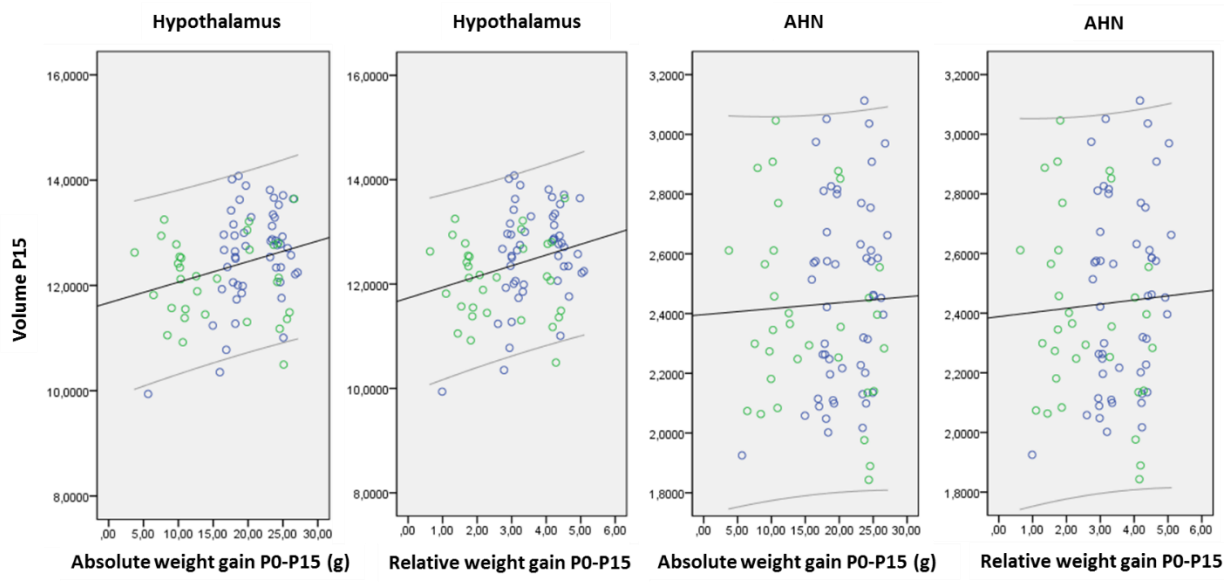

Figure 17 Scatter plots showing the correlation between absolute and relative weight gain from P0-P15 and the volumes of hypothalamus and AHN at P15. Black lines represent the fittet linear regression lines for all subjects, grey lines represent the individual 95% confidence intervals. Volumes are shown in units of mm3. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, P0; postnatal day 0, P15; postnatal day 15.

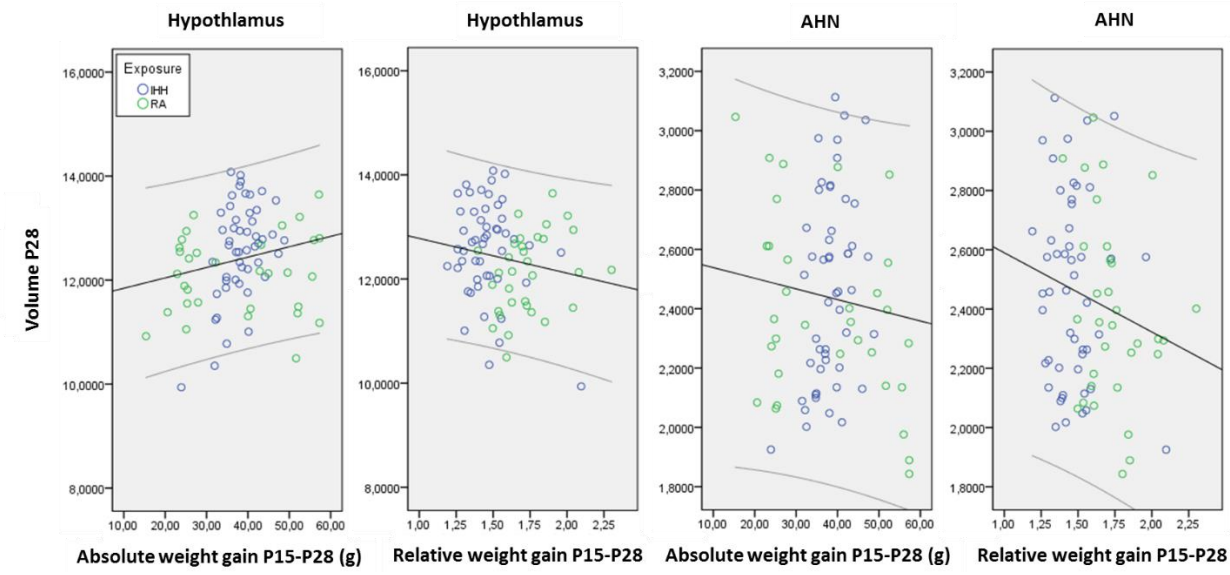

Figure 18 Scatter plots showing the correlation between the absolute and relative weight gain from P0-P15 and P15-P28 with the volumes of hippocampus and AHN. Black lines represent the fittet linear regression lines for all subjects, grey lines represent the individual 95% confidence intervals. Volumes are shown in units of mm3. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, P0; postnatal day 0, P15; postnatal day 15, P28; postnatal day 28.

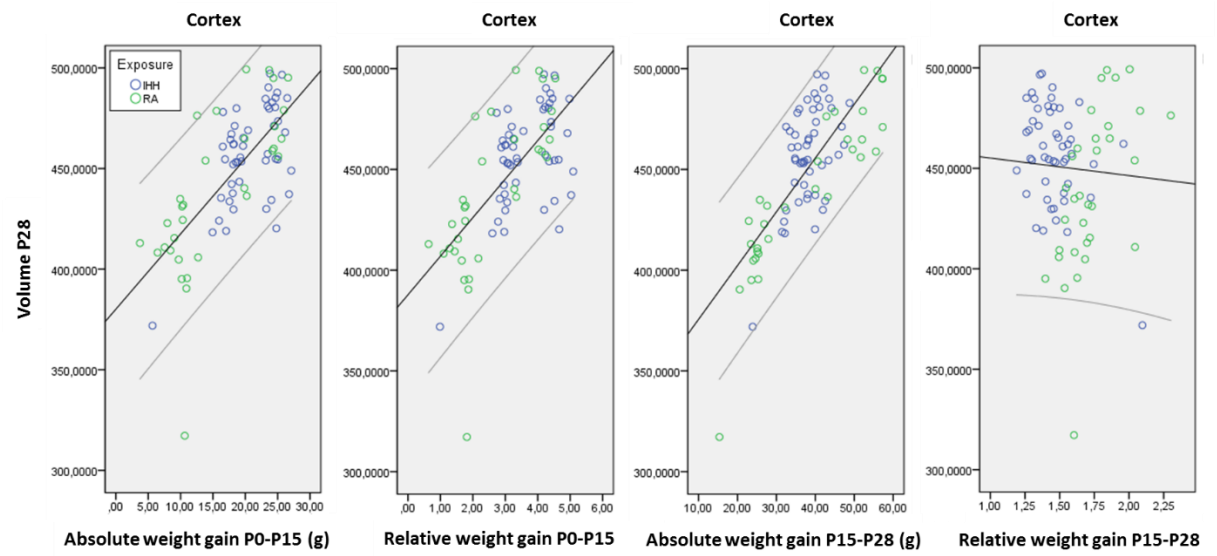

Figure 19 Scatter plots showing the correlation between absolute and relative weight gain from P0-P15 and P15-P28 and the volume of cortex at P28. Black lines represent the fittet linear regression lines for all subjects, grey lines represent the individual 95% confidence intervals. Volumes are shown in units of mm3. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air, AHN; anterior hypothalamic nucleus, P0; postnatal day 0, P15; postnatal day 15, P28; postnatal day 28.

Animals in the RA group had a higher brain volume to BW ratio compared with animals in the IHH group at both P15 and P28 (Figure 20A). This increased volume:BW ratio was present only in litter size 16 (Figure 20B). Animals in litter size 16 had higher volume:BW ratios compared with animals in litter size 8 in both the IHH and RA groups, and at both time points (Figure 20B). The IHH_{12} group also had higher volume:BW ratios than the IHH_8 group at both time points, but there was no difference in ratio size between IHH_{12} and IHH_{16} ($p_{15} = 0.157$, $p_{28} = 0.105$). Box plots of the volume:BW ratio for cortex are representative for the variations in volume:BW in all structures.

Overall, the brain volumes of all structures at both P15 and P28 varied inversely with litter size (Figure 21).

4.2.3 The additive effect of IHH and BW on brain volumes

Animals in the IHH group had a positive correlation between BW and volumes in all structures at both time points P15 and P28 (Table 2, Table 3). Animals in the RA group had a positive correlation between BW and volumes in all structures, except thalamus, hypothalamus and AHN at both P15 and P28 (Table 2, Table 3), and in substantia nigra at P15 only (Table 3). Additionally, all structures with a correlation between BW at P28 and brain volumes at P28 also had a correlation between BW at P15 and brain volumes at P28, within both the IHH and RA groups (Table 3).

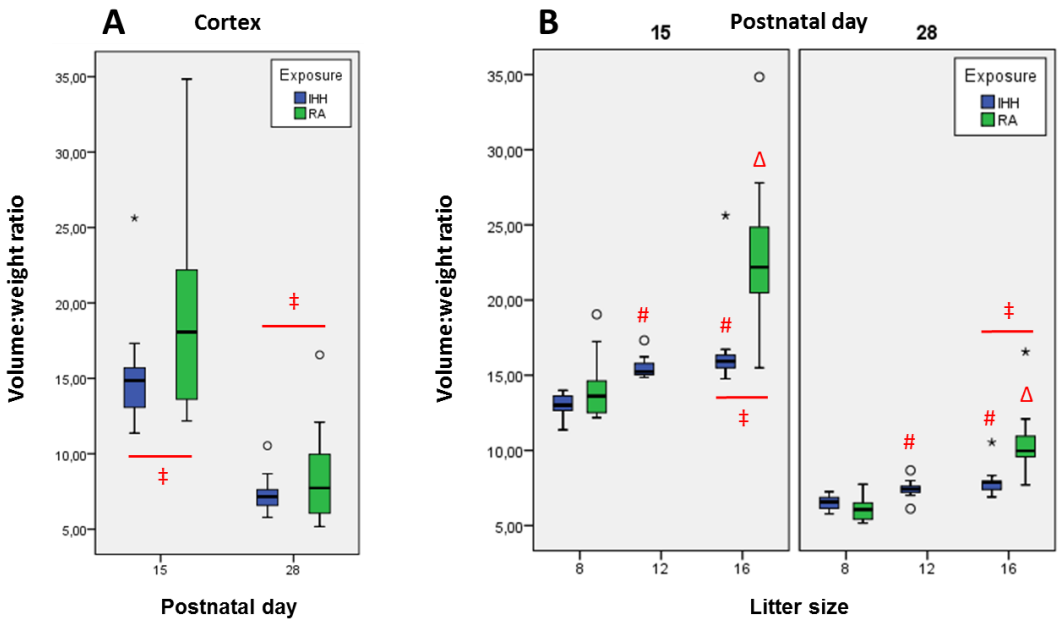

Figure 20 Box plots showing the volume:BW ratio distribution for cortex according to A) time points and exposure groups, and B) time points, exposure groups and litter size. Volume:BW index is shown in units of mm^3/g. Circles and stars represent outliers and extreme outliers, respectively. ‡p-value < 0.05 between IHH and RA. #p-value < 0.001 between IHH_8 and IHH_{12}, and IHH_8 and IHH_{16}. Δp-value < 0.001 between RA_8 and RA_{16}. Abbreviations: IHH; intermittent hyperoxia hypoxia, RA; room air.

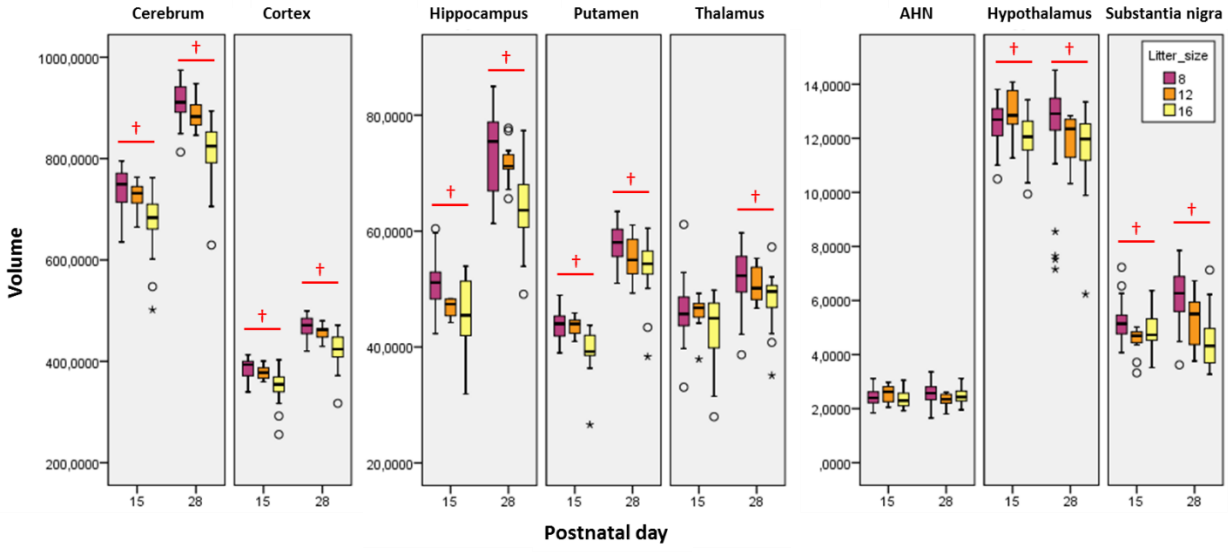

Figure 21 Box plots showing the volumes of cerebrum, cortex, hippocampus, putamen, thalamus, AHN, hypothalamus and substantia nigra for different litter sizes at time points P15 and P28. Volumes are shown in units of mm^3. Circles and stars represent outliers and extreme outliers, respectively. †p-value < 0.05 for correlation between litter size and brain volumes. Abbreviations: AHN; anterior hypothalamic nucleus.

There was a large degree of overlap between the IHH and RA group when looking at the correlation between BW and brain volumes, as seen in the scatter plots (Figure 15, Figure 16). However, data from the IHH group was more clustered together around higher values of BW, especially at P15, whereas data from the RA group was more spread out (Figure 15). The large spread in data in the RA group was mainly due to the small size of animals in the RA_{16} group (data not shown).

The distribution in brain volume according to absolute and relative weight gain appeared to be the same for both exposure groups at all time points, except in terms of the relative weight gain from P15-P28, where the IHH group retained a large brain volume despite having a smaller relative weight gain (Figure 19).

4.3 The influence of gender on BW and brain volumes

There was no difference in BWs between males and females at either time point P15 or P28, although males had a larger absolute weight gain from P15-P28 compared with females ($p = 0.012$). Males also had larger volumes than females in all structures, except AHN and substantia nigra, at both P15 and P28 (Figure 22). There was no difference in gender distribution between animals in the IHH and RA group.

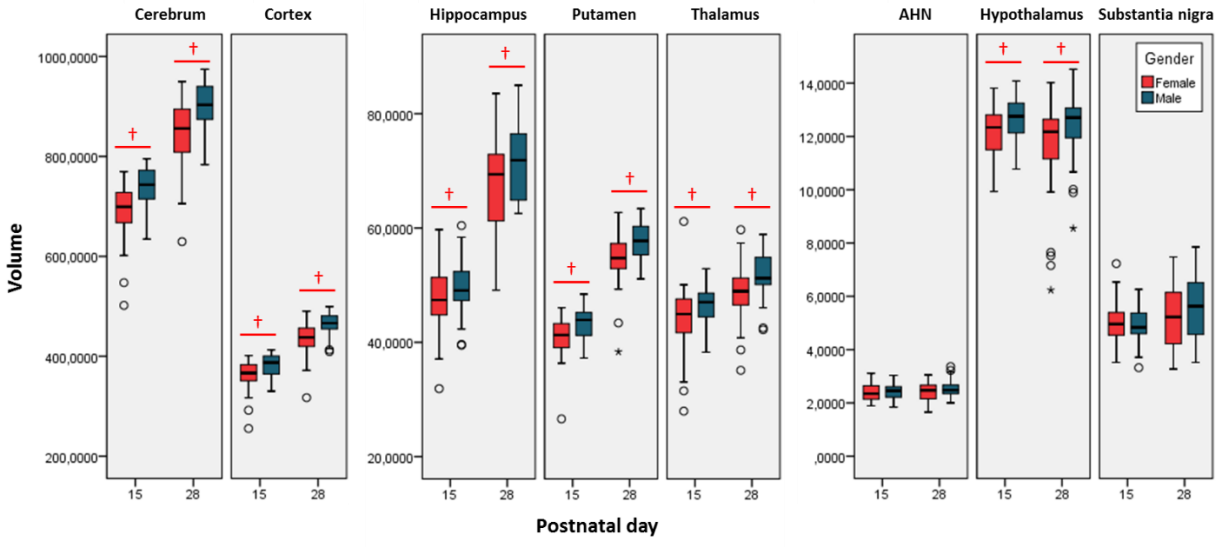

Figure 22 Box plots showing differences in brain volumes according to gender at time points P15 and P28. Volumes are shown in units of mm^3. Circles and stars represent outliers and extreme outliers, respectively. †p-value < 0.05 between males and females. Abbreviations: AHN; anterior hypothalamic nucleus.

5 Discussion

5.1 Methodological considerations

5.1.1 The animal model

A less severe oxygen paradigm

The IHH paradigm was chosen as it provides a clinically relevant profile of the fluctuations in tissue oxygen concentrations that may occur in premature and/or growth restricted children during the perinatal period[5]. Studies have shown that more frequent fluctuations and larger extremes in the air oxygen concentrations produce more severe pathological changes in retinal vasculature when compared with longer lasting episodes of high or low air oxygen concentrations[68-70]. As the retina may be considered a window to the brain, it is conceivable that such fluctuations in oxygen concentrations also have a negative effect on brain vasculature[30], and possibly other structural components in the brain as well.

In this study we did not obtain the IHH profile that we first intended. The long ramping times between high and low air oxygen concentrations caused a shorter exposure time to the low oxygen levels, and hence also a less severe exposure profile. As newborn rats have higher levels of arterial oxygen concentrations compared with preterm infants[71], they most likely also tolerate lower air oxygen levels before becoming oxygen deprived. The reduced severity in the IHH profile combined with the resilience of neonatal rats could therefore explain the subtleness of our results. The IHH profile used in this study was however more severe than the IHH profile used by Morken et al.[30], where animals were exposed to 12% O_2 every six hours, instead of every three hours . Despite this, the degree of inflicted damage did not appear to be more severe.

Rats as a model organism

Rats were used as a model organism for several reasons. First, rats are ideal models in studies on brain development, as they are altricial animals born with an underdeveloped brain. A significant part of the brain development occurs postnatally during the weaning period, and therefore the maturity of a rat brain at birth is comparable to that of a human brain at 22-23 weeks of gestation[72]. After 10-14 days, the maturity of the rat brain is comparable to that of a full-term born baby[73]. This delay in brain development make rats exceptionally useful as a model in studies on brain

development in preterm born children. Second, rats are generally a beneficial model organism used in research concerning human physiology[74]. Although some studies point out that small-animal models are not able to replicate the full scope of pathology and behavioural outcomes observed in relation to brain damage in humans[75], as a complex higher order organism, rats do provide a good system for the modelling of more specific, intricate events within neurological research. Rats are also cost and time-effective compared with larger model organisms. Third, most research concerning the influence of variations in oxygen concentrations on brain development is performed with rats. Using the same species as other studies opens up for comparison of result.

Possible variations in methodology between the experimental groups

The majority of the experimental parameters in this study were similar between the experimental groups. Points of variation between and within the experimental groups were limited to housing conditions during the first two weeks, duration of exposure to the IHH profile and duration of stay in the MR animal room.

The housing of animals in the IHH group was different from the housing of animals in the RA group, as animals in the IHH group were kept in lidless cages placed in A-chambers. This allowed the animals in the IHH group to have slightly more contact with the external environment, both in terms of physical parameters such as smells, sounds and variations in temperature and humidity, and in terms of social interactions between the two litters located in the same A-chambers. However, as animals in the RA group were housed in the same room as animals in the IHH group, they also experienced the same external protrusions, only to a lesser degree due to the IVC. There could also have been variations in the speed of fluctuations in oxygen concentrations between different litters in the IHH group. As one litter always was placed closer to the gas output in the A-chambers, it could have experienced more rapid changes in oxygen fluctuations and a higher noise level from the pressurised oxygen as it was released from the gas output. As a counteractive measure, cage positions within the A-chambers were frequently, but not regularly, altered between the two litters. Changes in the position of single cages in the A-chambers were however not performed, and these litters were therefore continuously placed directly under the gas output. Still, the effect of differential position in the A-chambers on the BW and brain volume outcomes has been evaluated to be very small, as the A-chambers were made to alter the oxygen levels evenly in the entire box, and as the distance between the litters was minimal.

The time period before animals in the IHH group were put into the A-chambers varied both within and between litters. Some litters had to wait around twelve hours before being exposed to the IHH regime, while other litters only had to wait around one hour. The difference in time before exposure was simply due to litter culling, where births occurred at different time points during the day, causing some animals to wait in RA before two litters could be put together. However, as twelve hours made up only a fraction of time compared with the two weeks long exposure to IHH, the variation in time before being exposed is considered to be of little importance. Also the three occasions where the air oxygen concertation was unable to reach 12% O_2 due to system failure would have reduced the exposure of rats to low oxygen levels. The time without exposure to low oxygen levels was estimated to be around twelve hours per occasion. However, as the three occasions were spread out in time, and as thirty-six hours in total made up only a minor part of the entire IHH profile duration, the reduced exposure to low oxygen levels therefore most likely did not affect the experimental outcomes.

Differences between the litters regarding how long the animals stayed in the animal room in the MR centre could have led to different levels of stress. Animals that arrived at the same day of scanning, instead of the night before, could have had a slightly increased stress level due to the additive effect of stress from the transportation and being prepared for scanning. Also, some litters were kept in the MR animal room for a longer time period, as it took longer to scan larger litters compared with smaller litters. Longer duration of stay in the animal room during scanning would have led to more exposure to noise from the MR scanner, smells from the anaesthesia and smells from the dissection at P28. However, the duration of stay in the animal room did not appear to have any impact on neither the animals nor the experiment outcomes. An increasing level of stress in the animals of larger litters was however noticed with the gradual removal of animals for euthanization at P28. The increased stress level could have affected BW outcomes in animals in litter size 16, as more stress could have caused less feeding towards the end. It was not possible to measure how much influence this stress had on weight differences, but the influence of one or two missed feedings on the total BW of the animal would not have been enough to drastically alter the weight results.

5.1.2 Uncertainties related to the MR scanning

The main point for variation during MR scanning was the positioning of animals in the water-heated bed, and the positioning of the MR slice pack. These procedures had to be individually prepared for each animal, and were performed by three different persons at alternating time points due to a shift rotation in the work. Furthermore, the varying size of animals according to the litter size introduced a variation in the position of the head on the water-heated bed and a variation in the position of the slice package between different litters. To counteract variation, positioning of animals on the bed was performed according to a set routine, and anatomical markers were used for slice positioning.

There was also a change in the size of the head coil between scanning at P15 and at P28, where the head coil at P15 had to be exchanged for a larger one at P28 due to the growth in the rats. The larger coil introduced a lower signal-to-noise ratio, giving a slightly lower overall quality of the MR images.

5.1.3 Uncertainties related to the data analysis

Some measurement uncertainties were also present regarding the drawing of brain volumes. Differential positioning of animals in the water-heated bed and of the slice package made it impossible to draw according to a set template, and the brain volumes of each animal had to individually be drawn by hand. Also, despite a good general image quality, some images were of poorer quality and contained image artefacts, making it hard to distinguish the true boarders of the structures. To assure as much standardization between each drawing as possible, simple and easily identifiable anatomical markers were used, allowing for the same volumetric measures to be drawn of each structure in each animal. Despite some structural integrity being compromised because of this, the assurance of a high level of replication in drawing between each animal was more important than attaining the exact brain volume. The anatomical markers were also set to encapsulate as much of the true structural volumes as possible.

In terms of the statistical analysis, one major point of inconsistency was the number of litters in each experimental group. There were only two litters consisting of 16 animals, and each litter belonged to a separate exposure group. There was also only one litter consisting of 12 animals. All the statistical measures including data from litter size 12 and 16 could therefore have shown reflections of litter differences, in addition to showing results from the actual experimental

parameters. Possible litter differences were taken into consideration when evaluating and interpreting the results, and any conclusions based on litter size 16 were formed cautiously, and preferably with the backup of additional results.

5.2 Growth of animals varies with litter size and oxygen exposure

The inverse relationship between weigh and litter size confirms that an increased litter size induces postnatal growth restriction. Animals that were raised in larger litters became more growth restricted and had lower average BW than their peers raised in smaller litters. Lower BW and reduced growth in animals that have experienced postnatal food restriction has also been found in other studies[76-80]. Growth restriction probably occurred as a result of the limited milk availability being divided across more individuals. Growth restriction could also have occurred independently of nutrient availability, where maternal behaviours varied with different litter sizes[81]. There is more grooming time and heat transfer from the dam to the pups in smaller litters, which again improves growth as the pups receive more maternal care and use less energy in thermoregulation. The thermoregulatory advantages are most prominent during the early postnatal period, as rats have a limited ability to regulate their body temperature during their first postnatal days[82].

The differences in BW and weight gain between animals in the two exposure groups coincided with the timing of the IHH paradigm. For the first two weeks, animals in the IHH group were exposed to IHH. During this time, they grew faster and weighed more compared with the animals kept in RA. For the next two weeks, animals in both the IHH and RA group were exposed to the same oxygen concentrations. During this time, animals in the RA group grew faster than animals in the IHH group. This trend indicates that the IHH paradigm had a direct influence on growth promotion, as the growth advantage in animals from the IHH group was concluded upon their removal from the IHH paradigm. Previous studies investigating the separate effects of hyperoxia and hypoxia on growth have however found results that show the opposite to what was discovered in this study. Rats and mice exposed to intermittent hypoxia demonstrated an early growth restriction and a later catch-up growth to controls, both while still under hypoxic exposure[80], and three weeks after completed exposure[83]. Moromisato et al.[84] also found reduced BW in rats exposed to hypoxia, where the weight reduction was correlated with total dam food consumption. Also hyperoxia alone has been found to decrease BW. Mice exposed to 100% O_2 for different periods of time showed a daily weight loss of about 4% of the initial BW[85], and increases in leptin levels

in the lungs[86] and in the blood[85] were found in mice following 100% O_2 exposure. High leptin levels stimulate a reduction in food intake and an increase in energy expenditure, which together lead to weight reductions[87]. Although the pathology inflicted by the use of 100% O_2 is not directly comparable to the outcomes in our study, hyperoxic insults are in general promotive towards forming reactive oxygen species[88], inflammatory responses[89] and a variety of other pathologies, all of which could contribute towards reduction in BW. Despite hyperoxia and hypoxia separately having a negative effect on weight gain, the combination of both hyperoxia and hypoxia as done in this study may have promoted an increase in BW. Perhaps the interchangeability in tissue oxygen concentrations lead to irregularities in the levels of growth hormones, which again stimulated an uncontrolled growth pattern and increased weight gain. Clustered episodes of hypoxia (12% O_2) during chronic hyperoxia (50% O_2) have been found to cause an upregulation of retinal vascular endothelial growth factors, resulting in uncontrolled growth in the retinal vasculature in rats[68], while chronic exposure to hyperoxia (95% O_2) for ninety hours has been found to cause a significant increase in the levels of insulin-like growth factor 1 (IGF)-1[90]. IGF-1 is implicated in the regulation of normal physiology, and plays a role in the promotion of cell proliferation and in the inhibition of cell death[91]. Another explanation for higher BWs in response to IHH could be that the larger oxygen availability in tissues during hyperoxia could have led to an increased cellular metabolism, thereby simulating cellular growth and increase in BW over time.

The large difference in absolute and relative weight gain from P0-P15 between the exposure groups in litter size 16 compared with litter size 8 suggests that variations in oxygen levels had a greater positive influence on growth in the smaller, more growth restricted individuals. The influence of IHH on the smaller animals was also apparent when considering that the average BWs of the IHH_8 and IHH_{16} groups were much more similar compared with the RA_8 and RA_{16} groups. Again, opposite trends have been found in previous research. Nutritional growth restriction was found to intensify the negative effects of hyperoxia on the pulmonary growth and architecture in rabbits[92], and on bone mass and bone load-carrying capacity in male rats[93], and hypoxia induced cerebral apoptosis in piglets was found to be increased in animals that had been exposed to intrauterine growth restriction compared with animals born with normal BW[94]. Why growth restriction therefore intensified the positive effect of IHH on BW in this study is difficult to explain. Perhaps the reduced growth in the smaller animals simply made them more vulnerable towards becoming influenced by the experimental factors.

The IHH_{16} group had higher average BW at P28 and larger absolute weight gain from P15-P28 compared with the RA_{16} group, despite animals in the RA group growing faster than animals in the IHH group, as indicated by their larger relative weight gain. One explanation for this could be that the IHH paradigm promoted growth enough for the animals to maintain a weight advantage even two weeks after the end of the IHH paradigm. The already large size of animals in the IHH group at P15 would have prompted a continuously high increase in BW over time, leaving the smaller animals in the RA group with an even greater amount of catch-up growth in order to equalize in BW. Furthermore, as the difference in BW between exposure groups was greater in litter size 16 than it was in litter size 8, it could have taken longer for the RA_{16} group to catch up with the IHH_{16} group then if would have taken the RA_8 to catch up with the IHH_8. Another explanation could be that the large size in animals in the IHH group allowed for an earlier weaning process, where they could supplement their food intake with pellets in addition to being fed with milk, thereby maintaining a growth advantage. BW at weaning has been found to be a good indicator of post-weaning growth and efficiency of converting milk into BW in rabbits[95]. Also litter differences between the two litters of 16 animals could have caused the animals in the IHH group to maintain a higher average BW than animals in the RA group. As the IHH_{16} and RA_{16} groups were made up of only one litter each, inter-litter differences, such as genetics, level of maternal care[96], length of nursing or social interactions[97] could have created the differences in BW between the two litters. However, litter differences were most likely not the cause of the observed BW differences, as the involvement of IHH in growth regulation was clear. First, the smaller relative weight gain from P15-P28 in animals within the IHH group compared with animals in the RA groups coincided with the ending of the IHH paradigm. Second, the smaller relative weight gain from P15-P28 was also present in IHH_8, which's measures were based on multiple litters.

The RA_8 group had much higher average BWs at P28 and larger weight gains from P15-P28 compared with the IHH_8 group. One explanation for this could been that the change in oxygen conditions that occurred for animals within the IHH group at P14 caused enough reduction in oxygen uptake to alter the animal's BWs. Whereas the RA animals remained exposed to the same air oxygen levels throughout the experiment, the IHH animals experienced a change from fluctuating high and low levels of oxygen to a constant medium level of oxygen at P14. The adaptation of animals to the IHH paradigm could have caused a reduced oxygen uptake and changes in the metabolic processes when exposed to constant RA levels of oxygen[98], negatively affecting

their growth rate. Furthermore, the critical periods for when changes in the air oxygen concentrations can induce long-lasting adaptive changes in an animals' respiratory physiology are during the first and second postnatal weeks[99,100], which is exactly when the animals in this study were exposed to IHH. Another explanation could be that the changes in housing conditions for animals in the IHH group increased the animal's stress levels, causing a reduced milk production and/or maternal care in the dam, again reducing the weight gain in pups. The transition of animals in the IHH group from being kept in lidless IVC in a large plexiform A-chamber to being kept in closed IVC appended to a cage rack undoubtedly brought with it changes in a range of other factors, such as noise levels from the surroundings, availability of smells from the environment, social interactions, air delivery method, etc.. However, as animals were still kept in the same room, and therefore not exposed to a completely new environment, the change in housing conditions most likely did not have a tremendous impact on the animal's welfare. Both the change in the oxygen paradigm and in the housing conditions probably contributed towards decreasing the growth rate in animals in the IHH group, although determining their exact influence on the weight reduction is difficult.

5.3 Influence of oxygen exposure and BW on brain volumes

5.3.1 Exposure to IHH leads to an increase in brain volumes

The volumes of brain structures in animals that had been exposed to IHH were generally larger than the volumes of animals exposed to RA. This trend was especially prominent in structures that had a large increase in brain volumes from P15-P28. The large increase in volumes most likely allowed for the IHH to have more influence, hence creating a difference in volumes between exposure groups.

The structure which had the largest increase in volume from P15-P28, and in which the effect of IHH on brain volume was most enhanced, was the hippocampus. At P15, the IHH_{16} group had volumes that levelled those of the IHH_8 group. The IHH therefore appeared to have promoted volumetric increase enough to even overcome the growth restrictions induced in litter size 16. The effect of IHH on hippocampal volume was further supported by the equalization of volumes between the IHH_{16} and RA_{16} groups at P28 coinciding with the removal of animals from the IHH paradigm into RA. Studies investigating the effect of variations in oxygen concentrations on hippocampal volume have however found opposite results. Both hyperoxia[26,101,102] and hypoxia[103]

have been found to lead to neuronal injury and hippocampal atrophy, which in turn lead to reduced hippocampal volume.

The effect of IHH on volumetric increase was to a lesser extent present in cerebrum and cortex. In these structures, IHH did promote a volumetric increase in animals in litter size 16 at P15, although this increase was not enough to level with the volumes of animals in litter size 8. At P28, the IHH_{16} group still had a greater volume than the RA_{16} group. One explanation for these results could be that the IHH paradigm provided the animals with a large enough momentum to maintain an increased volumes compared with the RA group up until P28. There are many studies that have investigated the effect of hyperoxia and hypoxia on cortical neurogenesis and volumes, and that have found different results. *In vitro* hypoxic exposure of rat cerebral cortical cells was found to increase the cell proliferation rate and reduce cell death[104,105], and in *in vivo* exposure of neocortical neurons to chronic hypoxia (9.5% O_2) from P3-P30 led to a 30% increase in the neuronal numbers[106]. Despite the increase in neuronal numbers, the exposure of animals to chronic hypoxia (9.5% O_2) was found to cause lower body and brain weight, as well as decreased cortical volumes[29,106,107]. Acute hyperoxia (80% O_2) at P6 in rats has also been found to increase apoptotic tendencies in cortical neurons[108]. On the other hand, Woodrow et al.[109] found that impaired cortical growth in growth restricted rats exposed to variable levels of hyperoxia did not affect the total brain volume or neuronal numbers.

Despite having a close to no difference in volume between P15 and P28, the volumes of hypothalamus and AHN also appeared to be influenced by IHH. At P15, the IHH_8 group had larger volumes than the RA_8 group, while animals in litter size 16 did not appear to be affected. Why oxygen variations would affect the hypothalamic volumes in the larger and stronger animals and not in the growth restricted more vulnerable animals is unclear. On possible explanation is that the increase in volumes in litter size 8 only occurred by chance. This is however not likely as both hypothalamus and AHN showed similar results, and as the measures of volume in litter size 8 were based on animals from several different litters. The literature also contradicts the findings in this experiment, as hyperoxia has been found to cause increased apoptotic tendencies in hypothalamus[108]. At P28 the trend reversed, and the RA_8 group appeared to have larger volumes than the IHH_8 group. This result is according to the general volume trend at P28, where the halted volumetric development in animals in the IHH group results from the loss of the growth advantage provided by IHH.

Our hypothesis was that the IHH paradigm would have a negative effect on brain volumes. It is however unclear if the increase in brain volumes in animals in the IHH group reflected a healthy growth in brain tissue or if it was a consequence of pathologies. As the majority of studies investigating the effects of hyperoxia and hypoxia on brain volume have found an impaired neuronal growth, it is more likely that the increase in volumes resulted from pathological processes. One pathology that could have caused the increased brain volumes is cerebral edema. Morken et al.[30] found increases in the T_2 relaxation times and mean diffusivity in cortex, hippocampus and thalamus in rats following IHH exposure, indicating an increased grey matter water content. They also found a long-term increase in vascular density and albumin leakage, hypothesizing that IHH could lead to alterations in angiogenesis and blood brain barrier (BBB)-formation. Also Julia Anna Adrian, another master student involved in this present study, found a correlation between mean diffusivity in cortex, hippocampus, thalamus and striatum and the BW of animals exposed to IHH, hypothesizing that the correlation resulted from increased extracellular water content (personal communication). The mean diffusivity was measured in the same animals as used in this study. LaManna et al.[110] investigated the cerebrovascular compensation responses of rats successfully adapted to three weeks of constant moderate hypoxia, and found an increase in angiogenesis leading to higher microvessel densities in the brain. Increased BBB permeability and cerebral edema has also been discovered following hypoxia (6% O_2) and reoxygenation at RA (21% O_2)[111]. Increase in vascularization and extracellular water content in grey matter within the brain could therefore be explanatory for the increase in brain volumes in response to IHH exposure as observed in this study. However, as the duration of cerebral edema is usually set to hours and days[112], it is doubtful that the increase in brain volumes in IHH animals two weeks after the end of the IHH exposure could have stemmed from increased water content. Furthermore, Bigdeli et al.[113] found that both intermittent and prolonged hyperoxia had a protective effect on brain tissue and the BBB against cerebral edema. In addition, it remains questionable if cerebral edema itself could have led to the large increase in BW that was found in IHH animals compared with RA animals, or if both the increase in BW and increase in brain volumes together result from a general promotion in growth induced by IHH.

5.3.2 BW is positively correlated with brain volumes

The positive correlation between the animals' BW and the brain volumes at both P15 and P28 was expected according to our hypothesis, as smaller animals naturally are less developed and have smaller brains. Reduced cerebral volume in rats has previously been found in response to reduced BW during the first twenty postnatal days[114], and smaller brain volumes are a common consequence of low birth weight in children[115-117]. The inverse variation between litter size and brain volumes was also expected, as BW reflects the litter size.

The positive correlation between brain volumes at P15 and the speed and amount of growth during the first two weeks, as shown by the absolute and relative weight gain from P0-P15, means that animals with the largest early growth had the highest BWs, and consequently also the largest brain volumes at P15. Similar results have also been found in the literature. For instance, the long-term effect of early excess weight gain was found to increase the risk of obesity in adulthood in both rats[118,119] and humans[120]. Also a correlation between birth weight and pre-weaning growth within litters has been found in rabbits, where animals with a higher birth weight had a higher milk intake and more efficient conversion of milk into body mass[95]. Since weight gain from P15-P28 also correlated with brain volumes at P28, the influence of the growth in animals on brain volumes was most likely present throughout the entire first four postnatal weeks.

Interestingly, the volume of hypothalamus at P28 did not correlate with neither BW at P28 nor absolute and relative weight gain from P15-P28, despite the volume at P15 correlating with both BW at P15 and absolute and relative weight gain from P0-P15. Also the volume of AHN did not correlate with any weight measures at any time point. The lack of correlation found in hypothalamus was most likely due to the five extreme outliers that fell outside the main volumetric range and disrupted the correlative significance. The reduced volumes in the five outliers most likely resulted from poor drawing, as three of the five outliers originated from litters raised in RA, and therefore in theory should not have had any pathological damage. The lack of correlation between the volume of AHN and the BW and weight gain of animals was however much more consistent to have arisen only by chance. Based on the scatter plots, the large spread in the volume of AHN occurred independently from the animal's BW at both P15 and P28, indicating that the volume of AHN had to be influenced by other factors. One plausible explanation is that the volume of AHN naturally varies between individuals, where differences in genetics between the animals

lead to a differential development of the AHN. In the arcuate nucleus of hypothalamus, the brain-derived neurotrophic factor (BDNF) is a potent regulator of neuronal development, and plays a crucial role in neuronal survival, synaptic plasticity, and neural circuit development[121]. Factors with similar traits could have been active within AHN and individually regulated the volume of AHN across the animals. Another explanation could be that activity dependent plasticity in the AHN changed the density and amount of neurons between individuals. Conformation of neurons in various hypothalamic nuclei have been found to vary with different external and internal protrusions, such as hormonal and synaptic input, photo-stimulation and body water content[122], and similar variations could have occurred in AHN. As AHN is involved in thermoregulation and sleep, one could imagine that animals with a more developed thermoregulatory or sleep pattern could have required more involvement of the AHN, leading to an increase in the AHN volume. Thermoregulation has however also been coupled to BW, where larger animals are able to both secure themselves more central positions in a litter huddle[123] and have a more favourable conversion of milk to body mass instead of heating processes[124]. Following the same logic, the influence of BW on thermoregulation should have altered the AHN volume according to its frequency of use. However, the lack of correlation between BW and AHN volume in this experiment argues against a variation in AHN volume based on thermoregulatory plasticity.

Surprisingly, almost no brain volumes at P28 had a correlation with relative weight gain from P15-P28 when considering animals from all the experimental groups. The lack of correlation was most likely a result of the reduced relative weight gain in the IHH_8 group, where the brain volumes maintained a large size despite a smaller weight gain. This caused a larger spread in the data distribution and an offset in the correlation for all animals. The possible causes for the smaller relative weight gain have been discussed previously, and include changes in oxygen paradigm and housing conditions. The only volumes that had a correlation with relative weight gain from P15-P28 were those of hippocampus and AHN. However, there is a high likelihood that these correlations just occurred by chance, as the correlations are quite weak.

All structures that had a correlation between brain volume at P28 and BW at P28 also showed a correlation between brain volume at P28 and BW at P15, both when considering all animals together and animals in the separate IHH and RA groups. It therefore appears that the animal's BW throughout the first four weeks after birth was fairly connected with the volumes of some structures at later time points. This also seems to be in accordance with previous studies, which have found

that a low early BW results in subtle white matter changes and poorer long-term neurodevelopment in rats[47], and that a lower birth weight results in smaller hippocampal volumes and poorer neurocognitive outcomes in young adult humans[125-127].

Interestingly, the trends in volume:BW ratios mirrored the trends in BW for all groups of animals at all time points. For instance, the large difference in volume:BW ratio between the exposure groups in litter size 16 reflected the large difference in BW between the exposure groups in litter size 16, both at P15 and P28. Also, the reversal in BW between the exposure groups in litter size 8, where animals in the IHH group went from having higher BWs at P15 to having lower BWs at P28, was also reflected in the volume:BW ratios. It therefore appears that the variations in the volume:BW ratios only demonstrate the variations in BW, and that brain volumes therefore are spared from being affected by growth restrictions. The same compensatory mechanism for conservation of the brain during reduced growth has been found in hypoxia exposed mice with very low BW[80,107] and in growth restricted rats[78].

5.3.3 Brain volumes are indirectly influenced by IHH through its effect on growth

The large degree of overlap in BW and brain volume distribution between the IHH and RA groups at both P15 and P28, as shown in the scatter plots, indicates that that there was essentially no difference in brain volume between the two exposure groups. The only difference between the exposure groups appeared to be the degree of spread in BW among the animals, where animals in the IHH group had more similar weights, and where animals in the RA group had less similar weights. The large spread in BW in animals in the RA group was caused by the exceptionally small size of animals in litter size 16, which again also reduced the group's overall average BW. Furthermore, as BW was correlated with brain volume, animals in the RA group necessarily also had smaller brain volumes compared with animals in the IHH group. The differential spread in BW within the exposure groups could therefore explain the differences in brain volume between the exposure groups. This also appears to be in accordance with the finding that the trends in volume:BW ratios mirrored the trends in the animal's BWs.

IHH therefore appeared to affect the growth of animals through a three-step cascade, where IHH first influenced the BW, and where animals with higher BWs had larger brain volumes: IHH → BW → volume.

5.3.4 Do the experimental factors influence postnatal neurogenesis?

There is one central question that remains unanswered by the variations in IHH and BW, and that is why the effects of the experimental parameters on brain volumes appeared to be dependent on the location of the structures within the brain. The vulnerability of a structure towards becoming affected by external parameters depends on how accessible the structure is to the parameters during the time when they are active. Therefore, any changes occurring in the brain after the start of the experiment could have become influenced by the experimental factors, which in turn could have affected the brain volumes. One such change is postnatal neurogenesis, and its timing with the exposure to IHH and growth restriction during the first two postnatal weeks could possibly offer an explanation for the anatomically dependent variations in brain volumes between animals in the IHH and RA groups.

Postnatal neurogenesis in the dentate gyrus occurs mainly during the first postnatal week, but neurogenesis can continue until adulthood[50-52]. The lengthy postnatal generation of cells in hippocampus fits well with the results obtained in this study, where the effect of experimental factors on hippocampal volumes were observed up until P28. Hippocampus also had a large increase in volume from P15 to P28, where neurogenesis in dentate gyrus could have made up a large part of this.

Cortical volume could have been influenced by neurogenesis and/or gliogenesis in the neocortex[54], piriform cortex[57] and the olfactory bulb[53]. The lengthy generation of interneurons in the olfactory bulb over the first postnatal weeks would have allowed the experimental factors to influence the growth process, explaining both the increase in cortical volume from P15 to P28, and the volumetric difference between the IHH and RA groups. The volume of cortex could also partially have been determined by the experimental factors influencing other growth processes occurring during the first postnatal weeks, such as for instance the ingrowth of thalamocortical axons[128]. The volumetric changes observed within the cerebrum were most likely a reflection of changes within the cortical volume, as cortex is the largest telencephalic structure[50], and to some extent also the hippocampal volume due to its peripheral location.

Also putamen has a postnatal neurogenesis occurring between P0-P4[60]. However, due to the small amount of neurogenesis, and the fact that it occurs for such a short amount of time so early in the postnatal development, it is questionable if the experimental factors would have had a possibility

to influence the volume of putamen. A reduced amount of influence by the experimental factors on the volume of putamen is also reflected in the subtleness of the differences in volume between the exposure groups.

The only two deep grey matter structures with a postnatal neurogenesis are thalamus and hypothalamus. The large scale neurogenesis in the ventrobasal thalamic nuclei during P3-P21[55] could have become influenced by the experimental factors. However, despite the large amount of neurogenesis occurring during the first three postnatal weeks, the difference in volume between the exposure groups at P15 was smaller compared with the other structures. One possible explanation for the subtle results could be that the influence of neurogenesis on volume of the ventrobasal nucleus was too small to have an extensive impact on the total thalamic volume. The postnatal neurogenesis in hypothalamus during the first postnatal week[64] could however have become influenced by the experimental factors, resulting in the difference in volume between the exposure groups in hypothalamus at P15.

Variations in the degree of postnatal neurogenesis between the individual structures could explain why the experimental factors influenced the volumes of only some structures and not others. However, not all structures with a large postnatal neurogenesis appeared to be influenced by the experimental factors, such as thalamus, and likewise not all structures that showed a difference in volume between exposure groups had a postnatal neurogenesis, such as AHN. The experimental factors therefore most likely influenced brain volumes through other pathways instead of, or in addition to, neurogenesis. Which pathways these might be requires further investigation.

6 Conclusion

This study investigated the effects of weight and intermittent hyperoxia hypoxia (IHH) on brain development in neonatal rats. Animals that had been exposed to IHH during the first fourteen postnatal days were found to have a higher average body weight (BW) compared with the room air (RA) controls, where the difference was greatest among the most growth restricted individuals. This suggests that IHH may have a positive effect on postnatal weight gain. It was also found that animals exposed to IHH had larger brain volumes compared with RA controls, and that this difference varied with different brain structures. This suggests that IHH had a positive effect on brain growth. The simultaneous increase in both BW and brain volumes in response to IHH exposure could be a sign of a general promotion in growth. We therefore hypothesize that IHH exposure causes an increase in body growth, which again leads to an increase in brain volumes.

The study could however not determine if the increase in brain volumes was a result of positive growth or if it was related to pathology. A potential explanation for the increase in brain volumes could be cerebral edema, as indications of extracellular water content have been found in both this and previous similar studies.

In order to fully understand what effects fluctuations in air oxygen level have on brain development in preterms, future studies should aim to investigate the mechanisms behind how exposure to IHH may lead to increased brain volumes. Future studies should also investigate which other parts of brain development, in addition to brain volumes, that may be affected by IHH

.

7 Literature

1. Osterman, M.J.K., K.D. Kochanek, M.F. MacDorman, et al., *Annual summary of vital statistics: 2012-2013.* Pediatrics, 2015. 135(6): p. 1115-1125.
2. McElrath, T.F., E.N. Allred, L. Van Marter, et al., *Perinatal systemic inflammatory responses of growth-restricted preterm newborns.* Acta Paediatr, 2013. 102(10): p. e439-42.
3. Henderson-Smart, D.J., *The effect of gestational age on the incidence and duration of recurrent apnoea in newborn babies.* Australian Paediatric Journal, 1981. 17(4): p. 273-276.
4. Hibbard, J.U., I. Wilkins, L. Sun, et al., *Respiratory morbidity in late preterm births.* Jama, 2010. 304(4): p. 419-25.
5. Vogel, E.R., R.D. Britt, Jr., M.C. Trinidad, et al., *Perinatal oxygen in the developing lung.* Can J Physiol Pharmacol, 2015. 93(2): p. 119-27.
6. Inder, T.E., S.K. Warfield, H. Wang, et al., *Abnormal cerebral structure is present at term in premature infants.* Pediatrics, 2005. 115(2): p. 286-94.
7. Inder, T.E., S.J. Wells, N.B. Mogridge, et al., *Defining the nature of the cerebral abnormalities in the premature infant: a qualitative magnetic resonance imaging study.* J Pediatr, 2003. 143(2): p. 171-9.
8. Nagy, Z., H. Lagercrantz, and C. Hutton, *Effects of preterm birth on cortical thickness measured in adolescence.* Cereb Cortex, 2011. 21(2): p. 300-6.
9. Peterson, B.S., B. Vohr, L.H. Staib, et al., *Regional brain volume abnormalities and long-term cognitive outcome in preterm infants.* Jama, 2000. 284(15): p. 1939-47.
10. Davis, E.P., C. Buss, L.T. Muftuler, et al., *Children's Brain Development Benefits from Longer Gestation.* Frontiers in Psychology, 2011. 2: p. 1.
11. Petrini, J.R., T. Dias, M.C. McCormick, et al., *Increased risk of adverse neurological development for late preterm infants.* J Pediatr, 2009. 154(2): p. 169-76.
12. Bhutta, A.T., M.A. Cleves, P.H. Casey, et al., *Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis.* Jama, 2002. 288(6): p. 728-37.
13. Van Hus, J.W., E.S. Potharst, M. Jeukens-Visser, et al., *Motor impairment in very preterm-born children: links with other developmental deficits at 5 years of age.* Dev Med Child Neurol, 2014. 56(6): p. 587-94.
14. Vederhus, B.J., T. Markestad, G.E. Eide, et al., *Health related quality of life after extremely preterm birth: a matched controlled cohort study.* Health Qual Life Outcomes, 2010. 8: p. 53.
15. Stjernqvist, K. and N.W. Svenningsen, *Ten-year follow-up of children born before 29 gestational weeks: health, cognitive development, behaviour and school achievement.* Acta Paediatr, 1999. 88(5): p. 557-62.
16. Johnson, S., C. Hollis, P. Kochhar, et al., *Psychiatric Disorders in Extremely Preterm Children: Longitudinal Finding at Age 11 Years in the EPICure Study.* Journal of the American Academy of Child & Adolescent Psychiatry, 2010. 49(5): p. 453-463.e1.
17. Fanaroff, A.A., B.J. Stoll, L.L. Wright, et al., *Trends in neonatal morbidity and mortality for very low birthweight infants.* Am J Obstet Gynecol, 2007. 196(2): p. 147.e1-8.

18. Askie, L.M., P. Brocklehurst, B.A. Darlow, et al., *NeOProM: Neonatal Oxygenation Prospective Meta-analysis Collaboration study protocol.* BMC Pediatrics, 2011. 11(1): p. 1-9.

19. Cole, C.H., K.W. Wright, W. Tarnow-Mordi, et al., *Resolving our uncertainty about oxygen therapy.* Pediatrics, 2003. 112(6 Pt 1): p. 1415-9.

20. Mach, W.J., A.R. Thimmesch, J.T. Pierce, et al., *Consequences of hyperoxia and the toxicity of oxygen in the lung.* Nurs Res Pract, 2011. 2011: p. 260482.

21. Michiels, C., *Physiological and Pathological Responses to Hypoxia.* The American Journal of Pathology, 2004. 164(6): p. 1875-1882.

22. Zhang, H., X. Wang, J. Lin, et al., *Reduced regional gray matter volume in patients with chronic obstructive pulmonary disease: a voxel-based morphometry study.* AJNR Am J Neuroradiol, 2013. 34(2): p. 334-9.

23. Lawley, J.S., N. Alperin, A.M. Bagci, et al., *Normobaric hypoxia and symptoms of acute mountain sickness: Elevated brain volume and intracranial hypertension.* Ann Neurol, 2014. 75(6): p. 890-8.

24. Deulofeut, R., A. Critz, I. Adams-Chapman, et al., *Avoiding hyperoxia in infants [les]1250[thinsp]g is associated with improved short- and long-term outcomes.* J Perinatol, 2006. 26(11): p. 700-705.

25. Ramani, M., T. van Groen, I. Kadish, et al., *Neurodevelopmental impairment following neonatal hyperoxia in the mouse.* Neurobiol Dis, 2013. 50: p. 69-75.

26. Porzionato, A., V. Macchi, P. Zaramella, et al., *Effects of postnatal hyperoxia exposure on the rat dentate gyrus and subventricular zone.* Brain Struct Funct, 2015. 220(1): p. 229-47.

27. Felderhoff-Mueser, U., P. Bittigau, M. Sifringer, et al., *Oxygen causes cell death in the developing brain.* Neurobiol Dis, 2004. 17(2): p. 273-82.

28. Schaeffer, E.L., F. Kuhn, A. Schmitt, et al., *Increased cell proliferation in the rat anterior cingulate cortex following neonatal hypoxia: relevance to schizophrenia.* J Neural Transm (Vienna), 2013. 120(1): p. 187-95.

29. Ment, L.R., M. Schwartz, R.W. Makuch, et al., *Association of chronic sublethal hypoxia with ventriculomegaly in the developing rat brain.* Developmental Brain Research, 1998. 111(2): p. 197-203.

30. Morken, T.S., A.K.G. Nyman, I. Sandvig, et al., *Brain development after neonatal intermittent hyperoxia-hypoxia in the rat studied by longitudinal mri and immunohistochemistry.* PLoS ONE, 2013. 8(12).

31. Bronson, R.T., *Brain weight-body weight scaling in breeds of dogs and cats.* Brain Behav Evol, 1979. 16(3): p. 227-36.

32. Schoenemann, P.T., *Brain size scaling and body composition in mammals.* Brain Behav Evol, 2004. 63(1): p. 47-60.

33. Schmidt, M.J., K.H. Amort, K. Failing, et al., *Comparison of the endocranial- and brain volumes in brachycephalic dogs, mesaticephalic dogs and Cavalier King Charles spaniels in relation to their body weight.* Acta Vet Scand, 2014. 56: p. 30.

34. Clark, R.H., P. Thomas, and J. Peabody, *Extrauterine growth restriction remains a serious problem in prematurely born neonates.* Pediatrics, 2003. 111(5 Pt 1): p. 986-90.

35. Cooke, R.J., S.B. Ainsworth, and A.C. Fenton, *Postnatal growth retardation: a universal problem in preterm infants.* Archives of Disease in Childhood - Fetal and Neonatal Edition, 2004. 89(5): p. F428-F430.

36. Clark, R.H., C.L. Wagner, R.J. Merritt, et al., *Nutrition in the neonatal intensive care unit: how do we reduce the incidence of extrauterine growth restriction?* J Perinatol, 2003. 23(4): p. 337-44.

37. Embleton, N.E., N. Pang, and R.J. Cooke, *Postnatal malnutrition and growth retardation: an inevitable consequence of current recommendations in preterm infants?* Pediatrics, 2001. 107(2): p. 270-3.

38. Lucas, A., R. Morley, T.J. Cole, et al., *Originally published as Volume 1, Issue 8704Early diet in preterm babies and developmental status at 18 months.* The Lancet, 1990. 335(8704): p. 1477-1481.

39. Lucas, A., R. Morley, and T.J. Cole, *Randomised trial of early diet in preterm babies and later intelligence quotient.* BMJ : British Medical Journal, 1998. 317(7171): p. 1481-1487.

40. Shan, H.M., W. Cai, J.H. Sun, et al., *[Extrauterine growth retardation and correlated factors in premature neonates].* Zhonghua Er Ke Za Zhi, 2007. 45(3): p. 183-8.

41. Brescianini, S., S. Giampietro, R. Cotichini, et al., *Genetic and environmental components of neonatal weight gain in preterm infants.* Pediatrics, 2012. 129(2): p. e455-9.

42. Ehrenkranz, R.A., N. Younes, J.A. Lemons, et al., *Longitudinal growth of hospitalized very low birth weight infants.* Pediatrics, 1999. 104(2 Pt 1): p. 280-9.

43. Stark, A.R., W.A. Carlo, J.E. Tyson, et al., *Adverse effects of early dexamethasone in extremely-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network.* N Engl J Med, 2001. 344(2): p. 95-101.

44. Vuguin, P.M., *Animal models for small for gestational age and fetal programming of adult disease.* Horm Res, 2007. 68(3): p. 113-23.

45. Holemans, K., L. Aerts, and F.A. Van Assche, *Fetal Growth Restriction and Consequences for the Offspring in Animal Models.* Journal of the Society for Gynecologic Investigation, 2003. 10(7): p. 392-399.

46. Widdowson, E.M. and R.A. McCance, *Some Effects of Accelerating Growth. I. General Somatic Development.* Proceedings of the Royal Society of London B: Biological Sciences, 1960. 152(947): p. 188-206.

47. Alexeev, E.E., B. Lonnerdal, and I.J. Griffin, *Effects of postnatal growth restriction and subsequent catch-up growth on neurodevelopment and glucose homeostasis in rats.* BMC Physiol, 2015. 15: p. 3.

48. Binder, M.D., N. Hirokawa, and U. Windhorst, eds. *Encyclopedia of Neuroscience*. T, ed. C.E. Chapman, et al. 2009, Springer Berlin Heidelberg: Berlin. 3989-4150.

49. Aboitiz, F. and J. Montiel, *Origin and evolution of the vertebrate telencephalon, with special reference to the mammalian neocortex.* Adv Anat Embryol Cell Biol, 2007. 193: p. 1-7.

50. Bayer, S.A. and J. Altman, *Chapter 2 - Development of the Telencephalon: Neural Stem Cells, Neurogenesis, and Neuronal Migration A2 - Paxinos, George*, in *The Rat Nervous System (THIRD EDITION)*. 2004, Academic Press: Burlington. p. 27-73.

51. Altman, J. and G.D. Das, *Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating*

tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J Comp Neurol, 1966. 126(3): p. 337-89.

52. Cameron, H.A. and R.D.G. McKay, *Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus.* The Journal of Comparative Neurology, 2001. 435(4): p. 406-417.

53. Bayer, S.A., *3H-thymidine-radiographic studies of neurogenesis in the rat olfactory bulb.* Experimental Brain Research, 1983. 50(2): p. 329-340.

54. Levison, S.W., *Mammalian subventricular zones: Their roles in brain development, cell replacement and disease*. Mammalian Subventricular Zones: Their Roles in Brain Development, Cell Replacement and Disease. 2006. 1-307.

55. Mooney, S.M. and M.W. Miller, *Postnatal generation of neurons in the ventrobasal nucleus of the rat thalamus.* Journal of Neuroscience, 2007. 27(19): p. 5023-5032.

56. Gould, E., N. Vail, M. Wagers, et al., *Adult-generated hippocampal and neocortical neurons in macaques have a transient existence.* Proceedings of the National Academy of Sciences of the United States of America, 2001. 98(19): p. 10910-10917.

57. Yuan, T.F., Y.X. Liang, and K.F. So, *Occurrence of new neurons in the piriform cortex.* Front Neuroanat, 2014. 8: p. 167.

58. Bernier, P.J., A. Bedard, J. Vinet, et al., *Newly generated neurons in the amygdala and adjoining cortex of adult primates.* Proc Natl Acad Sci U S A, 2002. 99(17): p. 11464-9.

59. Kaplan, M.S., *Neurogenesis in the 3-month-old rat visual cortex.* The Journal of Comparative Neurology, 1981. 195(2): p. 323-338.

60. Bayer, S.A., *Neurogenesis in the rat neostriatum.* International Journal of Developmental Neuroscience, 1984. 2(2): p. 163-175.

61. Yamashita, T., M. Ninomiya, P. Hernandez Acosta, et al., *Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum.* J Neurosci, 2006. 26(24): p. 6627-36.

62. Altman, J. and S.A. Bayer, *Development of the rat thalamus: IV. The intermediate lobule of the thalamic neuroepithelium, and the time and site of origin and settling pattern of neurons of the ventral nuclear complex.* Journal of Comparative Neurology, 1989. 284(4): p. 534-566.

63. Altman, J. and S.A. Bayer, *Development of the diencephalon in the rat. I. Autoradiographic study of the time of origin and settling patterns of neurons of the hypothalamus.* J Comp Neurol, 1978. 182(4 Pt 2): p. 945-71.

64. Seress, L., *Postnatal neurogenesis in the rat hypothalamus.* Developmental Brain Research, 1985. 22(1): p. 156-160.

65. Westbrook, C., C.K. Roth, and J. Talbot, *MRI in practice*. 4th ed. 2011, West Sussex: Wiley-Blackwell.

66. Paxinos, G. and C. Watson, *The rat brain in stereotaxic coordinates*. 2nd ed. 1986, San Diego, California: Academic Press.

67. Swanson, L.W., *Brain maps: structure of the rat brain*. 3rd ed. 2004, San Diego, California: Academic Press.

68. Coleman, R.J., K.D.A. Beharry, R.S. Brock, et al., *Effects of Brief, Clustered Versus Dispersed Hypoxic Episodes on Systemic and Ocular Growth Factors in a Rat Model of Oxygen-Induced Retinopathy.* Pediatr Res, 2008. 64(1): p. 50-55.

69. Penn, J.S., M.M. Henry, P.T. Wall, et al., *The range of PaO2 variation determines the severity of oxygen-induced retinopathy in newborn rats.* Invest Ophthalmol Vis Sci, 1995. 36(10): p. 2063-70.

70. Penn, J.S., M.M. Henry, and B.L. Tolman, *Exposure to Alternating Hypoxia and Hyperoxia Causes Severe Proliferative Retinopathy in the Newborn Rat.* Pediatr Res, 1994. 36(6): p. 724-731.

71. Cunningham, S., J.R. McColm, J. Wade, et al., *A novel model of retinopathy of prematurity simulating preterm oxygen variability in the rat.* Invest Ophthalmol Vis Sci, 2000. 41(13): p. 4275-80.

72. Dobbing, J. and J. Sands, *Comparative aspects of the brain growth spurt.* Early Human Development, 1979. 3(1): p. 79-83.

73. Romijn, H.J., M.A. Hofman, and A. Gramsbergen, *At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby?* Early Human Development, 1991. 26(1): p. 61-67.

74. Iannaccone, P.M. and H.J. Jacob, *Rats!* Disease Models & Mechanisms, 2009. 2(5-6): p. 206-210.

75. Silbereis, J.C., E.J. Huang, S.A. Back, et al., *Towards improved animal models of neonatal white matter injury associated with cerebral palsy.* Dis Model Mech, 2010. 3(11-12): p. 678-88.

76. Eleftheriades, M., P. Pervanidou, H. Vafaei, et al., *Metabolic profiles of adult Wistar rats in relation to prenatal and postnatal nutritional manipulation: the role of birthweight.* Hormones (Athens), 2014. 13(2): p. 268-79.

77. Jou, M.Y., B. Lonnerdal, and I.J. Griffin, *Effects of early postnatal growth restriction and subsequent catch-up growth on body composition, insulin sensitivity, and behavior in neonatal rats.* Pediatr Res, 2013. 73(5): p. 596-601.

78. Yeh, Y.Y., P. Zee, G.L. Owen, et al., *Effect of postnatal undernutrition on ketone metabolism and its relation to metabolic changes in brain and liver of rats.* J Nutr, 1982. 112(6): p. 1162-8.

79. Houdijk, M.E., M.T. Engelbregt, C. Popp-Snijders, et al., *Long-term effects of early postnatal food restriction on growth hormone secretion in rats.* JPEN J Parenter Enteral Nutr, 2003. 27(4): p. 260-7.

80. Farahani, R., A. Kanaan, O. Gavrialov, et al., *Differential effects of chronic intermittent and chronic constant hypoxia on postnatal growth and development.* Pediatr Pulmonol, 2008. 43(1): p. 20-8.

81. Schanberg, S.M. and T.M. Field, *Sensory deprivation stress and supplemental stimulation in the rat pup and preterm human neonate.* Child Dev, 1987. 58(6): p. 1431-47.

82. Blumberg, M.S. and G. Sokoloff, *Thermoregulatory competence and behavioral expression in the young of altricial species--revisited.* Dev Psychobiol, 1998. 33(2): p. 107-23.

83. Pozo, M.E., A. Cave, Ö.A. Köroğlu, et al., *Effect of postnatal intermittent hypoxia on growth and cardiovascular regulation of rat pups.* Neonatology, 2012. 102(2): p. 107-113.

84. Moromisato, D.Y., M.Y. Moromisato, J.A. Brasel, et al., *Effect of growth hormone therapy in mitigating hypoxia-induced and food restriction-induced growth retardation in the newborn rat.* Critical Care Medicine, 1999. 27(10): p. 2234-2238.

85. Barazzone-Argiroffo, C., P. Muzzin, Y.R. Donati, et al., *Hyperoxia increases leptin production: a mechanism mediated through endogenous elevation of corticosterone.* Am J Physiol Lung Cell Mol Physiol, 2001. 281(5): p. L1150-6.

86. Bellmeyer, A., J.M. Martino, N.S. Chandel, et al., *Leptin resistance protects mice from hyperoxia-induced acute lung injury.* Am J Respir Crit Care Med, 2007. 175(6): p. 587-94.

87. Halaas, J.L., K.S. Gajiwala, M. Maffei, et al., *Weight-reducing effects of the plasma protein encoded by the obese gene.* Science, 1995. 269(5223): p. 543-6.

88. Brueckl, C., S. Kaestle, A. Kerem, et al., *Hyperoxia-induced reactive oxygen species formation in pulmonary capillary endothelial cells in situ.* Am J Respir Cell Mol Biol, 2006. 34(4): p. 453-63.

89. Nagato, A.C., F.S. Bezerra, M. Lanzetti, et al., *Time course of inflammation, oxidative stress and tissue damage induced by hyperoxia in mouse lungs.* International Journal of Experimental Pathology, 2012. 93(4): p. 269-278.

90. Kim, T.H., Y.H. Chow, S.E. Gill, et al., *Effect of insulin-like growth factor blockade on hyperoxia-induced lung injury.* Am J Respir Cell Mol Biol, 2012. 47(3): p. 372-8.

91. Kurmasheva, R.T. and P.J. Houghton, *IGF-I mediated survival pathways in normal and malignant cells.* Biochim Biophys Acta, 2006. 1766(1): p. 1-22.

92. Mataloun, M.M., C.R. Leone, R.S. Mascaretti, et al., *Effect of postnatal malnutrition on hyperoxia-induced newborn lung development.* Braz J Med Biol Res, 2009. 42(7): p. 606-13.

93. Lezon, C., C. Bozzini, A. Agûero Romero, et al., *Effect of chronic undernutrition on body mass and mechanical bone quality under normoxic and altitude hypoxic conditions.* British Journal of Nutrition, 2016: p. 1-9.

94. Burke, C., K. Sinclair, G. Cowin, et al., *Intrauterine growth restriction due to uteroplacental vascular insufficiency leads to increased hypoxia-induced cerebral apoptosis in newborn piglets.* Brain Research, 2006. 1098(1): p. 19-25.

95. Rodel, H.G., A. Bautista, E. Garcia-Torres, et al., *Why do heavy littermates grow better than lighter ones? A study in wild and domestic European rabbits.* Physiol Behav, 2008. 95(3): p. 441-8.

96. Guerra, R.F. and C.R.d.O. Nunes, *Effects of litter size on maternal care, body weight and infant development in golden hamsters (Mesocricetus auratus).* Behavioural Processes, 2001. 55(3): p. 127-142.

97. Bautista, A., J.A. Zepeda, V. Reyes-Meza, et al., *Contribution of within-litter interactions to individual differences in early postnatal growth in the domestic rabbit.* Animal Behaviour, 2015. 108: p. 145-153.

98. Bavis, R.W., E.S. van Heerden, D.G. Brackett, et al., *Postnatal development of eupneic ventilation and metabolism in rats chronically exposed to moderate hyperoxia.* Respir Physiol Neurobiol, 2014. 198: p. 1-12.

99. Bavis, R.W., E.B. Olson, Jr., and G.S. Mitchell, *Critical developmental period for hyperoxia-induced blunting of hypoxic phrenic responses in rats.* J Appl Physiol (1985), 2002. 92(3): p. 1013-8.

100. Bisgard, G.E., E.B. Olson, Jr., Z.Y. Wang, et al., *Adult carotid chemoafferent responses to hypoxia after 1, 2, and 4 wk of postnatal hyperoxia.* J Appl Physiol (1985), 2003. 95(3): p. 946-52.

101. Ahdab-Barmada, M., J. Moossy, E.M. Nemoto, et al., *Hyperoxia produces neuronal necrosis in the rat.* J Neuropathol Exp Neurol, 1986. 45(3): p. 233-46.

102. Pomper, J.K., J. Graulich, R. Kovacs, et al., *High oxygen tension leads to acute cell death in organotypic hippocampal slice cultures.* Brain Res Dev Brain Res, 2001. 126(1): p. 109-16.

103. Cooper, J.M., D.G. Gadian, S. Jentschke, et al., *Neonatal hypoxia, hippocampal atrophy, and memory impairment: evidence of a causal sequence.* Cereb Cortex, 2015. 25(6): p. 1469-76.

104. Studer, L., M. Csete, S.H. Lee, et al., *Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen.* J Neurosci, 2000. 20(19): p. 7377-83.

105. Cai, M., Y. Zhou, B. Zhou, et al., *Hypoxic conditioned medium from rat cerebral cortical cells enhances the proliferation and differentiation of neural stem cells mainly through PI3-K/Akt pathways.* PLoS One, 2014. 9(11): p. e111938.

106. Stewart, W.B., L.R. Ment, and M. Schwartz, *Chronic postnatal hypoxia increases the numbers of cortical neurons.* Brain Research, 1997. 760(1–2): p. 17-21.

107. Salmaso, N., M. Dominguez, J. Kravitz, et al., *Contribution of maternal oxygenic state to the effects of chronic postnatal hypoxia on mouse body and brain development.* Neurosci Lett, 2015. 604: p. 12-7.

108. Endesfelder, S., I. Zaak, U. Weichelt, et al., *Caffeine protects neuronal cells against injury caused by hyperoxia in the immature brain.* Free Radic Biol Med, 2014. 67: p. 221-34.

109. Woodrow, E., P. Georgala, J. Wade, et al., *Cortical Growth is Impaired by Variable Hyperoxia in a Preterm Model of Growth Restriction.* Pediatr Res, 2011. 70(S5): p. 126-126.

110. LaManna, J.C., L.M. Vendel, and R.M. Farrell, *Brain adaptation to chronic hypobaric hypoxia in rats.* J Appl Physiol (1985), 1992. 72(6): p. 2238-43.

111. Witt, K.A., K.S. Mark, K.E. Sandoval, et al., *Reoxygenation stress on blood-brain barrier paracellular permeability and edema in the rat.* Microvasc Res, 2008. 75(1): p. 91-6.

112. Ho, M.-L., R. Rojas, and R.L. Eisenberg, *Cerebral Edema.* American Journal of Roentgenology, 2012. 199(3): p. W258-W273.

113. Bigdeli, M.R., S. Hajizadeh, M. Froozandeh, et al., *Prolonged and intermittent normobaric hyperoxia induce different degrees of ischemic tolerance in rat brain tissue.* Brain Research, 2007. 1152: p. 228-233.

114. Angulo-Colmenares, A.G., D.W. Vaughan, and J.W. Hinds, *Rehabilitation following early malnutrition in the rat: Body weight, brain size, and cerebral cortex development.* Brain Research, 1979. 169(1): p. 121-138.

115. Juranek, J., P.A. Filipek, H. Taylo, et al., *Anomalous brain development in adolescent survivors of very low birth weight: A structural imaging study.* Annals of Neurology, 2005. 58(S9): p. S88-S90.

116. Parikh, N.A., R.E. Lasky, K.A. Kennedy, et al., *Perinatal factors and regional brain volume abnormalities at term in a cohort of extremely low birth weight infants.* PLoS One, 2013. 8(5): p. e62804.

117. Martinussen, M., D.W. Flanders, B. Fischl, et al., *Segmental Brain Volumes and Cognitive and Perceptual Correlates in 15-Year-Old Adolescents with Low Birth Weight.* The Journal of Pediatrics, 2009. 155(6): p. 848-853.e1.

118. Xiao, X.Q., S.M. Williams, B.E. Grayson, et al., *Excess weight gain during the early postnatal period is associated with permanent reprogramming of brown adipose tissue adaptive thermogenesis.* Endocrinology, 2007. 148(9): p. 4150-9.

119. Prior, L.J., E. Velkoska, R. Watts, et al., *Undernutrition during suckling in rats elevates plasma adiponectin and its receptor in skeletal muscle regardless of diet composition: a protective effect[quest].* Int J Obes, 2008. 32(10): p. 1585-1594.

120. Fernandes, M.T.B., A.A. Ferraro, A. Pires, et al., *Early-life weight and weight gain as predictors of obesity in Brazilian adolescents.* Clinics, 2013. 68(11): p. 1408-1412.

121. Liao, G.-Y., K. Bouyer, A. Kamitakahara, et al., *Brain-derived neurotrophic factor is required for axonal growth of selective groups of neurons in the arcuate nucleus.* Molecular Metabolism, 2015. 4(6): p. 471-482.

122. Theodosis, D.T. and D.A. Poulain, *Activity-dependent neuronal-glial and synaptic plasticity in the adult mammalian hypothalamus.* Neuroscience, 1993. 57(3): p. 501-535.

123. Scantlebury, M., N.C. Bennett, J.R. Speakman, et al., *Huddling in groups leads to daily energy savings in free-living African Four-Striped Grass Mice, Rhabdomys pumilio.* Functional Ecology, 2006. 20(1): p. 166-173.

124. Gilbert, C., S. Blanc, S. Giroud, et al., *Role of huddling on the energetic of growth in a newborn altricial mammal.* Am J Physiol Regul Integr Comp Physiol, 2007. 293(2): p. R867-76.

125. Aanes, S., K.J. Bjuland, J. Skranes, et al., *Memory function and hippocampal volumes in preterm born very-low-birth-weight (VLBW) young adults.* NeuroImage, 2015. 105: p. 76-83.

126. Ostgard, H.F., J. Skranes, M. Martinussen, et al., *Neuropsychological deficits in young adults born small-for-gestational age (SGA) at term.* J Int Neuropsychol Soc, 2014. 20(3): p. 313-23.

127. Ostgard, H.F., G.C. Lohaugen, K.J. Bjuland, et al., *Brain morphometry and cognition in young adults born small for gestational age at term.* J Pediatr, 2014. 165(5): p. 921-7.e1.

128. Agmon, A., L.T. Yang, D.K. O'Dowd, et al., *Organized growth of thalamocortical axons from the deep tier of terminations into layer IV of developing mouse barrel cortex.* J Neurosci, 1993. 13(12): p. 5365-82.