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Mouse maternal protein restriction around conception and during gestation permanently alters brain cytoarchitecture and memory

by

Joanna Mary Gould

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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MOUSE MATERNAL PROTEIN RESTRICTION AROUND CONCEPTION AND DURING GESTTATION PERMANENTLY ALTERS BRAIN CYTOARCHITECTURE AND MEMORY

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Poor maternal nutrition during pregnancy is detrimental to foetal development and increases the risk of chronic diseases in later life, including neurological disorders such as schizophrenia. Previous studies have shown that diet restriction during gestation influences foetal brain development causing structural, biochemical and pathway alterations leading to impaired motor and cognitive function. However, the effect of diet restriction solely during embryogenesis on brain development is unknown. We have previously shown that maternal low protein diet confined to the preimplantation period with normal diet thereafter during gestation and postnatally is sufficient to induce cardiometabolic and locomotory behavioural abnormalities in adult mouse offspring. In this study female mice were fed from mating to term either (1) normal protein diet (NPD), (2) low protein diet (LPD) or (3) embryonic LPD (Emb-LPD; LPD for 0-3.5 days (E3.5), NPD thereafter) and a comprehensive analysis of foetal and adult brain development was performed for each diet. Emb-LPD and sustained LPD reduced neural stem cell and progenitor cell numbers through suppressed proliferation rates in both ganglionic eminences and cortex of foetal brain at E12.5, E14.5 and E17.5. Emb-LPD additionally caused rapid differentiation of remaining NSCs resulting in increased differentiated neurons. Emb-LPD adult offspring exhibited a deficit in short-term and long-term memory, whilst only LPD males show poor long-term memory. Female Emb-LPD adult offspring show increased cortical thickness, male and female Emb-LPD and sustained LPD showed changes in the proportion of different neuronal populations. Additionally, Emb-LPD and LPD diets affected the glial populations of the hippocampus with increased astrocytic cells in female LPD but a reduction in the Emb-LPD offspring. These data reveal, for the first time, that poor maternal nutrition around conception, even with good nutrition thereafter, compromises fundamental cellular processes coordinating early brain development and differentiation, correlated with adult offspring memory deficits.

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Academic Thesis: Declaration Of Authorship

I,.....Joanna Mary Gould

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Mouse Maternal Protein Restriction Around Conception And During Gestation Permanently Alters Brain Cytoarchitecture And Memory

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: .....

Date: .....

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Definitions and Abbreviations

ADHD	Attention deficit hyperactivity disorder
BCAA	Branched chain amino acids
BDNF	Brain derived neurotrophic factor
CA	Cornu ammonia
CHD	Coronary Heart Disease
ChIP	Chromatin Immunoprecipitation
COMT	Catechol-O-methyltransferase
CREB	cAMP response element binding protein
CP	Cortical plate
DAPI	4’,6’-diamidino-2-phenylindole
DOHaD	Developmental origins of health and disease
DCM	Dichloromethane
DG	Dentate Gyrus
DHA	Docosahexanoic acid
DI	Discrimination Index
DMSO	Dimethyl sulfoxide
E12.5	Embryonic day 12.5
EC	Entorhinal Cortex
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
Emb-LPD	Embryonic Low Protein Diet
EPM	Elevated Plus Maze
ESC	Embryonic stem cells
EWAS	Epigenome wide association study
FACS	Fluorescence activated cell sorting
FGF2	Fibroblast growth factor 2
FGFb	Fibroblast growth factor beta

GE	Ganglionic eminences
GFAP	Glial fibrillary acidic protein
IBA1	Ionized calcium binding adapter molecule 1
ICM	Inner cell mass
IGF1	Insulin-like growth factor 1
IGF2	Insulin-like growth factor 2
IHD	Ischemic heart disease
IUGR	Intrauterine growth restriction
LPS	Low protein diet
MZ	Marginal zone
mTORC1	Mammalian target of rapamycin complex 1
NCD	Non-communicable disease
NeuN	Neuronal nuclei
NOR	Novel object recognition
NPD	Normal protein diet
NSC	Neural stem cell
OFT	Open field test
PE	Primitive endoderm
PFA	Paraformaldehyde
PND	Postnatal day
PBS	Phosphate buffered saline
SGZ	Sub-granular zone
SOX2	Sex determining region Y box 2
SVZ	Sub-ventricular zone
TBS	Tris buffered saline
TE	Trophectoderm
TP	Timepoint
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone
VYS	Visceral yolk sac
WHO	World health organisation

Chapter 1 General Introduction

Malnutrition is the greatest threat to public health according to the World Health Organization. 13% of the global population are malnourished, which represented 925 million people in 2010, and malnutrition is responsible for 54% of child mortality [1]. The 'perfect storm' scenario proposed by Professor John Beddington in 2014 forecast the global population to reach a staggering 8.3 billion by 2030, which will require 50% more energy, 50% more food and 30% more water than the current population[2]. Therefore, the greater food demand coupled with a growing population is predicted to lead to a higher incidence of famine by 2030.

Nutrition directly affects cell metabolism and growth and therefore it is vital for women during pregnancy and lactation to ingest sufficient nutrients for themselves and their babies to prevent the risk of abnormal development and disease in offspring [3]. Recent epidemiological studies have associated maternal malnutrition with programming consequences such as cognitive decline and neurodevelopmental disorders in the offspring [4 5]. A concept called 'The Developmental Origins of Health and Disease' (DOHaD) hypothesises a link between environmental exposure *in utero* and the programming of disease in later life[6]. This DOHaD paradigm put forward by Professor David Barker has been modelled and verified by human epidemiological studies[6 7]. Non-communicable diseases (NCDs), such as diabetes and coronary heart disease have been well studied [8]. Furthermore, Susser & Stein provide evidence that malnutrition specifically occurring only during the early stages of pregnancy can also lead to brain damage [9]. These studies provide evidence that maternal diet during early pregnancy, defined here as the first trimester can shape the development of offspring. Howevear, the first trimester is comprised of several crucial developmental stages; going from preimplantation period through to gastrulation and the effects of maternal malnutrition during these individual stages is poorly understood.

Many studies have identified the preimplantation period to be a particularly sensitive timepoint during embryo development [10]. During the preimplantation period, many cellular changes take place including embryonic genome activation and the emergence of distinct differentiation pathways at the beginning of morphogenesis [10 11]Previous studies using a low-protein diet (LPD) confined to the preimplantation period (Emb-LPD) showed that protein restriction during this sensitive period results in developmental changes including increased glucose tolerance, weight and hypertension [12]. This study also identified neurodevelopmental alterations based on behavioural changes in adult offspring related with anxiety and/or poor memory that are particularly relevant for this thesis, which focuses on the association of maternal protein restriction

during the preimplantation period with postnatal behavioural phenotypes in offspring and the corresponding morphological changes in the adult and embryonic offspring brains.

This project aims to determine whether low protein diet during pregnancy and the preimplantation period, can induce changes to the foetal and offspring brains. The long- term vision of this project will be to advise women of the importance of protein during pregnancy, in order to suppress adverse embryo programming so that the health of the next generations brains would be protected.

1.1 The Preimplantation period, DOHaD and the Brain

In the last 20 years there have seen considerable advances in our understanding of intrinsic developmental mechanisms associated with embryogenesis and nuclear reprogramming. However, recently research has been more concerned with how the homeostatic regulation of embryonic cells may impact developmental programming leading to altered phenotypes in adult offspring. Recently, the preimplantation stage of development has been identified in a range of studies as a critical period for developmental programming [13]. The preimplantation period in all mammalian species exhibits a relatively synchronous doubling of cell numbers until the 8-cell stage, followed by asynchronous cell divisions thereafter. At the 8- to 16-cell stage the embryo enters the uterine environment [14], and there is subsequent epithelial differentiation in outer cell layer, which forms the extra-embryonic trophectoderm (TE) of the developing blastocyst (32-cell stage in mouse) [15 16]. During further development, the embryo requires more sophisticated metabolic and nutrient uptake pathways in order to survive [17]. TE trans-epithelial transport activity then forms the blastocoel cavity[15]. At the blastocyst stage the embryo hatches from the surrounding zona pellucida and subsequently implants in the uterus, thus ending the preimplantation period just before placental interactions have occurred. The preimplantation period is seen as a sensitive 'window', many studies have shown that in vitro and in vivo manipulations, such as culture conditions or maternal diet, may have critical consequences for offspring health in later life. Many studies have shown how environmental conditions, mediated via a range of epigenetic, cellular and metabolic mechanisms in the preimplantation embryo, may alter the pattern of cell division, gene expression, morphology leading to poorer health in foetal and adult offspring.

The embryo has the ability to exhibit a remarkable plasticity during development and is able to influence the phenotype of offspring postnatally. This concept is known as the developmental origins of health and disease (DOHaD) or the "Barker Hypothesis", which has been refined over a number of years [18]. embryonic Professor David Barker first highlighted that the intrauterine environment could affect not only *in utero* foetal development, but could programme permanent changes in physiology and metabolism that increase risks of disease later in life. There is now substantial evidence that *in utero* environment may influence postnatal health and disease risk. Epidemiological studies on diverse human populations show low birth weight and early catch-up growth during infancy is associated with increased chronic disease in adulthood [5 6]. However, many animal and human studies have identified the early embryo and the preimplantation period during pregnancy as the critical period when environmental factors cause permanent changes in development leading to adult onset disease[12]. This programming may lead to many physiological consequences illustrated in Figure 1.1. Abnormal cognitive decline and neurodevelopmental

disorders following environmental changes during preimplantation are particularly relevant for this thesis [4 5].

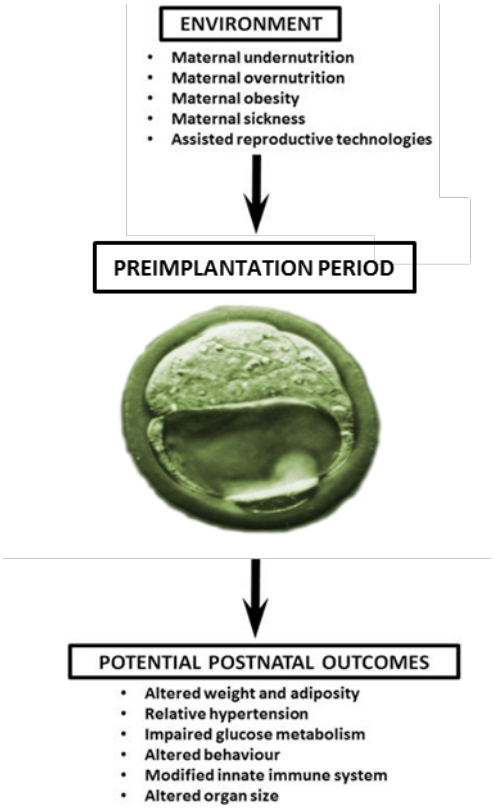


Figure 1.1Environmental changes during the preimplantation period can impact on the early embryo.

Illustration of environmental factors that have been shown to result in abnormal postnatal outcomes when altered during the preimplantation period. [19].

A trio of papers in *The Lancet* by Barker and colleagues form the origin of the DOHaD hypothesis [7 20 21]. Barker had hypothesised that malnutrition in early life increased the susceptibility to affluence-associated diseases later in life. The first article in the Lancet showed that although the rise in ischaemic heart disease in England and Wales could be associated with increasing prosperity, it did not answer fully why mortality rates are highest in the least affluent areas. However, when specific regions of the country were looked at, a strong geographical relation was found between ischaemic heart disease mortality rates in 1968-78 and infant mortality in 1921-25 [20]. Heart disease was not the only disease to be correlated with infant mortality, as bronchitis, stomach cancer, and rheumatic heart diseases were similarly related. These diseases were associated with poor living

conditions and mortality, suggesting that poor nutrition in early life increases risk of diseases more typically associated with an affluent diet [20].

The second of the three Lancet papers showed that increased birth weight and growth during the first postnatal year reduced the incidence of ischaemic heart disease mortality throughout life [7]. Thus, Barker proposed the hypothesis that adult disease was not just influenced by genetics and current environmental conditions but also by developmental programming [21].

The ability to reprogram the developing foetus is seen as an advantageous mechanism, as a foetus can alter its physiological parameters in response to maternal malnutrition to prepare for scarcity once born, providing the idea of a 'thrifty' embryo. This mechanism only becomes maladaptive when a malnourished foetus is subsequently born into a plentiful environment [22]. The DOHaD hypothesis was further supported by Barker's third Lancet paper showing that maternal malnutrition at any point of pregnancy reduced birth weight and caused placental abnormalities [20]. Further studies linking low birth weight with an increased risk of hypertension, diabetes and metabolic syndrome have been completed in diverse populations to further support this hypothesis [23].

One key study to further support the DOHaD hypothesis was the Dutch hunger winter which was a five-month period of famine due to the Nazi occupation between 1944-1945 in Amsterdam. Adults who were exposed to poor in utero nutrition due to this occupation were later found to have increased incidence of numerous health conditions compared to other groups, indication that maternal malnutrition can have a profound effect later in life [24]. The supplies of rations during the occupation were 400-800 calories a day mainly consisting of bread and potatoes. This was supplied to all residents including pregnant women regardless of social class. Detailed medical records of these people provided an excellent example to later prove the DOHaD hypothesis. Immediately following the occupation, food became plentiful indicating that poor nutrition solely during in utero development was responsible for these changes. This study highlighted the importance of nutrition during critical periods of development that corresponded with diseases in later life. For example, glucose intolerance was prevalent regardless of timing of the period of famine during pregnancy; however, those exposed to famine only during in early gestation had an increased likelihood of obesity and neurodevelopmental disorders [25 26].

One of the first indications that the Dutch hunger winter influenced neural development among the cohort conceived during the height of the famine was the observation of an increase in congenital neural defects, especially neural tube defects including spina bifida and anencephaly [27]. This alteration in foetal neurodevelopment supported the hypothesis that prenatal famine could cause neurological problems later in life. In addition to clear neurodevelopmental conditions, this

cohort also displayed more subtle and long-term changes such as an increased risk of schizophrenia in adulthood [24 28 29]. The exposed cohorts were chosen when the height of the famine corresponded to the periconceptional period or early gestation for this cohort. The Dutch psychiatric registry was used to compare psychiatric outcomes in adulthood for exposed and unexposed birth cohorts as illustrated in Figure 1.2. The study found a twofold increase in the risk of schizophrenia in the exposed birth cohort during early gestation (EX1) compared to late gestation and unexposed cohorts [30]. Moreover, a subsequent study showed a twofold increased risk of schizoid personality disorder in the same exposed birth cohort [31]. Later analysis of the disease risks for successive birth cohorts of 1944–1946 revealed striking peaks in the incidence of schizophrenia, schizoid personality, and congenital neural defects in this same birth cohort [30].

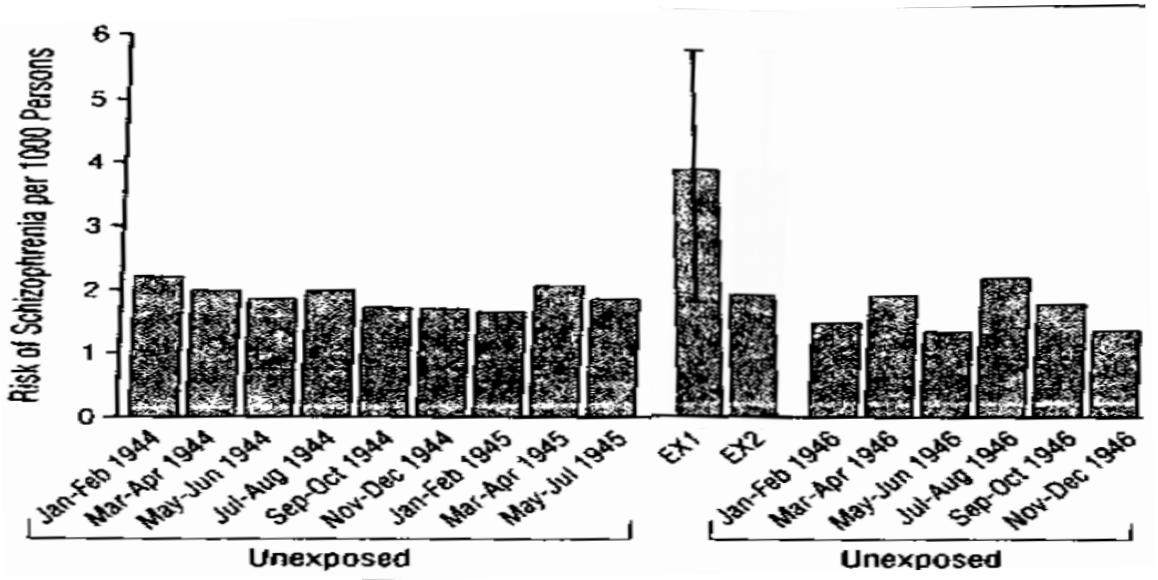


Figure 1.2. The risk of schizophrenia per 1000 persons during the Dutch Hunger Winter

EX1 shows offspring exposed to famine in early pregnancy and EX2 shows offspring exposed in late pregnancy, EX1 cohort have 2-fold increase risk of schizophrenia in adulthood (defined as hospitalised subject of 24-48 years of age) in Dutch birth cohorts of 1944 through 1946. EX1 and EX2 indicate cohorts born October 15 through December 31, 1945, and August through October 14, 1995 respectively. X^2 statistics was used to test the null hypothesis showing no association between exposure and disease (unexposed n= 136,691, exposed EX1=5446 and EX2=4190). Risk of schizophrenia was calculated by finding the risk for the EX1, EX2 and unexposed cohorts and dividing the number of cases by the number of births minus deaths up to 18 years. Risk was then multiplied by 1000 to derive the risk per 1000 persons. The relative risk in the exposed compared to unexposed were calculated and 95% confidence limits were derived using the Taylor series. Taken from [32]

Famine is not a rare global phenomenon; however, it is rare that it is often restricted to a defined time and space and that appropriate and detailed health records have been kept enabling a detailed

analysis of nutrition and corresponding long-term health implications. In the Chinese Famine Study the relationship between prenatal famine and risk of schizophrenia was successfully examined in a cohort in the Wuhu region of Anhui Province, China in the late 1950s [33]. A massive famine was precipitated in China by the marked social and economic change as the country farming communities radically changed and people went to work in the industrial cities. In the Chinese study, data on caloric rations were not available, but they could examine whether the risk of schizophrenia was increased in the birth cohorts conceived during the height of this famine. Accordingly, the Wuhu birth cohorts of 1960 and 1961 were defined as the exposed group, being conceived in the period of most severe famine for this region. The schizophrenia outcomes were obtained from systematic review of the health records and saw a marked increase in the incidence of schizophrenia [28].

Calorie restriction in pregnant women is not a thing of the past unfortunately, as there are many regions of famine across the world. A more recent phenomenon appears to be occurring in Japan currently where birth weights have decreased steadily over the last 30 years due to intentional dieting of young Japanese women during pregnancy, which has resulted in foetal malnutrition and it will be interesting to see the outcome for these babies later in life [34].

It is important to consider that neurodevelopment is multidimensional and also includes cognitive, motor, social and emotional domains. However, all are interdependent with a change in one affecting the others. These domains can be influenced by the genetics, the health and nutrition of the individual and the environment the individual has been brought up in. However, the Dutch hunger winter and the Chinese winter studies really do highlight the importance of maternal nutrition in the development of the offspring nervous system.

1.2 The development of the embryo and the nervous system

After fertilisation, the zygote undergoes five reduction cleavage divisions to form a blastocyst. The blastocyst then moves into the uterus and implants [35]. This process takes 3.5 days post conception (PC) in the mouse and 6.5 days PC in the human [36]. As the blastocyst is implanting, the formation of an indentation appears which is known as the primitive pit, this then elongates to form the primitive streak. The primitive streak indicates the caudal midline of the embryo which defines the body axes and allows gastrulation to occur. Gastrulation is a vital part of development as this then transforms the blastocyst into the gastrula [36] from a simple spherical ball of cells into a multi-layered organism developing the three primitive germ layers, endoderm, mesoderm and ectoderm as shown in **Error! Reference source not found.** [37].

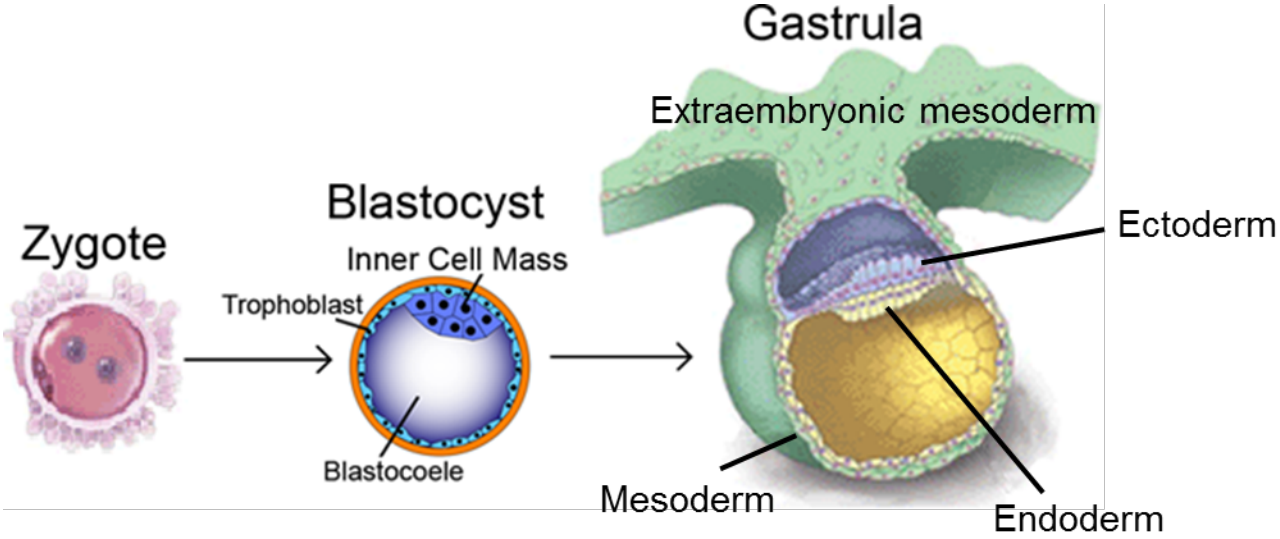


Figure 1.3. The three germ layers form during human gastrulation

> The three layers are determined by their physical position in the gastrula. This stage follows the zygote and blastocyst stages; the gastrula forms when the embryo is approximately 14-16 days old in the human[38].

A number of highly orchestrated processes are required for brain development with the formation of the general brain region architecture complete by birth. The process of brain development starts with neurulation from the ectoderm of the embryo. Neurulation is a highly regulated process of apoptosis and cell proliferation that occurs in two phases, primary and secondary neurulation [39]. Primary neurulation is essential in humans while secondary neurulation appears only to play a key role in tailed animals [40]. By the end of gastrulation, the neuroepithelial cells have differentiated

and are positioned along the rostral-caudal midline of the upper layer of the three-layered embryo. The region of the embryo containing the neuroepithelial cells is referred to as the neural plate. The first appearance of neural tube formation is the appearance of two ridges on either side of the neural plate. The neuroepithelial cells lie between the two ridges. Over the course of several days the ridges rise, fold inward and fuse to form a hollow tube [41]. Fusion begins in the centre of the developing neural tube and then proceeds in both the rostral and caudal directions almost like a 'zip' as shown in Figure 1.4, failure to close this 'zip' can lead to neural tube defects (NTDs)[37 39].

The NT forms the embryonic neuroepithelial cells and is patterned by distinct organising centres, which secrete morphogens, inducing differentiation to distinct cell fates (Figure 1.5). The process is concentration gradient dependant and provides positional information to the cells across the NT. The notochord and prechordal plate express sonic hedgehog (Shh) while the ectoderm adjacent to the neural plate, expresses bone morphogenetic protein (BMP) and Wnt. By acting at opposing poles of the neural tube they define the dorsoventral axis. The anteroposterior axis is defined by Fibroblast growth Factor FGF, Wnt and retinoic acid gradients, expressed from discrete local organisers such as the midbrain-hindbrain boundary and anterior neural boundary. Organiser centres and their soluble factors also have mitogenic functions; regulating cell growth and proliferation, apoptosis and axon guidance in the developing CNS [42].

Neurulation in humans is usually completed between 21 and 28 days post conception and in mice neurulation occurs at day 8.5 post conception and is complete by day 10.5 post conception[43]. When the NT is complete, the neuroepithelial cells form a single layer of cells that lines the centre of the NT immediately adjacent to its hollow centre; as well as the neural stem cells that will give rise to the neurons and glia (astrocytes and oligodendrocytes) of the CNS.

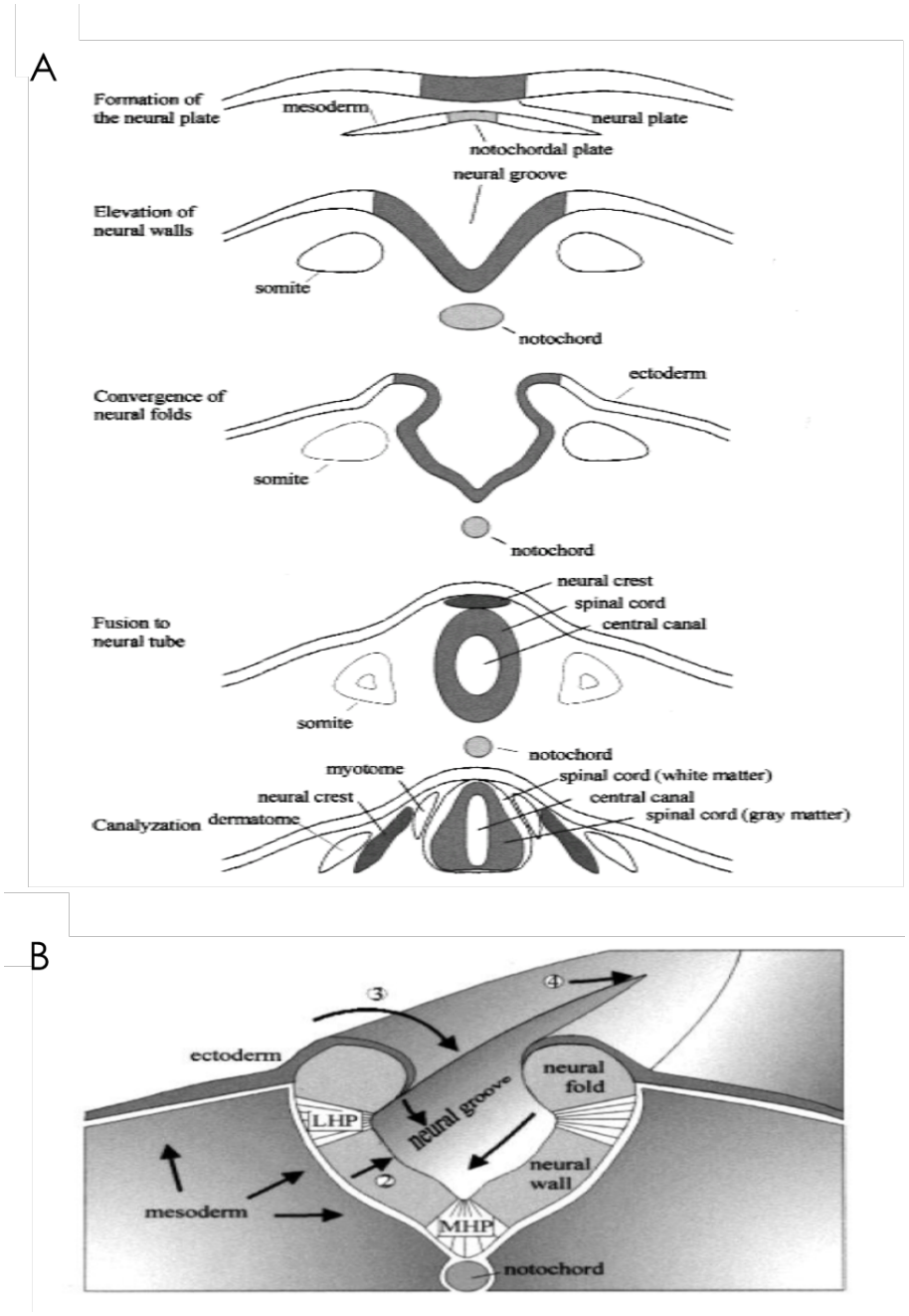


Figure 1.4. Formation of the neural tube

(A) The four phases of primary neurulation. (B) The orientation of how the processes of primary neurulation overlap spatially and temporally in the following stages: 1. narrowing and elongation of the neural tube; 2. elevation of the neural wall; 3. convergence of the neural folds; 4. fusion of the neural folds. Originally used in Detrait et al [37].

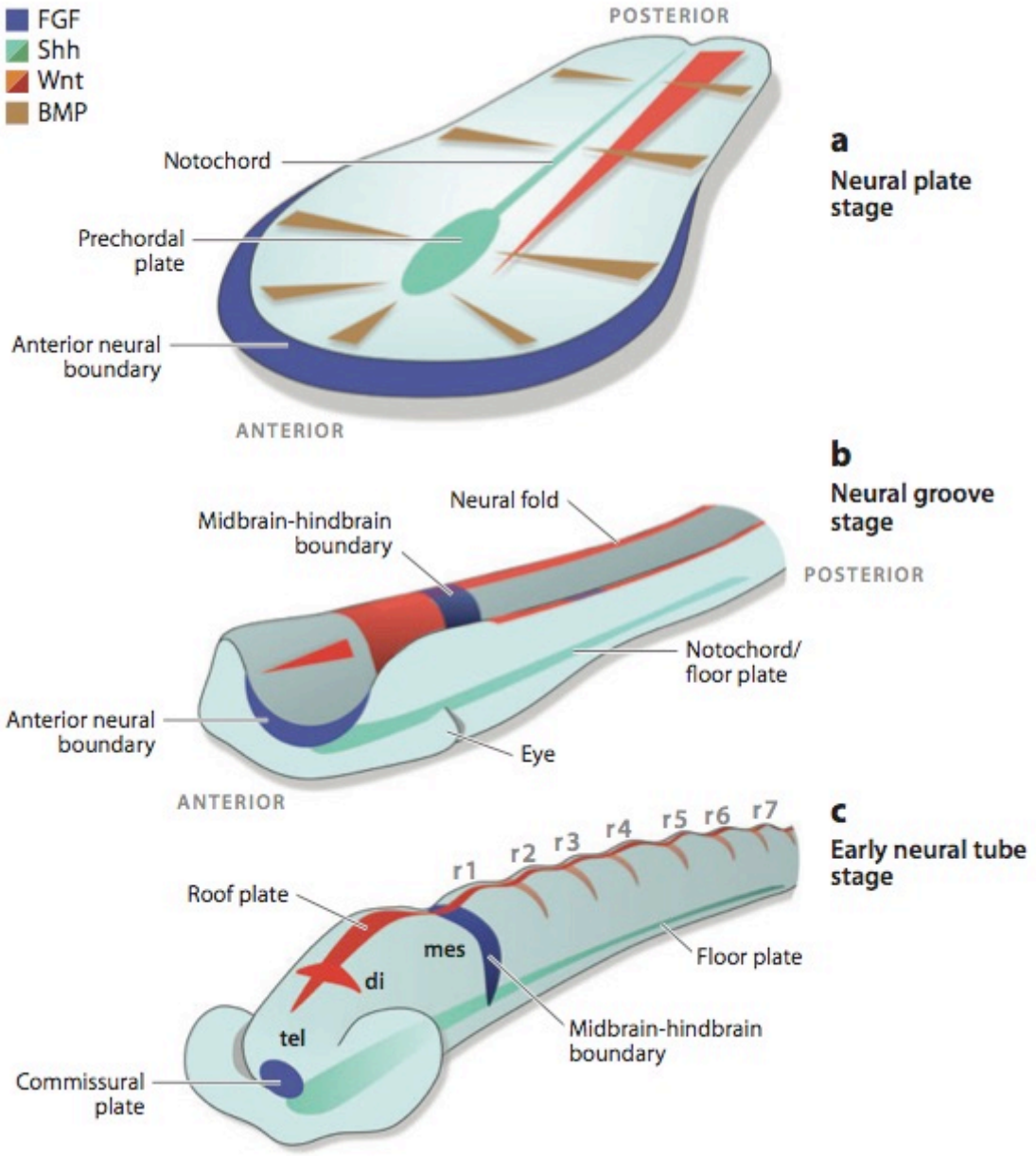


Figure 1.5. Sites of morphogen production during neurulation

The pattern of release of morphogens Fibroblast growth factor (FGF), Sonic hedgehog (Shh), Wnt and Bone morphogenic protein (BMP) during (a) neural plate development, (b) neural groove development and (c) early neural tube development is critical to guide growth of the developing CNS. Taken from [42].

As the brain increases in size and complexity this fluid filled tube must adapt to form the ventricular system of the brain. Because the neuroepithelial cells are located in the region that will become the ventricles, the region is called the “ventricular zone” (VZ). The neuroepithelial cells at the ventral midline of the NT, which are closest to the notochord, receive inductive signals allowing them to differentiate into a special strip of epithelial-like cells called the floorplate. The floorplate position at the ventral midline determines the dorso-ventral polarity of the neural tube and further influences the differentiation of neuroepithelial cells. The floorplate secretes inductive signals that lead to the differentiation of the cells in the VZ of the neural tube giving rise to the spinal and hindbrain motor neurons[44].

Neural progenitor cells located further from the ventral midline give rise to the sensory neurons within the spinal cord and hindbrain. At the most dorsal limit of the neural tube, a third population of cells emerges in the region where the edges of the folded neural plate join the neural crest. The neural crest cells migrate away from the neural tube along different specific pathways and therefore give rise to a variety of progeny, including the neurons and glia of the sensory and visceral motor (autonomic) ganglia, the neurosecretory cells of the adrenal gland, and the neurons of the enteric nervous system. They also contribute to variety of non-neural structures such as pigment cells, cartilage, and bone[45].

1.3 Neural Stem Cells and Corticogenesis

Neural stem cells (NSCs) are multipotent, capable of self-renewal and differentiate into 3 cell types in the CNS[46] neurones, astrocytes and oligodendrocytes[47]. As discussed earlier the NT is lined with neuroepithelial cells, the cells develop the cerebral cortex or as it is called in mammals the neocortex that distinguish the region from the more ancient paleo cortex and the telencephalon. The neocortex is essential for higher cognitive functions in the mammal and has a very complex architecture.

In the developing cortex neuroepithelial cells extend long processes throughout the entire cortical wall with their apical feet attaching to the ventricular surface and the basal end feet attach to the pial lamina. The neuroepithelial cells divide via symmetric cell division to create an adequate pool of NSC. As the number of neural stem cells increase, the NT thickens and becomes a pseudostratified epithelium. This initial proliferation phase affects both lateral extensions and radial extensions as they migrate from the ventricle and return to divide forming the Ventricular zone (VZ). A disturbance as this point has significant effects on the final surface area and thickness of the neocortex [44 48]. This is a cell cycle dependant process in which the cell bodies migrate away from the ventricle and then return to divide, forming the VZ[48].

Asymmetric division follows this process, early in cortical neurogenesis Radial Glia (RG) cells differentiate from neuroepithelial cells and are ubiquitous in the CNS. Like neuroepithelial cells, RG cells populate the VZ, maintain apical-basal polarity and function as NSCs. The primary type of division is asymmetric divisions, producing a new a RG and a neuron (direct neurogenesis) or an intermediary neural cell that gives rise to neurons (indirect neurogenesis), which populate the subventricular zone (SVZ). Subsequent division forms neurons or glial cells (Figure 1.6). This serves to amplify the number of cells produced from a single NSC division[49].

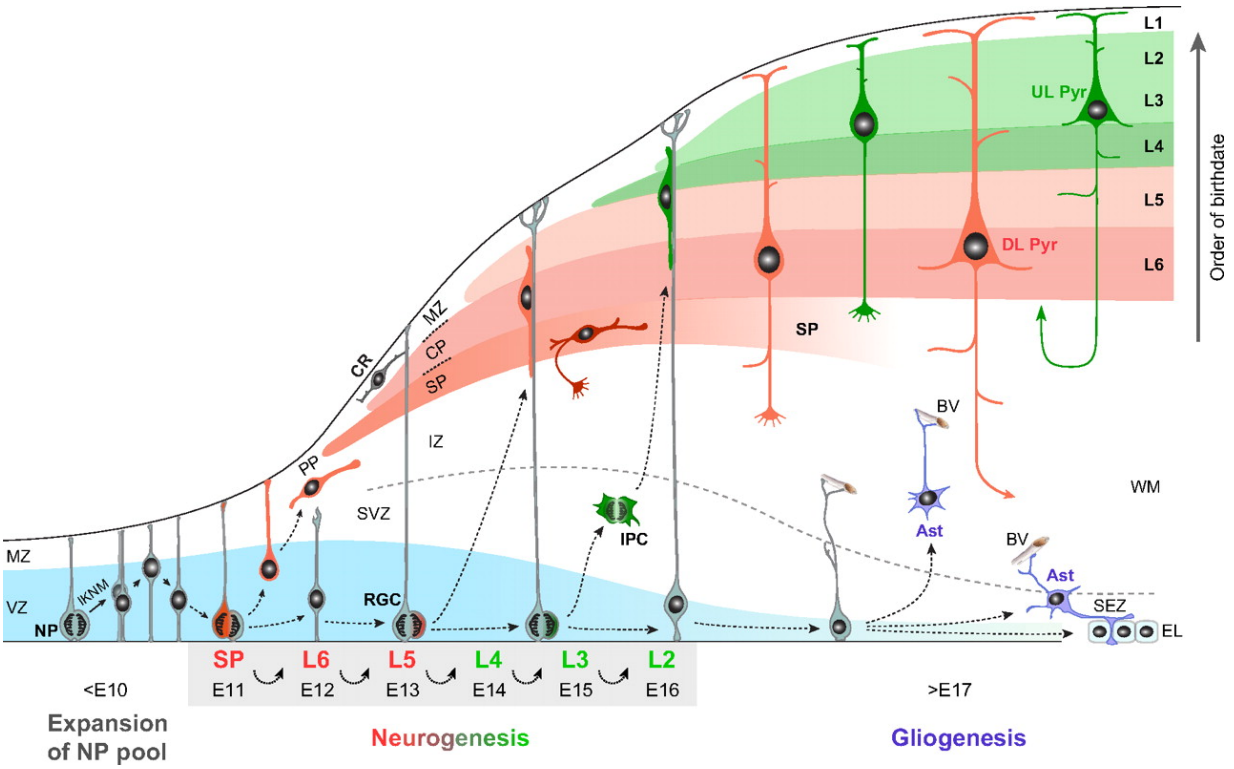


Figure 1.6. Schematic of projection neuron generation and migration in the mouse neocortex

Prior to the onset of neurogenesis, neuroepithelial cells (NPs) in the ventricular zone (VZ; blue) of the developing neocortex divide symmetrically to expand the progenitor pool, undergoing interkinetic nuclear migration (IKNM) as they progress through the cell cycle. Starting at ~E11.5, NPs assume radial glial morphology and begin dividing asymmetrically to generate neurons, which migrate from the germinal zones guided by radial glia cells (RGC) to reach the mantle layers. The first projection neurons settle within the preplate (PP) to form the nascent cortical plate (CP), which will subsequently become layers (L) 2 to 6 of the neocortex. Additional incoming CP neurons then split the PP into the marginal zone (MZ) and the subplate (SP). As neurogenesis progresses, diverse subtypes of projection neurons are generated sequentially through successive asymmetric divisions of NPs. The end of neurogenesis occurs at ~E17.5, the radial scaffold is dismantled and NPs become gliogeneic, generating astrocytes (Ast) and giving rise to a layer of ependymal cells (EL). BV, blood vessel; CR, Cajal-Retzius neuron; DL Pyr, deep-layer pyramidal neuron; IZ, intermediate zone; UL Pyr, upper-layer pyramidal neuron, WM, white matter [50].

The prevalence of indirect neurogenesis increases markedly as neurogenesis progresses. Mouse data has shown that each RG cell can give rise to 8–9 neurons stochastically distributed throughout the different layers [51]. They delaminate from the VZ to settle in the SVZ, where they divide symmetrically to self-renew before undergoing a terminal division that gives rise to two neurons [52]. The final neuronal output is sequentially impacted by the size of the initial pool of founder of neuroepithelial cells, by the progressive switch from symmetric replicative to neurogenic divisions, and finally by the duration of the neurogenic phase[53]. In the mouse, SVZ progenitors, principally comprising IPCs, undergo at most two rounds of divisions[52], whereas they undergo significantly more rounds in humans and other primates [54].

As corticogenesis progresses neurons migrate from the VZ and SVZ to populate the neocortical layers. The neurons formed first populate the deeper layers and subsequent neurons the more superficial layers[48 55]

The cortex is composed of two major subsets of neuron, the glutamatergic excitatory projection neurons and GABAergic inhibitory interneurons. Cortical projection neurons migrate from the VZ to the cortical plate via somal translocation or radial migration. Inhibitory interneurons undergo tangential migration from the SVZ and preoptic area, inhibitory neurons originate from the ganglionic eminences (Figure 1.7). Populating the neocortex in an inside out fashion, the final position is determined by place and time of birth[56].

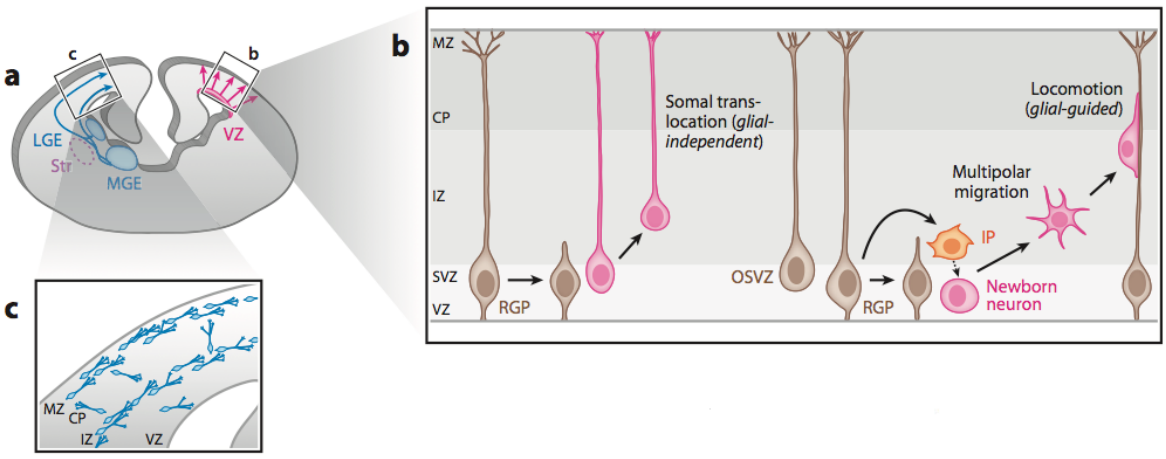


Figure 1.7. Neuronal migration in the developing brain.

A) Projection neurons (PINK) and Interneurons (BLUE) originate from the VZ And SVZ respectively. (B) Projection neurons generate from RG or IPCs and migrate via somal translocation or radial migration. (C) Tangential migration of interneurons. Taken from [56].

Neurogenesis arrests in the VZ prior to birth, however, the SVZ continues to provide new neurons throughout the lifetime of the organism. In the adult, NSCs are found in two neurogenic regions the SVZ and the subgranular zone (SGZ) of the hippocampal dentate gyrus[36]. Evidence shows that the NSCs of the adult SVZ are derived from RG cells[57]sharing morphological and molecular features of RG cells and expressing glial fibrillary acidic protein (GFAP) an astrocytic marker expressed in RG cells. Migration of cells from the SVZ continuously supply the olfactory bulb with new neuroblasts, migrating via the rostral migratory stream[55].

The Ganglionic eminences (GE) is a transitory brain structure in the foetal brain, which is essential for the generation of GABAergic neurones. The GE is found in the ventral ventricular zone of the telencephalon, where they facilitate tangential cell migration during embryonic development. Tangential migration does not involve interactions with radial glial cells; instead the interneurons migrate perpendicularly through the radial glial cells to reach their final location (Figure 1.8) [58]. The characteristics and function of the cells that follow the tangential migration pathway seem to be closely related to the location and precise timing of their production. The medial and caudal ganglionic eminences (MGE and CGE), and the preoptic area (POA) ventral to the MGE give rise to distinct populations of neocortical interneurons, with only minor overlap in phenotypes. This diversity is driven by the ventral pallium transcription factor SOX6 [59] .

The GABAergic interneurons migrate tangentially, and the GEs contribute significantly to building up the GABAergic cortical cell population. Another structure that the GEs contribute to is the basal ganglia. The GEs also guide the axons growing from the thalamus into the cortex and vice versa.

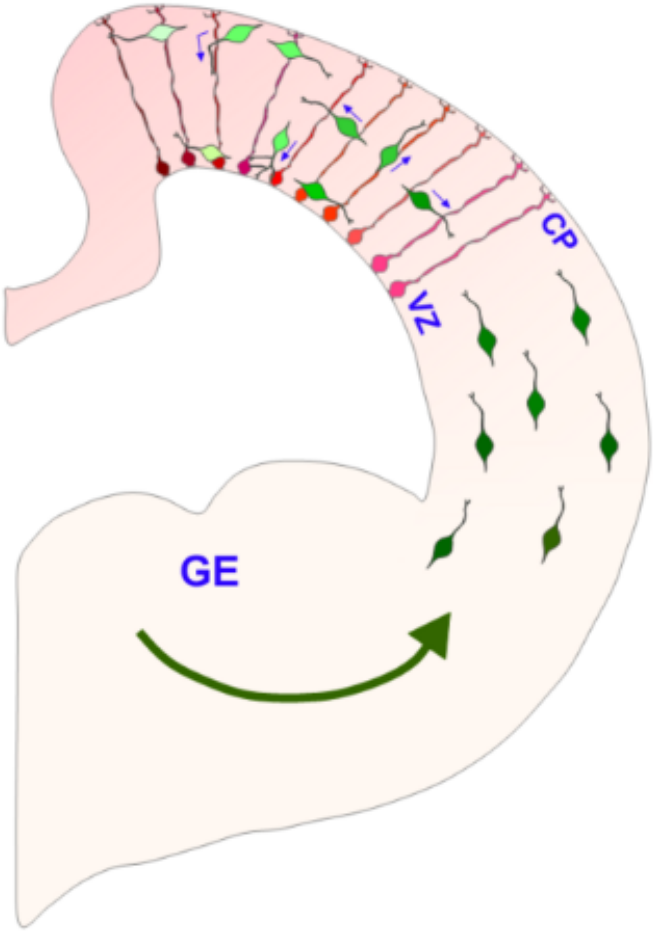


Figure 1.8. Interneurone migration from the ganglion eminences to the developing cerebral cortex

Interneurones (green) migrate into the cerebral cortex from the ganglionic eminence (GE) and then interact with the radial glia (red) and change direction of migration once they come into contact with the radial glia. These interneurons then use the radial glia as a scaffold to migrate towards the cortical plate (CP) or descent to the ventricular zone(VZ)[60]

1.4 Development of the hippocampus

The hippocampus develops from the telencephalon's caudomedial edge of the dorsal telencephalic neuroepithelium which is next to the cortical hem [61]. At the start of its development the hippocampus is a structure called the hippocampal primordium which consists of Cajal-Retzius cells and radial glial cells [61]. These two cell types then proliferate within the dentate neuroepithelium and produce embryonic neural stem cells which later in hippocampal development migrate into the sub granular zone (SGZ) of the dentate gyrus (DG). The developmental period of the dentate gyrus in mice starts at embryonic day 13 and ends on postnatal day 15 and it is these stem cells which will give rise to the DG granule neurons [61]. The first granule cells arise in the ventricular germinal zone and migrate using a scaffold created by the primordial radial glia towards the pial surface. Most granule neurons are formed in the prenatal period as well as early postnatal period [62]. This radial glia scaffold disappears near birth and a second radial glia scaffold develops; the cell bodies of these radial glia are based in the sub granular zone (SGZ), which is the innermost layer of the dentate gyrus[62]. The processes of the radial glia extend across the granule layer situated above the SGZ and into the molecular layer of the dentate gyrus. Correct wingless (WNT) and β-catenin signalling is crucial for the correct size and organisation of the DG [61]. Sonic hedgehog (SHH) signalling has also been shown to be crucial in proper expansion of the granule cell neural progenitor population and impairments in SHH signalling reduces the amount of granule cells in the DG when compared with other regions of the hippocampus [63].

The mature hippocampus or hippocampal formation is made from the hippocampal proper which consists of the cornu ammonis subfields (CA1, CA2, CA3) as well as the dentate gyrus, entorhinal cortex and subiculum. Granule neurons found in the granule cell layer of the dentate gyrus project their axons through the hilus, an area with high numbers of interneurons also called the polymorphic layer, to the CA3 region [64]. These projections are referred to as "Mossy fibres". The CA3 region of the hippocampus consists of pyramidal neurons and many inhibitory interneurons, the classical example being the basket cell, which synapse onto the pyramidal neurons[64]. These pyramidal neurons project to the CA1 with their projections termed the Schaffer collaterals. The CA1 region also receives input from layer 3 of the entorhinal cortex and the thalamus and integrates the information from these different pathways. The CA1 then projects to the subiculum and deep layers of the entorhinal cortex (EC) [64 65].

1.5 Mechanisms controlling neural progenitors and neural stem cells.

The nucleus of the VZ progenitor cells extends through the entire cytoplasm in neuroepithelial cells, but spans only the VZ in RGCs. This process is called interkinetic nuclear movement (INM), and is cell cycle dependent and creates a pseudo-stratified structure, in which synchronized nuclei located at the basal end of the cell during S phase, undertake basal to apical migration towards the ventricular surface during G2, undergo M phase at the ventricular surface and then migrate back to the basal side during G1 [66]. This coordination between INM and cell cycle phases plays an essential role in the maintenance of the progenitor pool [66]. A disturbance in this process can lead to the loss of progenitors due to abnormal ventricular mitosis and consequent apoptosis or exit from the cell cycle. The balance between symmetric and asymmetric divisions also has a clear impact on the final neuronal output. Symmetric divisions are important to produce an adequate pool of founder neuroepithelial cells, whose progeny will give rise to all cortical projection neurons. Therefore, even slight alterations in this pool can have a substantial impact on the final neuronal output. On the other hand, asymmetric divisions are important to maintain the proper distribution of the progenitor pools, including RGCs and IPCs, in coordination with the sequential generation of neurons populating the six layers of the cortical plate [53 54]. During symmetric neuroepithelial cell division, the cleavage plane is oriented vertically and perpendicular to the ventricular surface. Alterations in the centrosome cycle affect spindle positioning, which is detrimental to division symmetry and can drive cells to prematurely exit the cell cycle and differentiate, or even to undergo apoptosis[67].

Cell signalling mediated by the meninges and Cajal–Retzius cells through contacts with the basal membrane of neuroepithelial cells and RG cells participate in the activation of proliferation[67]. This activation is also promoted by Notch signalling[68], which is sustained by high Par3 expression [69]. The Notch pathway activates Hes1, which in turn represses neurogenic genes i.e. neurogenins, and therefore inhibits proneural gene transcription and is required in both the embryonic germinal zones and the adult SVZ to maintain the NSC population. Hence, the unequal divide of Par3 during asymmetric divisions results in cell cycle exit and differentiation of the daughter cell which then receives the lower amount of Par3 [69]. Notch signalling is further regulated by other pathways, such as factors secreted by the choroid plexus into the cerebrospinal fluid and known to play an important role in the control of progenitor amplification such as IGF2 [45], FGFs[70], Wnts [71], and Shh, which acts on the primary neuralepithelial cells and RG cells[72]. Shh signalling plays a role in neurogenesis in the SVZ and SGZ. The NSCs respond to Shh in late embryonic stages, *in vivo* genetic fate glycogen mapping demonstrates quiescent NSCs are regulated by Shh. Site specific recombinase techniques were used to induce cytoplasmic LacZ expression following tamoxifen administration and can be detected in the NSCs of the SVZ and SGZ[73].

Growth factor signalling promotes NSC proliferation and survival. Epidermal growth factor (EGF) and FGF2 are required for the survival and proliferation of NSCs in culture, facilitating symmetrical cell division and maintenance of an undifferentiated state[74]. Both EGF and FGF2 are required for optimal proliferation whereas EGF is critical to NSC survival. Both activate the ERK/MAPK signalling pathway, but EGF also signals via the canonical PI3K/Akt pathway[75]. Both pathways increase cell cycle progression and proliferation, but PI3K/Akt signalling results in caspase inhibition and is antiapoptotic. IGFs appear to be vital to normal brain development with IGF1 overexpression or under-expression resulting in increased or decreased brain growth respectively. IGF1 receptors are expressed in all neural cells and IGF stimulates proliferation of NSC and progenitor cells and has been implicated in development of all CNS cell lineages[76]. IGF2 has recently been implicated in control of NSCs in the dentate gyrus of the hippocampus, with IGF2 being highly expressed and regulating proliferation of hippocampal NSCs via Akt signalling through IGF1 receptors[77]. Signalling via many growth factors and cellular adhesion molecules converge on the prosurvival and proliferative ERK/MAPK and PI3K/Akt signalling pathways (Figure 1.9), making the quantification of the influence that individual signalling molecules have on NSC growth and survival troublesome. Understanding the role of these molecules and how they interact is vital to developing effective future therapeutic interventions.

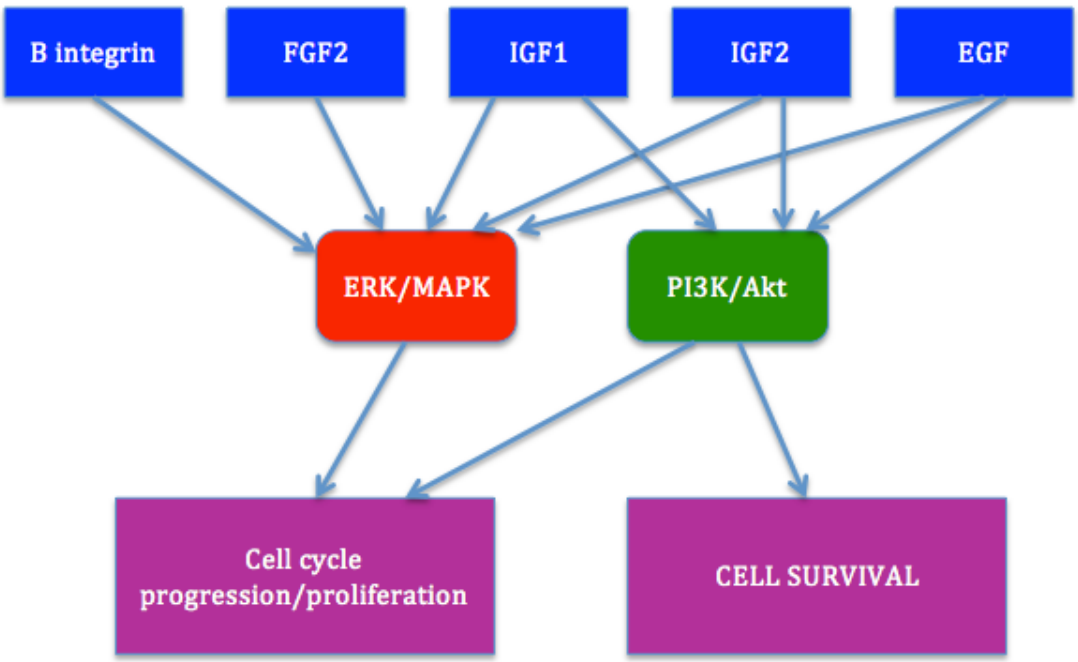


Figure 1.9. Growth factor adhesion molecules have convergent effects on cell proliferation and survival.

Growth factor adhesion molecules (blue) converge on the MAPK and PI3K pathways to drive cellular proliferation and survival.

1.6 Neurogenesis in the adult brain.

In the mammalian brain the hippocampus is one of the main sites of adult neurogenesis along with the olfactory bulb and subventricular zone (SVZ) of the lateral ventricle. The area of the hippocampus involved with neurogenesis is called the dentate gyrus, and radioactive carbon dating techniques of the human brain have shown that the human dentate gyrus has an annual turnover rate of 1.75%, meaning 700 neurons are generated and integrated into existing circuitry daily [78]. Postnatal neurogenesis is modulated by a number of factors including trauma, exposure to an enriched environment, exercise and dietary restriction[79], with an increase in the amount of BDNF stated as the mechanism leading to enhancements in neurogenesis for the last two factors[80].

NSCs reside in the SGZ of the dentate gyrus and give rise to new neurons and glial cells, making this layer a main area of neurogenesis throughout adult life [62]. Progression through cell proliferation, neuronal differentiation, migration, dendritic and axonal growth with synapse formation leads to progenitor cells becoming granule neurons and integrating with the existing circuitry, extending their axons along the mossy fibre tract to the CA3 region of the hippocampus [81]. Neurons are the building blocks of the nervous system and allow for transmission of information, signalling and connectivity within different regions of the central and peripheral nervous system. Glial cells such as astrocytes and oligodendrocytes have supporting roles such as provision of nutrients and synaptic support making them crucial for the nervous system to function correctly[80], therefore the dentate gyrus has an integral role in neurogenesis and glial genesis that is critical for maintaining the health of the brain throughout the life of the organism. There are two types of neural progenitors which are present within the SGZ, type 1 and type 2 hippocampal progenitors. Radial glia otherwise termed type 1 cells, have radial processes and express glial fibrillary acid protein (GFAP), nestin and Sex determining region Y-Box 2 (SOX2).

In the SGZ the neural progenitors are positioned close to the dense layer of granule cells where the immature and mature neurons are positioned. Immature neurons and glial cells migrate outwards through the granule layer and immature glial cells that are destined to become astrocytes reach maturation in the molecular layer [62]. The astrocytes are also prominent within the hippocampus and those present in the SGZ promote the differentiation of progenitor cells into immature neurons and aid in the integration of these neurons into the existing circuitry[80]. The complex circuitry of the hippocampus creates a complex microenvironment of both excitatory and inhibitory inputs and exposure to many different neurotransmitters and neural peptides which may influence the development of neural stem cells [80], including cAMP response element binding protein (CREB) and vascular endothelial growth factor (VEGF) pathways. The granule neurons within the dentate gyrus receive excitatory inputs from the entorhinal cortex, which led to an avenue of research

investigating if the glutamatergic NMDA receptor plays a role in adult neurogenesis. It has been shown that the NMDA receptor activity is inversely correlated with proliferation in the hippocampus, but it is unclear whether the neural progenitors express functional NMDA receptors[80]. Adult neurogenesis is also influenced by many extrinsic factors such as brain derived neurotrophic factor (BDNF) which enhances the survival of new neurons. A reduction in BDNF or its receptor leads to a reduction in the survival of immature neurons and therefore impaired neurogenesis[80].

1.7 The role of memory

Formation of memory isn't a single process but instead a complex network with many different pathways[82]. An individual experiences an event and properties of this event become consolidated leading to a memory trace. This trace can be reactivated after some time and become labile, meaning it can be changed and new aspects of the experience or event can be added to the trace [83]. These processes require a great deal of synaptic plasticity and input from several regions in the brain, one of the main regions being the hippocampus. The hippocampus encodes and consolidates temporal and spatial information about objects and events rather than the event itself meaning it is key for spatial navigation and episodic memory. In the encoding and formation of a memory the entorhinal cortex receives input from a region in the neocortex, with the region depending on the type of event that has occurred i.e. when a song is heard by the organism, the auditory cortex sends the information to the entorhinal cortex. The entorhinal cortex projects to the dentate gyrus which performs pattern separation on these inputs through sending orthogonal and sparse signals via the Mossy fibre tract to the CA3 pyramidal neurons [83]. The CA3 region acts as temporary storage for the information. Each pattern of neocortex activity creates a unique input pattern activating a unique population of neurons in the CA3 and when these neurons are activated at the same time the connections between them are strengthened [84]. If a feature of the original event is experienced again, i.e. the melody of the song, some of the neurons which were activated in the first experience will be stimulated again and because of the connections between these and the rest of the neurons activated in the original memory, an entire memory can be recalled from a single feature of the original. However, a very similar experience to the original runs the risk of activating the same population of neurons again causing confusion in the circuit. The denate gyrus is proposed to act as a pattern separator of these inputs [83]. One method suggested is that the dentate granule neurons change their spiking frequency and the more frequently a dentate granule neuron fires action potentials the more neurotransmitters are released and the more likely the downstream CA3 pyramidal neuron is activated [84]. By changing their rate of fire they can activate the correct unique populations of cells. As the entorhinal cortex also projects to the CA1, the CA1 also receives the cortical pattern, leading to long term potentiation between the CA3 and CA1 which are connected

through the Schaffer collaterals occurs. CA1 helps to consolidate the memory and sets up back projections to the original neocortical areas via the entorhinal cortex which facilitates recall of that memory[83].

Neurotransmitters are crucial for a normal functioning nervous system, they are stored in synaptic vesicles beneath the membrane of the axon terminal located in the presynaptic synapse. Neurotransmitters are released into and diffuse across the synaptic cleft where they bind to specific receptors in the membrane on the postsynaptic side of the synapse[85].

Dopamine is one of the key neurotransmitters involved in many pathways including reward and motor circuitry in the brain, with a loss of dopaminergic neurons in the substantia nigra implicated in the motor defects of Parkinson 's disease [86]. There is also a putative role for altered dopamine signalling in schizophrenia since many antipsychotics are antagonists or partial agonists for dopamine receptor D2, giving rise to the dopamine hypothesis of schizophrenia[87]. Aside from these functions, dopamine has also been implicated in mechanisms underpinning memory formation within the hippocampus[88].

The hippocampus has been shown to receive dopaminergic innervation from several regions [89] including the ventral tegmental area (VTA), locus coeruleus (LC) and the substantia nigra. It has been suggested that the hippocampus and the VTA form a functional loop which controls the entry of information into long term memory [88]. Many components of dopaminergic transmission machinery are present within the hippocampus including dopamine uptake sites, both D1 and D2 dopamine receptor families as well as catechol-O-methyltransferase (COMT) enzyme which metabolizes dopamine [88]. In this system dopamine release within the hippocampus, particularly in the CA1 region, enhances long term potentiation and allows entry of the new information into long term memory [88]. The subiculum, a part of the hippocampal formation which is important for regulating the interaction between the cortex and the hippocampus [65], sends excitatory glutamatergic projections to the nucleus accumbens. The nucleus accumbens then sends inhibitory input to the ventral pallidum which removes a tonic inhibitory influence from VTA dopaminergic neurons[88]. The upward phase of this loop is when the dopamine, which is released from the VTA dopaminergic neurons, increases LTP in the hippocampus. This process is selective for hippocampal regions, acting at CA1 but not in the DG [88]. While measuring hippocampal dopamine release in living rats using microdialysis, it was shown that placing a rat in a novel environment leads to an increase in dopamine and an enhancement of LTP [90], suggesting that novel stimuli can lead to dopamine dependent increase in LTP, contributing to the incorporation of this novel stimuli into long term memory. With an enhancement of LTP it is expected that increased dopamine leads to the enhancement of learning with studies showing systemic dopamine agonists improve spatial

memory in aged rats [91] and that depleting dopamine within the hippocampus impairs spatial navigation in the Morris water maze test[92].

1.8 The role of Glia

The brain contains supportive cells termed glia, which include astrocytes, microglia, oligodendrocytes and radial glia. Astrocytes have a wide range of functions in the central nervous system including nutrient provision, energy metabolism, neurotransmission and blood flow regulation [93] but also have critical roles in neurodevelopment. At the beginning of neural development, neural precursor cells first generate neurons and then astrocytes, but by the early postnatal stages the generation and expansion of astrocytes is nearly completed. For the development of neurons from neural progenitor cells (NPCs), WNT signalling is essential to activate *Neurogenin 1* and *2* which lead differentiation of NPCs into neurons[94]. To allow for glial genesis, epigenetic changes are made to histone H3 acetylation and trimethylation at the *Neurogenin* promoters in NPCs which changes the chromatin conformation and leads to a polycomb group complex dependent suppression of *Neurogenin*[94]. This allows the development of astrocytes when WNT signalling is still active. Extrinsic signals also play a role in switching between neurogenesis and glial genesis; the JAK-STAT pathway is well categorised in its role of activating astrocyte cell fate in NPCs and can be switched on by a number of cytokines including cardiotrophin-1 (CT-1) and ciliary neurotrophic factor (CNTF)[95]. Notch signalling is also required to initiate glial genesis, Notch ligands JAG1 and DLL1 activate the Notch signalling in the NPCs[95]. Notch causes DNMT1 maintenance methyltransferase to leave the *Gfap* promoter and leads to increased expression of NFIA which inhibits neurogenesis by binding the *Gfap* promoter preventing it from DNMT1 induced methylation[95].

From the cell body of an astrocyte, primary branches extend outwards in multiple directions and then smaller and smaller projections radiate out from these branches. This complex morphology means in some neural regions a single rodent astrocyte occupies a spatial domain between 20,000-80,000μm^3, can associate with 300-600 neuronal dendrites and make contact with ~100,000 synapses[95]. The amount of contact a single astrocyte can make with multiple neurons shows there is communication between the two cell populations. During the amplification of astrocytes in development the recently generated astrocytes at P7 show significant overlap of processes between different astrocytes but by P21 there is a clear domain for each astrocyte suggesting mechanisms similar to pruning seen in synapse formation are active during this stage[96]. This configuration allows the astrocytes to form a supportive network across the brain ensuring all regions are covered by their processes[96] .

More than just passive support cells, astrocytes have been shown to be highly engaged in synaptogenesis and maintenance of synaptic contacts. For example, retinal ganglion cells cultured in the absence of glia form few synapses but their presence or use of glia conditioned media causes the retinal ganglion cells to create 10 times the number of excitatory synapses[97]. Evidence for the interaction between neuronal populations and astrocytes is apparent in live preparations where changes in astrocytic Ca^{2+} signalling is observed near synapses and cell bodies of neurons as well as neuronal stimulation causing increased glial ensheathment of synapses[98]. Astrocytes also possess several receptors for neurotransmitters and transporters and it has been suggested they can sense neuronal activity and morphology. These attributes allow astrocytes to interpret and coordinate signals from all the synapses within its defined area[95] and make this glial population an important population to study within neurodevelopmental models.

Microglial development is distinct from neurons and astrocytes due to their origin from mesodermal progenitors in the yolk sac, which is an extra embryonic structure, rather than originating from the neuroectoderm. Microglia comprise 10% of the glial population within the adult brain and are the tissue-resident macrophages of the CNS, actively searching out areas of damage as well as protecting neuronal tissue [99]. Microglia infiltrate the CNS early in development and are present within the developing brain by E8, proliferating until late in gestation. 95% of microglia are born by 2 weeks after birth[99] and once they have entered the CNS it is thought they are cut off from their yolk sac progenitors and form a self-renewing colony [100]. Due to the fact that systemic monocytes/macrophages are prevented from entering the brain under healthy conditions due the the blood-brain barrier, it is the microglia that takes on their role and gives the brain the ability to mount an innate immune response. In a healthy brain the microglia are in a resting quiescent state, but in response to injury or detection of pathogenic events in the brain they can cause inflammatory responses in the brain changing to an activated “amoeboid” state[100]. Microglia have the ability to migrate to sites of damage and carry out phagocytosis and express macrophage markers such as CD11b, CD11c and CD68. As well as functioning as immune cells, microglia have also been shown to influence synaptogenesis as well as directing new blood vessels during development[100]. Microglia contact neuronal synapses through their processes, which are motile and actively surveil the environment. These transient synaptic contacts have been shown to increase in duration after cerebral ischemia[101] and seem to function to inform the microglial cell of neuronal condition or damage. Microglia have also been shown to participate in apoptosis during neurodevelopment by actively controlling neuronal death through secretion of apoptotic factors such as Tumour Necrosis Factor α (TNFα) and Fas Ligand (FasL)[102]. Due to their many functions microglia are an important cell population in neurogenesis and the maintenance of health in the adult brain[100].

1.9 The importance of animal models in pregnancy and development research

Mouse models of pregnancy are extremely important to allow the role of individual genes and environmental factors to be studied in the pathogenesis of pregnancy complications [103]. Mouse models have broadened our understanding of foetal development in pregnancy complications; the mouse model allows the *in utero* environment to be easily altered by maternal diet, allowing insights into the role of nutrient balance and homeostasis in the aetiology of pregnancy [104]. Mice are a particularly useful model in this PhD project as they have a similar implantation process to humans and 80% of the genes known to cause placental phenotypes are conserved from mouse to human [105 106]. They also have a similar type of placentation to humans, being haemochorial; albeit monochorial for human and trichorial for mouse[38]. This allows the mouse genome to be manipulated, and candidate genes for normal and abnormal pregnancy outcomes such as Foetal growth restriction (FGR), congenital malformations and pre-eclampsia to be explored as biomarkers[88 91]. For example, deletion of the placental-specific transcript (P0) of the insulin-like growth factor-2 (Igf2) gene in mice results in a reduction of placental growth by embryonic day 12 of pregnancy (E12), with FGR apparent from E16 onwards. Deletion of P0 Igf2 caused an adaptive up regulation of system A amino acid transporter activity to maintain foetal growth until E16, but as this was not sustained and FGR ensued thereafter. This suggests that placental system A amino acid transporter activity can be modulated and is an important determinant of foetal growth.

Animal models of protein restriction produce effects similar to human health problems found in underdeveloped countries [107 108]. Consequently, the Fleming lab has developed mouse models of maternal low protein diet (LPD) during either the entire gestation period or just during the preimplantation period (Emb-LPD), a developmental window the Fleming group has shown to be particularly sensitive for long-term offspring health [109 110]. Our LPD model known as the Southampton LPD challenge demonstrated adverse cardiovascular, metabolic and behavioural outcomes in adult offspring [11 12 109]. The Southampton LPD challenge is recognised as a suitable model for developmental programming of disease and has been used extensively for almost 20 years across species and internationally [3].

Early studies using conditions of malnutrition over extended pre- and post-natal periods illustrate adverse effects on overall brain growth and cell cycle rate [111-114], but the underlying causes have received less attention. A large range of literature suggests a change in the maternal nutrition [107], either global under-nutrition or a lack of specific nutrients, during the early period of brain development is likely to impact foetal brain development and lead to permanent deficit in learning and behaviour[115] throughout adult life.

Human studies have shown impaired foetal and postnatal brain growth, often encountered in preterm infants following poor maternal diet, are strong risk factors for the development of cognitive difficulties at school age [116] [117]. Accordingly, animal models of under-nutrition or deficiency in specific nutrients were used to demonstrate the profound impact of maternal nutrition during pre-conception, gestation and lactation on the development of the brain, most notably the hippocampus, resulting in impaired cognitive performance[115]. As the brain is a highly plastic organ, it is particularly sensitive to major metabolic sensors i.e insulin.

1.10 Maternal nutrition effect on Central Nervous System development and developing foetus.

Nutrients and growth factors regulate brain development during fetal and early postnatal life. The developing brain is particularly vulnerable to nutritional insults because of the rapid trajectory of several neurologic processes, including synapse formation and myelination. All nutrients are important for neuronal and glial cell growth and development. The effect of nutrient deficiency or supplementation on the developing brain is a function of the brain's requirement for a nutrient in specific metabolic pathways and structural components. The effects are regionally distributed within the brain on the basis of which areas are rapidly developing at any given time [24]. A nutrient that promotes normal brain development at one time may be toxic at another point in development. Similarly, a nutrient that promotes normal brain development at one concentration may be toxic at another. Several nutrients, including iron, are regulated within a relatively narrow range, where either an excess or a deficiency induces abnormal brain development. Others have wider ranges of tolerance.

Nutrients can affect not only neuroanatomy, but also neurochemistry and neurophysiology as summarised in table 1.1. Neurochemical alterations include changes in neurotransmitter synthesis, receptor synthesis, and neurotransmitter reuptake mechanisms. Neurophysiologic changes reflect changes in metabolism and signal propagation. Long-term changes in form and function can occur if the altered nutrient state changes the trajectory of brain development in a substantive anatomical or neurochemical way, beyond the period where repair can occur.

Nutrient	Brain requirement for the nutrient	Predominant brain circuitry or process affected by deficiency
Protein-energy	Cell proliferation, cell differentiation	Global
	Synaptogenesis	Cortex
	Growth factor synthesis	Hippocampus
Folate	Neurulation	Neural tube defects
Iron	Myelin	White matter
	Monoamine synthesis	Striatal-frontal
	Neuronal and glial energy metabolism	Hippocampal-frontal
Zinc	DNA synthesis	Autonomic nervous system
	Neurotransmitter release	Hippocampus, cerebellum
Copper	Neurotransmitter synthesis, neuronal and glial energy metabolism, antioxidant activity	Cerebellum
Long-chain poly unsaturated fatty acids	Synaptogenesis	Eye
	Myelin	Cortex
Choline	Neurotransmitter synthesis	Global
	DNA methylation	Hippocampus
	Myelin synthesis	White matter

Table 1.1 Summary of embryonic perturbations on the fetal brain

The mechanisms underpinning how maternal nutritional may affect foetal growth and development are now being studied [118]. One potential mechanism is epigenetic processes, which mediate changes in gene expression by regulating DNA methylation. Changes in DNA methylation of cytosine residues and histone modification are passed to offspring [22] whilst the DNA sequence remains unchanged. Many human and animal studies support the theory that epigenetic modification plays a role in development of pathological phenotype in the DOHaD model [22 118].

Malnutrition during gestation in rats decreases DNA methylation and increases expression of the peroxisome proliferator-activated receptor (PPAR) alpha gene in the liver, which may increase risks of metabolic disorders [119] This supports findings in humans showing that altered DNA methylation status was identified in the blood of individuals who suffered malnutrition during the Dutch hunger winter. The DNA methylation of key loci involved in growth and metabolism such as *INSIGF* and *IGF2* was lower among individuals who were periconceptionally exposed to the famine compared with their unexposed same-sex siblings, whereas methylation of *IL10, LEP, ABCA1, GNASAS* and *MEG3* was higher [120].

Antonow-Schlorke's team has shown in the baboon that a moderate maternal under-nutrition in early pregnancy led to major disturbances in the architecture of the foetal SVZ, before they migrate outwards to their final locations within the brain[121]. The SVZ provides interneurones for the cortex and the primates who received poor in utero nutrition early in development went on to show

delayed maturation of the brain cortical neuronal network [122]. Potential mechanisms for this effect included neurotrophic factor suppression, cell proliferation and cell death imbalance, impaired glial maturation and neuronal process formation, downregulation of gene ontological pathways, and up-regulation of transcription of cerebral catabolism. Additional mechanisms mediating the effects of maternal undernutrition on foetal brain development may be secondary to detrimental effects of excess glucocorticoid exposure on the developing central nervous system[123 124]. A similar level of maternal under nutrition led to significant elevations in circulating glucocorticoids in the baboon foetus [123].

1.11 The role of protein in foetal development and adult neurogenesis.

Many studies have been carried out to investigate maternal protein restriction and its effects on offspring health in animal models as described earlier[107]. Animal models of protein under nutrition are extremely useful as they provide effects similar to human health problems found in developing countries[107 108]. As stated earlier studies carried out at Southampton demonstrate adverse cardiovascular, metabolic and behavioural outcomes in adult offspring who have had maternal restriction of protein [11 12 109]. The LPD challenge as discussed earlier is recognised as a suitable model for developmental programming of disease [3 125].Offspring from dams fed a LPD during pregnancy were shown to have increased systolic blood pressure (SBP), however, maternal LPD during periconceptional period[109 110] showed offspring had reduced kidney size and increased adipose tissue [68] demonstrating that events pre-conception can contribute to developmental programming. This rise in hypertension is also seen in offspring from dams fed the LPD for the first 3.5 days of pregnancy (Emb-LPD)[12]. Both maternal LPD and Emb-LPD also alters expression of metabolic hormones[126]. However, the effects of maternal preimplantation protein restriction on adult disease phenotype appear to be gender-dependent, with female offspring displaying increased growth rate and altered anxiety-related behaviour[12].

LPD throughout pregnancy impacts neural stem cells by inhibiting DNA methylation, increasing the expression of astrocytic GFAP and decreasing the expression of the neuron-specific doublecortin gene, suggesting an impact on cell differentiation [127]. Protein restriction during gestation has been linked to changes in astrocytes and mature neurons in postnatal brain [107 128]. However, studies to date have not examined the primary upstream events where the effects of protein restriction on brain development may originate. Neural development relies on the tight regulation of neural stem cell proliferation and differentiation to generate the appropriate number of progeny (neurons, glial cells) to correctly populate the nervous system. Some animal studies to date show maternal malnutrition during pregnancy and lactation may affect diverse aspects of brain development associated with impaired physical and coordinated movement, hyperactivity, altered social activity

and motivation, as well as reduced mental and cognitive function, potentially in a gender-specific manner [107] [129] [130]. These consequences may derive from detriments on the maturation and functioning of specific brain tissues such as the hippocampus, cortex and hypothalamus leading to altered neurotransmitter and hormone release [131-133]. Further study has revealed that maternal protein restriction in mice may also affect the proliferation and differentiation capacities of NSCs [127].

It has also been shown that protein restriction prior to mating can also have an effect, as offspring from female rats fed a 8% protein diet one month before mating and throughout conception had significantly reduced DNA in their brains which was taken to indicate a reduction in the number of cells present in these offspring [134]. Lipid content and composition of free fatty acids in the brain also changes after exposure to a maternal low protein diet (10% casein), resulting in a reduction in docosahexaenoic acid levels (DHA) [135]. DHA is most abundant omega-3 fatty acid in the brain, comprising 50% of the weight of the plasma membrane for neurons, and is therefore important in neurodevelopment and brain structure. Changing the composition of plasma membranes can alter cell signalling as permeability and membrane fluidity is changed [134] [135]. As well as global changes, changes in behaviour, neurotransmitter levels and neurodevelopmental processes such as neurogenesis have also been observed in maternal protein restriction models, indicating that suboptimal environments *in utero* can upset the delicate processes in neurological function.

Many studies have investigated the effect of dietary restriction on neurogenesis. It has been shown that restriction of all macronutrient groups leads to alterations in neurogenesis. Dietary restriction in adult animals has been shown to stimulate neurogenesis, which is thought to be in part stimulated by increased expression of BDNF[136], while also making the neurons more resistant to dysfunction and damage. There is also some epidemiological evidence through longitudinal studies demonstrating that a low-calorie diet throughout a person's lifetime reduces the chance of Parkinson's disease and Alzheimer's disease [137] and rats fed a restricted diet perform better in learning and memory tasks [138]. Maternal dietary restriction has also been shown to impact the offspring neurogenic potential. In one study pregnant rats had their food intake reduced by 50% during gestation and lactation and their pups were then injected with BrdU, a marker of proliferating cells. A decrease in BrdU labelled cells was seen in the dentate gyrus of these pups at 90, 97 and 112 days of age. Out of the newborn cells within the hippocampus, a higher proportion of cells exhibiting a neuronal phenotype was also seen compared to the controls shown by colabeling BrdU with NeuN, a neuronal specific marker [81]. This data suggests perinatal nutrient restriction causes a deficit in the number of new neurons in the adult offspring but does not impact the neuronal differentiation process for granule neurons [81].

Another study using a rat model investigated the effect of maternal malnutrition on the development of dentate gyrus in the foetal offspring, with the diet consisting of protein restriction through a 6% casein diet implemented 5 weeks before conception and throughout gestation, compared to the control 25% casein diet [139]. No change in the numbers of pyramidal neurons at E12, E16 or E20 was found in the protein restricted offspring but the number granule neurons was significantly decreased in these rats at E20[139]. The highest proportion of neurogenesis occurs postnatally with only 15% of the adult population generated before birth [139] so the impact of this maternal malnutrition diet was then investigated in the early postnatal period. On postnatal day 8 (P8) a significant decrease in the neurogenesis of granule cells was seen in the protein restricted rats while an increase of tritiated thymidine labelled granule cells at P30 was also apparent. This suggests a compensatory mechanism whereby the initially reduced pool of neurons is increased by P30 and shows maternal prenatal protein restriction alters the pattern of neurogenesis in the early life of the offspring [139] .

Other studies have limited the protein restriction to only during gestation and weaning to investigate the *in-utero* programming effects [79]. Rats whose mothers were fed a diet of 8% protein during gestation showed a reduction in cell proliferation, number of hippocampal progenitors in adult offspring and number of proliferating cells in the lateral wall of lateral ventricle[79]. The discrepancy between this finding and the aforementioned study where P30 rats showed an increase in granule neurons maybe due to the age of the animals as neurogenesis slows down with age, or due to the differences in diet and exposure time, i.e. the previous study started the diet 5 weeks before conception. Despite the differences it is clear from studies like these that maternal malnutrition and particularly protein restriction impacts the functionality and supply of NSC in the offspring, leading to changes in neurogenesis which persist into adulthood. However, the consequences of maternal protein restriction specifically during early embryonic development on later brain development are unknown.

1.12 The role of maternal protein restriction on offspring behaviour

Malnutrition during gestation and early postnatal life has been linked to many neurological conditions and altered behavioural phenotypes, even deficiencies in micronutrients such as vitamin D have been linked to conditions such as autism, schizophrenia and ADHD [140 141]. As discussed earlier in the Dutch Hunger Winter during world war two, women who conceived during the famine had children with a much higher incidence of schizophrenia and depression than those who conceived before the famine [24]. Delivering smaller babies is a common and well recognised consequence of maternal under-nutrition [142] and reduced foetal growth has been linked to a higher risk of behavioural conditions such as ADHD.

There have been many diet models specifically designed to be restrict protein in an isocaloric manner that have shown behavioural consequences for offspring. In a study of gestational protein restriction, where mothers were fed a diet of 8% protein, the offspring displayed depressive behaviour at both 21 days of age and in adulthood [79]. This was measured with the tail suspension test, where the time the animal remains immobile is taken as an indication of depression. Here male protein-restricted offspring remained immobile significantly longer than control offspring [79]. In the same study, it was shown that the male protein restricted offspring spent less time exploring a novel object in the long-term novel object test which measures the working memory capability of the animals[79]. This suggests that gestational protein restriction impaired the ability of the offspring to recognise a new object and induced a working memory deficit.

Work has also been done to look at both prenatal and early postnatal protein restriction with some dams receiving a 10% casein isocaloric diet during gestation and lactation, some just in pregnancy or just in lactation. Using the elevated plus maze, where the time an animal spends in the open arms is taken as a measure of anxiety, with more time indicating less anxiety, offspring from all three of the dietary regimes showed significantly increased time in the open arms compared to the controls [136]. This suggests altered risk-taking behaviour with a more exploratory phenotype, decreased avoidance and reduced anxiety. However, the same paper reported the offspring with protein-restriction during pregnancy and lactations spent less time in the centre zone of an open field arena which suggests more anxiety compared to other groups [136]. This casts doubt in regard to whether the anxiety of the offspring has been changed but suggests the offspring whose mothers had the low protein exclusively in either pregnancy or lactation show behavioural changes. In this case it may be due to developmental programming preparing the offspring for a particular nutritional environment e.g. low protein and then exposure to a different environment such as suitable protein leading to maladaptation in brain development or function causing altered behavioural phenotypes.

1.13 The role of maternal protein restriction on neurotransmitters pathways.

Maternal under-nutrition models have shown fundamental changes in the levels of neurotransmitters and their related circuitry. A reduction in the level of serotonin (5-HT) release suggesting a reduction in the density of serotonergic neurons has been shown in prenatally undernourished rats [143]. Tryptophan is an essential amino acid which acts as a precursor for neuroactive compounds in the serotonergic pathway, as protein restriction limits the exposure of the developing foetus to this amino acid impaired tryptophan metabolism is suggested to

contribute to changes seen in neurodevelopment. As 5-HT is also important in memory formation and behaviour[144] it may contribute to the changes seen protein restriction models such as the previously discussed study where in utero protein restricted animals displayed impaired memory and depression[79].

As well as 5-HT, in mouse models dopamine circuitry has also been shown to change for offspring whose mothers were given a protein restriction diet during pregnancy. Dams fed an isocaloric diet of 8.5% protein throughout pregnancy and lactation had offspring with a substantial increase in the expression of dopamine related genes in mesocorticolimbic and hypothalamic pathways [145]. Quantitative real-time PCR showed that tyrosine hydroxylase had a 6-8-fold increase in expression within the VTA, prefrontal cortex, nucleus accumbens and the hypothalamus of protein restricted offspring [145]. DAT, a dopamine re-uptake transporter had a 3-4-fold increase within the VTA and levels in the nucleus accumbens was 8 times greater than the offspring from dams fed a normal protein diet[145]. The VTA of protein-restricted animals showed an increase in the number of tyrosine hydroxylase reactive cells. A change was also seen in the epigenetic profile of the protein restricted animals; hypomethylation was seen on the Cdkn1c gene promoter leading to over- expression of this molecule which is critical for the proper development and differentiation of dopaminergic neurons[145]. Changes in expression of this gene have been suggested to underpin the altered dopamine circuitry for in utero growth restriction models and explain some of the altered behaviours such as hyperactivity[145].

1.14 Summary

In summary, we have highlighted Maternal malnutrition during pregnancy is detrimental to foetal development and increases the risk of many chronic diseases in later life i.e. neurological consequences such as increased risk of schizophrenia. Studies have shown maternal protein malnutrition during pregnancy and lactation compromises brain development in late gestation and after birth, affecting structural, biochemical and pathway dynamics with lasting consequences for motor and cognitive function. However, this thesis will compare the effects of maternal low protein diet during conception and sustained low protein diet on mouse brain development and consequences in adult offspring. This study shows maternal dietary quality from conception to be a critical factor in brain developmental capacity with enduring consequences on brain organization and adult behavior.

1.15 Aims and Hypothesis

We propose that maternal LPD during gestation or just the periconceptional period (Emb-LPD) affects the development of the brain by disturbing the regulation of neural stem cells proliferation and differentiation across life. This may contribute to the behavioural phenotype in adult offspring [12 109] and represent the origin of adverse postnatal neurobehavioral traits.

The project aim is therefore to evaluate the effects of protein restriction on 'upstream' events in the development windows of the brain that we already know lead to adult offspring behavioural dysfunction[71 68].

The project objectives are to assess the effect of the maternal low protein diet (during gestation and just the preimplantation period and to characterise as follows:

Aim 1: To characterise the effect of Emb-LPD and sustained LPD maternal diets on the development of foetal brains, focusing on the NSC and neuronal cell populations.

Aim 2: To identify any long-term neurodevelopmental consequences in the adult offspring of mothers given Emb-LPD and sustained LPD diets by assaying a range of behaviours over life.

Aim 3: To assess the effect of Emb-LPD and sustained LPD maternal diets on the morphology and gene expression of adult offspring brains

Chapter 2 The effect of maternal low protein diet on the foetal offspring brain

2.1 Introduction

The concept that in utero environment may influence postnatal neurological health is now a well recognised following the original epidemiological studies on diverse human populations Such programming consequences also included cognitive decline and other neurodevelopmental disorders[4] [5]. The key dataset that supports this is particularly the Dutch Hunger Winter, demonstrating cardiometabolic and neurological dysfunction associate with in utero maternal nutrient depravation during pregnancy[26]. Both human studies and animal models further demonstrate the particular vulnerability of the periconceptional period in DOHaD-related programming. For example, people who were conceived during the Dutch famine (rather than experienced it during later gestation) had increased risk of schizophrenia and depression together with poorer cognitive capacity in later life as well as cardiometabolic consequences[26].

Watkin's had previously shown in a mouse model of maternal low protein diet fed exclusively during preimplantation development with control diet fed thereafter and postnatally (Emb-LPD), was sufficient to induce cardiometabolic and behavioral abnormalities in adult offspring[12]. Early embryos are incredibly vulnerable, the quality of maternal diet plays a key roles in influencing the developmental plasticity, which ultimately can affect fetal growth and metabolism with prevailing maternal conditions, However, if conditions change during the early embryo period, this may result in maladaptive responses and ultimately may have consequences for disease risk in adulthood[146].

A number of animal studies have currently shown maternal malnutrition during pregnancy may affect diverse aspects of brain development associated with impaired coordination and movement, hyperactivity, altered social activity and motivation, as well as reduced mental and cognitive function, potentially in a gender-specific manner[107] [129] [130]. These phenotypes may have derived as a consequence of the diet having detrimental effects on the maturation and functioning of brain tissues such as the hippocampus, cortex and hypothalamus during foetal development therefore affecting neurotransmitter and hormonal release[131-133]. Maternal protein restriction may also affect the proliferation and differentiation capacities of neural stem cells (NSCs) [127].

2.2 Aims

The novelty of this study is to investigate maternal protein restriction just during the early embryonic period unlike previous studies looking at sustained protein restriction throughout pregnancy and/or lactation.

Therefore, the aim of this study was to examine the effects of maternal Emb-LPD and sustained LPD (fig2.1) on mouse brain development and investigage if NSC proliferation and maintenance are adversely affected by both treatments leading to altered rates of neuronal differentiation.

2.3 Methods

2.3.1 Experimental Animals

Mice were kept in plastic cages under a 12-hour light-dark cycle at 19-24°C. Virgin MF1 female mice were kept in in groups of 2-10 prior to mating and were placed individually with male MF1 mice for mating. Following mating, pregnant female MF1 mice (aged 6.5–8.5 weeks) were identified by the presence of a vaginal plug. Pregnant mice were previously maintained on standard Chow diet and were randomly allocated on the morning of the vaginal plug to one of three isocaloric dietary treatment groups. Maternal dietary treatments were conducted as shown in the regime described in Figure 2.1 [12]. Diets were produced by Special Diet Services, UK and their compositions are detailed in Table 2.1. Mice were fed ad libitum with free access to water.

Male mice were obtained from Charles River (Margate, UK) while female mice originally obtained from Charles River were bred by the Biomedical Research Facility, SGH, UK. All mouse procedures were conducted using protocols approved by, and in accordance with, the UK Home Office Animal (Scientific Procedures) Act 1986, local ethical approval from the University of Southampton and were carried out under the authority of UK Home Office Project Licenses PPL30/2467 and PPL30/3001.

Figure 2.1. Schedule of dietary changes for NPD, LPD and Emb-LPD models.

All pregnant female mice previously maintained on standard laboratory chow were allocated a diet after vaginal plug to either low protein diet (LPD; 9% casein) or normal protein diet (NPD; 18% casein) [11], throughout gestation only (termed LPD and NPD respectively) or exclusively during preimplantation development (from vaginal plug to day 3.5 post gestation termed E3.5) before being switched to NPD for the remainder of gestation (termed Emb-LPD).

Component	LPD	NPD
Casein	90	180
Corn Starch	485	425
Sucrose	243	213
Corn oil	100	100
Fibre	50	50
AIN-76-mineral mix	20	20
AIN-76-vitamin mix	5	5
DL-methionine	5	5
Choline Chloride	2	2

Table 2.1. Composition of normal and low protein diets.

Components of low protein diet (LPD; 9% protein) and normal protein diet (NPD; 18% protein) are expressed as grams per kilogram.

2.3.2 Tissue collection of foetal brains.

Pregnant dams were sacrificed by cervical dislocation and pups harvested at days 12.5, 14.5 or 17.5 post gestation (E12.5, E14.5 and E17.5 respectively). The uterine horn was surgically extracted and placed in ice cold PBS (Phosphate-Buffered Saline) for 10 minutes, ensuring metabolic activity within the foetuses was completely inhibited prior to foetal dissection. Foetuses were then randomly selected and removed from the uterine horn for all techniques. The foetal cortex and ganglionic eminences (GE) were dissected using an optical microscope (Leica EZ4) as shown in Figure 2.2. The GE and cortex were taken from the same mouse either for neurosphere culture, flow cytometry and immunohistochemistry. Mice from each litter were randomly allocated to one of the techniques.

The E12.5 time point was chosen to study very early neurodevelopment. E14.5 time point was picked as it is the peak of neural stem cell proliferation and E17.5 was picked as it is the end of neurogenesis and ongoing gliogenesis [147].

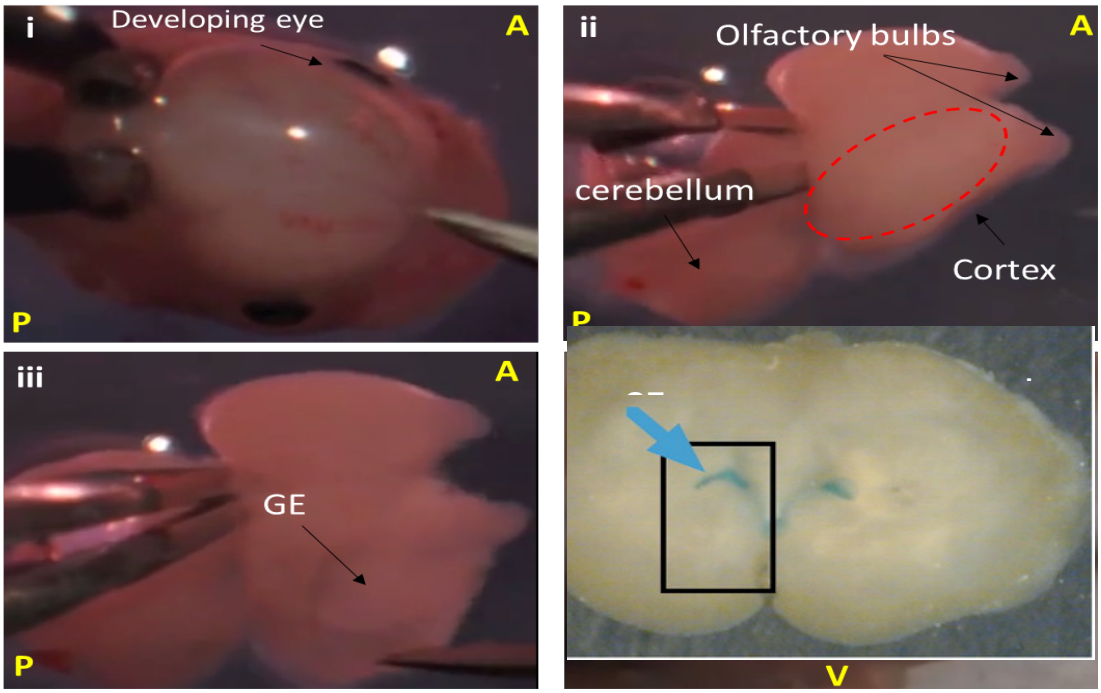


Figure 2.2 Dissection protocol for the isolation of foetal cortex and ganglion eminences (GE).

(i) The first step in the dissection protocol involves the isolation of the whole foetal brain. An incision is made along the midline of the developing cranium, exposing the foetal brain and allowing its subsequent removal. (ii) Once removed the cortex is dissected from both hemispheres (circled in red) and stored in 0.5ml neurosphere media. (iii) Removal of the cortex exposes the ventrally located GE. (iv) The GE are visible due to their slightly darker more off-white colour. Both the medial and lateral GE (within black box) are then isolated

from both hemispheres, pooled together and stored within 0.5ml PBS for subsequent culture. i and ii-iii indicate a dorsal view of a decapitated mouse foetal head and foetal brain respectively. The images are orientated so the anterior region of both the head and brain are facing right. iv indicates a cranial view of the GE after the removal of the cortex. The image is orientated so that the dorsal region of the brains is located at the top of the image. A – anterior, P – posterior, D - dorsal and V - ventral.

2.3.3 Neurosphere culture.

Following harvesting, the foetal cortex and GE were mechanically dissociated to single cell suspensions according to established protocols for culture of foetal NSCs [148 149]. Live cell number for each sample was calculated using a haemocytometer and dead cells were excluded using 0.4% trypan blue solution (Sigma-Aldrich, UK). 5000 live cells were then plated in 0.5ml neurosphere growth medium for an initial cell density of 10 cells/µL. Neurosphere growth medium consisted of Neurobasal-A medium (Invitrogen) supplemented with 1x B27, L-glutamine 2 mM, penicillin/streptomycin 100x preparation, and growth factors (human FGFb 10 ng/mL, human EGF 20 ng/mL and heparin 2 µg/mL). Cortex and GE from three foetuses per dam, were plated within the same 24 well plate (Costar, Sigma-Aldrich, UK). The cells were stored in a 37˚C, 5% CO_2 incubator (NU-5510E, Nuaire, Wales) for 7 days. Sphere counts were performed at day 7. Neurospheres >100µm in diameter were counted and classed within three groups; 100 - 199µm, 200 - 399µm and >400µm. Sphere counts were performed in duplicate and averaged.

2.3.4 Flow cytometry.

2.3.4.1 Sample preparation

Flow cytometry reagents were obtained from Sigma-Aldrich, UK, unless otherwise stated. FACS buffer was prepared as 3% heat inactivated foetal calf serum, 0.09% NaN_3, 0.005M EDTA in 1xPBS pH 7.4, followed by filter sterilisation. Cortex, GE or neurospheres from foetuses harvested at E12.5, E14.5 and E17.5 were analysed by flow cytometry. Cortex and GE dissected from each foetus or pools of neurospheres were mechanically dissociated and washed 3 times with FACS buffer cooled on ice. Cells were incubated in Live/Dead Violet Viability stain (Invitrogen, Paisley, United Kingdom) for 30 minutes at 4°C. Samples were treated with BD Cytofix/Cytoperm kit (BD Biosciences, UK) according to manufacturer's instructions and were then washed 3 times in FACS buffer. Cells were then incubated with mouse anti-nestin IgG conjugated to phycoerythin (PE) (IC2736P, R&D systems) and mouse anti-Beta-III-tubulin IgG conjugated to allophycocyanin (APC) (Biolegend) or isotype

control antibodies for 30 minutes at 4°C. Cells were then washed 3 times in FACS buffer on ice and re-suspended in 250 µl FACS buffer for analysis.

2.3.4.2 Sample acquisition and analysis

Samples (n=3 foetuses per litter) were analysed by a FACSAria II cell sorter (BD Biosciences, UK) with the following laser/detector configuration: 405nm laser - 450/40 for Live/Dead Violet Viability Stain; 488nm laser - 488/10 for SSC and 576/26 for PE; 633nm laser - 660/20 for APC. Sequential firing of each lasers ensured only low levels of compensation between lasers was required. Samples were acquired at a constant flow rate and 10,000 cell events per tube were recorded. The cell population was selected by on a FSC vs SSC plot to exclude debris and aggregated cells. Dead/dying cells were excluded based on uptake of fixable live/dead dye. Data was recorded using FACSDiva Software (version 5.0.3; BD Biosciences, Oxford, United Kingdom) and the percentage of positive cells for each marker (see Figure 2.3) were analysed in FlowJo v10 (OR, USA).

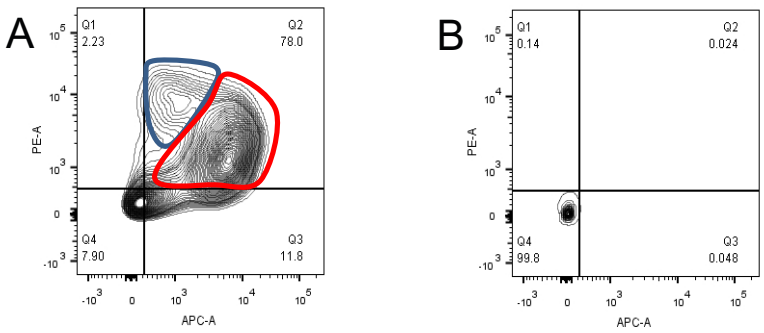


Figure 2.3 FACS plot gating method.

Example FACS plot showing cells from ganglionic eminences (GE) stained with (A) antibodies against Nestin (PE) and Beta-III-tubulin (APC) and (B) isotype control antibodies. Cells were defined and gated as: Nestin PE-A only positive cells (Q1), double positive cells (Q2), Beta-III-tubulin APC-A only positive cells (Q3) and negative cells (Q4). Double positive cells (Q2) were further separated into Nestin+ Beta-III-tubulin dim (Q2N outlined in blue), and Nestin dim Beta-III-tubulin+ (Q2B outlined in red).

2.3.5 Immunohistochemistry

2.3.5.1 Sectioning

14µm thick brain sections were cut from brains embedded in OCT using a cryostat at -20°C (chamber temperature) and -16°C (object temperature). Sections were transferred onto SuperFrost slides (ThermoFisher Scientific, UK) which were air dried for 1 hr and then stored at -20°C until use.

2.3.5.2 Immunofluorescence staining of adult and foetal brains

Slides were thawed for 45mins at 37°C, adult brain sections were fixed in 4% PFA at room temperature for 20 mins, while foetal brain sections skipped this step. Citrate buffer was used for heat shock antigen retrieval using an 800w microwave at half power for 25mins (ThermoFisher Scientific, UK). Slides were then placed under cold running water for 3 minutes, tapped off onto a piece of tissue and washed in PBS 3 x 2 minutes. Slides were then washed in 1X PBS-T for 15 mins and sections were drawn around with a wax pen to allow staining of different sections with different primary antibodies. Sections were blocked for 30mins using PBS containing 5% BSA and 10% DS (Donkey Serum; Sigma-Aldrich, UK) and were then washed 3x5mins in PBS-T. Sections were stained with primary antibodies in PBS-T at 4°C for 16-24 hours (Table 2.5). Slides were washed 3x5 mins in PBS-T and then stained with secondary antibodies in PBS-T for 60mins (Table 2.6). Slides were submerged in 1µg/ml DAPI solution for 10mins and were then washed for 3x10 mins in PBS-T and coverslipped with Mowiol mounting medium (Biomedical imaging unit, SGH).

Target	Antibody type	Distributor	Product ID (Clone)	Concentration (Dilution)
Sex Determining Region Y-Box 2 (SOX2)	Rabbit anti-mouse mAb	Santa Cruz	Sc-17320	(1:50)
DAPI		Calbiochem	CAS 28718-90-3	(1:2000)
Nestin	Rat anti-Rabbit mAb	Millipore, UK*	MAB353	(1:100)
Beta-III-tubulin	Rabbit anti-mouse pAb	Biolegend, USA	801202	(1:500)
Cleaved caspase3'	Rat anti-Rabbit pAb	Cell signalling, UK	Asp 175	(1:100)
Ki67	Donkey anti-rabbit pAb	Novusbio, UK	F2266	(1:400)

Table 2.2 Primary antibodies used in immunohistochemical techniques

Target (Conjugate)	Antibody type	Dilution	Product ID	Distributor
Rabbit IgG (Biotin)	Goat anti-rabbit pAb	1:200	BA-1000	Vector Labs, UK
Mouse IgG (Alexa Fluor 488)	Donkey anti-mouse mAb	1:200	A-21202	ThermoFisher Scientific, UK
Rabbit IgG (Alexa Fluor 568)	Goat anti-rabbit pAb	1:200	A11036	ThermoFisher Scientific, UK
Rabbit IgG (Alexa Fluor 568)	Donkey anti-rabbit mAb	1:200	A-21206	ThermoFisher Scientific, UK
Goat IgG (Alexa Fluor 568)	Donkey anti-goat mAb	1:200	A-11057	ThermoFisher Scientific, UK

Table 2.3 Secondary antibodies used in immunohistochemical techniques

2.3.5.3 Image analysis for foetal brain immunofluorescence staining

All staining of foetal brains was imaged using a DM5000 (Leica Microsystems) 3 channel fluorescence camera with a 10x objective. The same region of the cortex or ganglionic eminences were taken for each brain. All foetal markers were counted or analysed by placing boxes at random in three areas of the Ventricular zone (VZ) of the Cortex, three areas of the Intermediate zone (IZ) of the Cortex, three areas of the VZ of the Ganglionic eminences (GE) and three areas of the Sub-Ventricular zone (SVZ) of the GE. The boxes were standardized for each experiment, to include approximately 100 cells within each boxed area. The cells were labelled and cross checked to ensure that only positive cells were included as illustrated in Figure 2.4. Beta-III-tubulin was measured by stain intensity and all other foetal markers were manually counted for positive cells. All quantitative methods for intensity and cell count were performed using FIJI software.

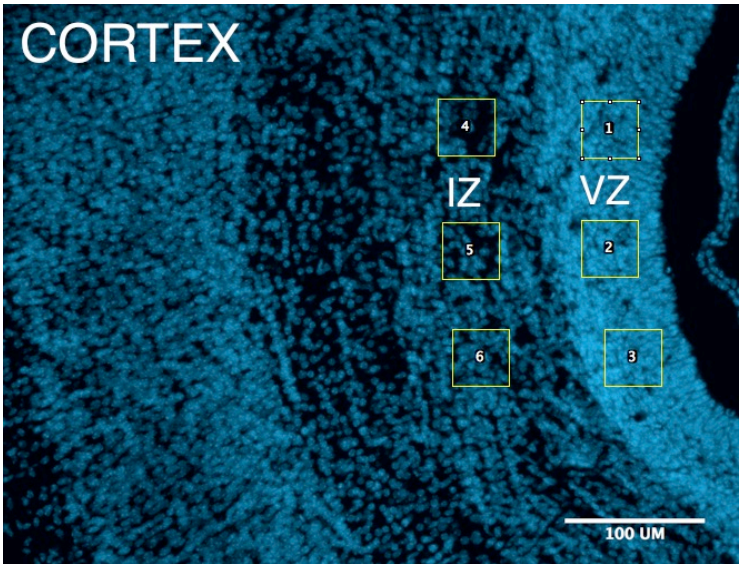


Figure 2.4 Example image analysis for foetal brain staining

Example shows the placement of 3 boxes in the ventricular zone (VZ) and 3 boxes in the intermediate zone of the cortex in FIJI, which can then be measured by stain intensity or cell counting.

2.3.6 Gender typing

Two techniques were used to isolate the DNA of ear and tail clippings from E12.5 and E17.5 mice following maternal NPD/LPD/Emb-LPD diets. The initial lysis method was as follows: To isolate the DNA from tail or ear sections of E12.5 and E17.5 mice, they were lysed by digesting in 100 µl lysis buffer (50 µl 1M Tris, 100 µl 0.5M EDTA, 100 µl 5M NaCl, 250 µl 10% SDS and 4.5 ml dH_2O) with 20 mg/ml Proteinase K at 55˚C for a minimum of 2 hours. Samples were then centrifuged at 13000 RPM for 15 minutes, then 30 µl 5M NaCl added to the supernatant. After 4 minutes at RT, samples were centrifuged at 1300 RPM for 15 minutes. The supernatant was then added to 100 µl isopropanol. Tubes were shaken then incubated at RT for 10 minutes. Samples were centrifuged for 30 minutes at 13000 RPM, then the supernatant was discarded. 1 ml ethanol was added to the pellet and then incubated at RT for 15 minutes. Before centrifuging at 13000 RPM for 30 minutes. The supernatant was removed and the pellet was resuspended in 50 µl nuclease free H_2O.

PCR cycle setting:

95˚C for 15 minutes, 40 cycles: 94˚C for 1 minute, 55˚C for 30 seconds, 72˚C for 1 minute. Incubation: 72˚C for 1 minute. 14˚C forever.

The optimised protocol used the KAPA Mouse Genotyping Kit (KAPA Biosystems), following the KAPA Express Extract Protocol: 88 µl PCR-grade water, 10 µl 10X KAPA Express Extract Buffer, 2 µl 1U/µl KAPA Express Extract Enzyme was added to the mouse tissue for 10 minutes at 75˚C followed by 5 minutes at 95˚C. Samples were centrifuged for 10 minutes before the supernatant was removed and diluted to a DNA concentration of 100 ng/µl before PCR. The initial PCR protocol is shown in the appendix. Following the KAPA Express Extract Protocol, the KAPA2G Fast Genotyping PCR Protocol was followed. The reaction setup was as follows: 9 µl PCR-grade water, 12.5 µl 2X KAPA2G Fast (HotStart) Genotyping Mix with dye, 0.625 µl 10 µM Sry Forward primer, 0.625 µl 10 µM Sry Reverse primer, 0.625 µl 10 µM DXNds3 Forward primer, 0.625 µl 10 µM DXNds3 Reverse primer, 1 µl Template

DNA. The PCR cycle was as follows: 95˚C for 3 minutes, then 40 cycles of: 95˚C for 15 seconds, 60˚C for 15 seconds, 72˚C for 15 seconds/kb. Finally, 72˚C for 1 min/kb.

Sry primers were used to determine males, whilst DXNds3 primers were used to determine females

Primers used were Sry2, Sry4, NDS3 and NDS4, forming fragments of 404 bp and 244 bp respectively. Following PCR, samples were run on a 1% agarose gel with 0.2 µg/ml ethidium bromide with a 100 bp DNA ladder for 30 minutes, followed by UV imaging.

2.3.7 Statistical analysis

Unless otherwise stated, data were analyzed using a multilevel linear regression model using PASW for Windows program version 21 (SPSS UK, Woking, Surrey, United Kingdom)**,** in which there was a random effect assigned to each litter. Thus we evaluated both between-litter and within-litter effects. We always included terms for the litter size and for the sex of the offspring, where appropriate. We used indicator variables to compare the Emb-LPD and the LPD with the NPD. This showed that differences identified between treatment groups are independent of maternal origin of litter and litter size *(44)*. Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Data was represented throughout this thesis as a box and whisker diagram due to the data being non-parametric.

2.4 Results

In order to minimise effect between mother variation in litter size influencing the data for any one group five to seven litters were generated per treatment group, and the data was analysed by muli level regression analysis showing the maternal litter size and embryo position in the uterus had no effect.

2.4.1 Maternal protein restriction reduces primary sphere formation from E12.5, E14.5 and E17.5 cortex and ganglionic eminences cells.

The effect of maternal low protein diet (fig2.5A) on neurosphere formation capabilities was investigated using primary cells from the ganglionic eminence and cortex. (fig2.5). We observed a significant decrease in the number of neurospheres formed after 7 days in culture, for both LPD

and Emb-LPD, compared to NPD at E12.5 (fig 2.5 C,D), E14.5 (fig 2.51E,F) and E17.5 (fig 2.5 G,H), with a further significant decrease between Emb-LPD and LPD in E12.5 ganglionic eminences and E14.5 ganglionic eminences and cortex cells. At E17.5, both the ganglionic eminences and cortex cells from the Emb-LPD group formed significantly fewer neurospheres than both NPD and LPD (fig 2.5 G,H). These results were independent of maternal litter size, the sex of the fetuses (fig 2.6). The neurospheres had a variety of sizes (fig 2.6.B), and were also grouped into 100-200 µm, 200-400 µm and over 400 µm in diameter sizes before analysis. All three size groups were reduced in number for LPD compared to NPD (significantly for 100-200 µm and 200-400 µm, a trend for over 400 µm). These results reveal that maternal LPD and Emb-LPD caused reduced neurosphere-forming capabilities from ganglionic eminences and cortex primary cells at three fetal ages.

The composition of the primary neurospheres were analysed to see if maternal diet had an effect. For this, cells from primary neurospheres generated from E14.5 cortex and ganglionic eminences were analysed by FACS for Nestin and Beta-III-tubulin positive cells (fig 2.6 C-E) and confirmed by confocal staining (fig2.6 F). This showed a significant increase in the proportion of double-labelled cells and reduced proportion of Beta-III-tubulin+ cells in the Emb-LPD group compared to NPD and LPD. This confirms that the neurospheres are mostly composed of NSCs and neural progenitors. This indicates that there is an increase in progenitor cells in the Emb-LPD group. It also suggests that the NSCs may have rapidly differentiated to neural progenitors and as neurons cannot survive within the culture media the decrease in beta 3 tubulin positive cells could be due to death of these excess neurons.

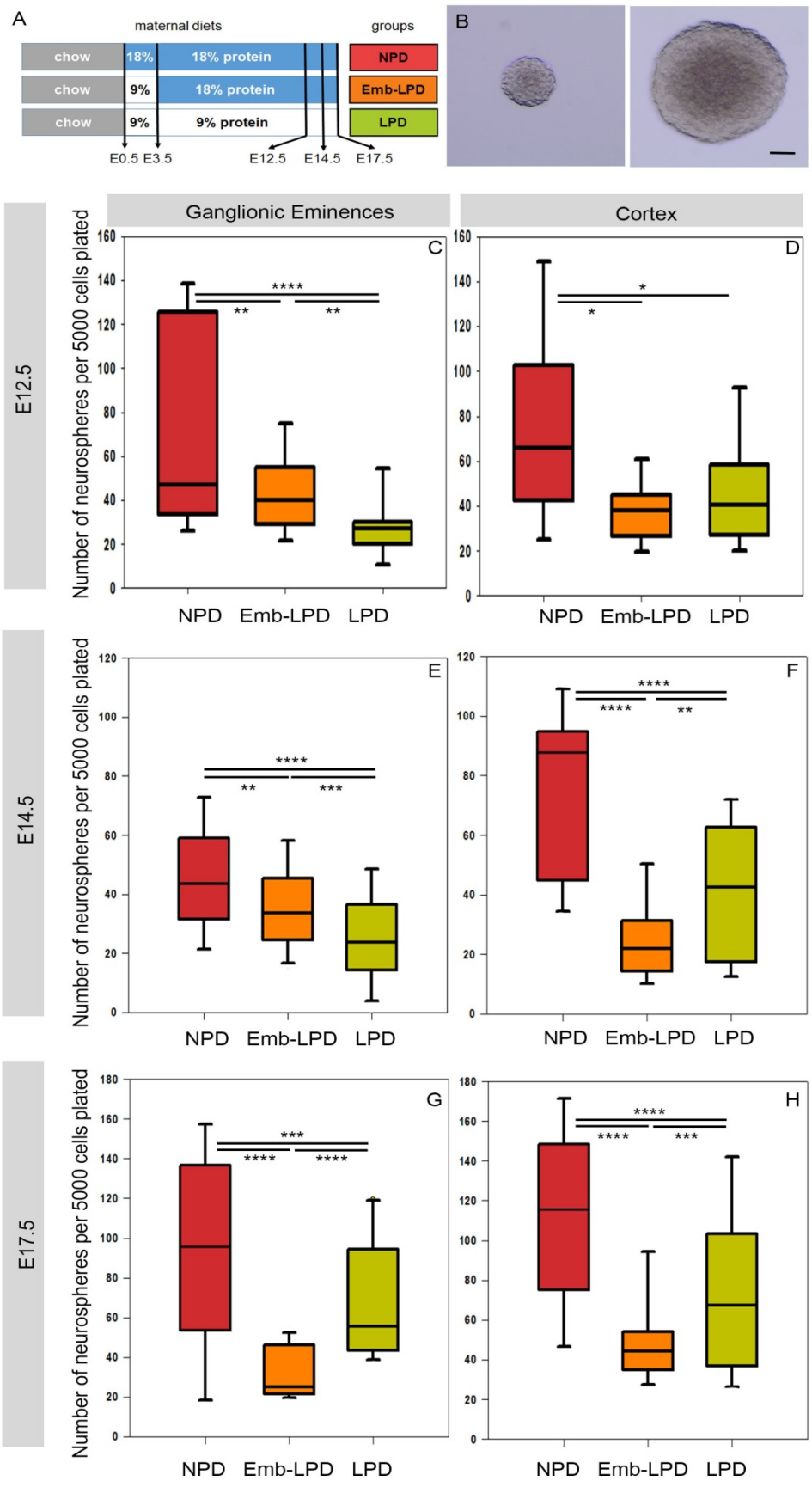

A
maternal diets
groups
chow
18%
18% protein
NPD
chow
9%
18% protein
Emb-LPD
chow
9%
9% protein
LPD
E0.5 E3.5
E12.5 E14.5 E17.5
B
Ganglionic Eminences
Cortex
E12.5
E14.5
E17.5
Number of neurospheres per 5000 cells plated
C
D
E
F
G
H
NPD Emb-LPD LPD

Figure 2.5 Maternal diet affects primary sphere formation from neural cells

(A) Experimental model describing the three diet groups with NPD group exposed to 18% protein diet throughout gestation, LPD group exposed to 9% protein diet from vaginal plug on E0.5 and Emb-LPD group exposed to 9% protein diet between E0.5 and E3.5 followed by 18% protein diet until time of analysis at E12.5, E14.5 or E17.5 with both genders included.

(B) Representative images of spheres generated from neural cells. Scale bar = 50µm.

Quantification of the number of primary spheres (over 100µm in diameter) per well after 7 days with 5000 cells plated from the ganglionic eminences (C-E-G) or cortex (D-F-H) from the three maternal diet groups.

E12.5 ganglionic eminences and cortex data represent n=24 (NPD), 18 (Emb-LPD), 21 (LPD) fetuses from 8 (NPD), 6 (Emb-LPD) or 7 (LPD) mothers.

E14.5 ganglionic eminences data represent n=131 (NPD), 125 (Emb-LPD), 124 (LPD) fetuses from 17 (NPD), 17 (Emb-LPD) or 18 (LPD) mothers.

E14.5 cortex data represent n=18 (NPD), 18 (Emb-LPD), 19 (LPD) fetuses from 6 (NPD), 6 (Emb-LPD) or 6 (LPD) mothers.

E17.5 ganglionic eminences data represent n=18 (NPD), 18 (Emb-LPD), 21 (LPD) fetuses from 6 (NPD), 6 (Emb-LPD) or 7 (LPD) mothers.

E17.5 cortex data represent n=18 (NPD), 18 (Emb-LPD), 24 (LPD) fetuses from 6 (NPD), 6 (Emb-LPD) or 8 (LPD) mothers.

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

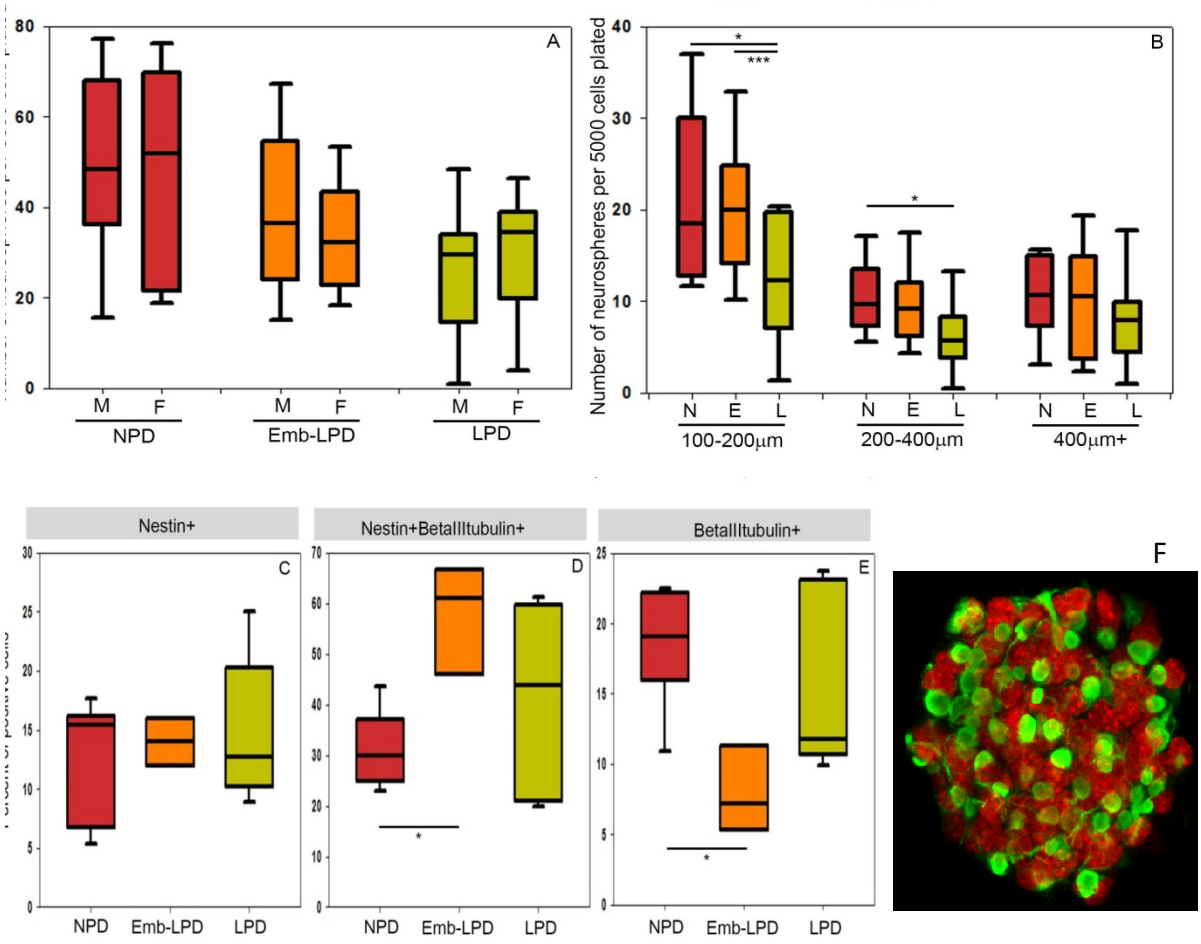


Figure 2.6 Maternal diet doesn't affect E14.5 ganglionic eminences primary and size distribution

(A) Quantification of the number of primary spheres per well after 7 days with 5000 cells plated from E14.5 ganglionic eminences from different maternal diets sorted by sex of the fetus. Data represent n=19 (NPD-M), 18 (NDP-F), 14 (Emb-LPD-M), 17 (Emb-LPD-F), 11 (LPD-M), 13 (LPD-F) fetuses from at least 7 mothers per group. Data analyzed by multi-level regression analysis ($p<0.001$) but not sex effect ($p=0.866$) and no interaction between diet and sex ($p=0.769$). (B) Primary sphere size was analyzed by grouping ganglionic eminences E14.5 primary spheres into size groups: 100 and 200µm in diameter, 200 and 400µm in diameter, over 400µm in diameter. Data represent n=12 (NPD), 13 (Emb-LPD), 10 (LPD) mothers with at least 9 wells from at least 3 fetuses. (C-E) Quantification by flow cytometry of E17.5 cortex primary spheres dissociated cells stained for Nestin and Beta-III-tubulin. Nestin only positive cells (C), double positive cells (D) and Beta-III-tubulin only positive cells (E) were quantified. Data represent n=6 (NPD), 3 (Emb-LPD), 5 (LPD) mothers with spheres from 3 fetuses

pooled for each mother.(F)) Representative image illustrating the staining in a neurosphere Nestin (green), Beta-III- Tubulin (red).

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N=NPD; E=Emb=LPD; L=LPD; M=male; F=female.

2.4.2 Maternal protein restriction alters the expression of neural stem cells and neuronal differentiation markers in ganglionic eminences and cortex in vivo

To assess whether the stem and differentiation qualities of the cells in the fetal brain were affected by different maternal diets, ganglionic eminences and cortex primary cells were stained for Nestin and Beta-III-tubulin and flow cytometry sorted. The cells analyzed separated into four main populations: Nestin+ only (Q1, representing neural stem/progenitor cells), double positive cells (Q2, representing neuronal progenitors), Beta-III-tubulin+ only (Q3, representing differentiated neurons), and double negative cells (Q4), compared to isotype control stained section 2.5.2. When analysing the FACS plots (fig 2.9), it was apparent there was two different populations in the double positive cell population. These were separated as Nestin+ Beta-III-tubulin dim (representing early neuronal progenitors, Q2N), and Nestin dim Beta-III-tubulin+ (representing late neuronal progenitors Q2B).

Nestin only positive cells represented only a small percentage of the whole population and showed in ganglionic eminences (fig 2.8) and cortex (fig 3.5) cells a significant decrease in Emb-LPD at E12.5, E14.5 and E17.5, as well as in LPD at E12.5 and E14.5, whereas E17.5 LPD Nestin+ cells did not differ from NPD (fig 2.8 A,E,I and fig 3.5 A,E,I). The decrease in Nestin+ cells confirms the decrease in sphere-forming cells observed in the sphere assay and suggests a decrease in stem cells at E12.5 and E14.5 in both Emb-LPD and LPD and at E17.5 in Emb-LPD.

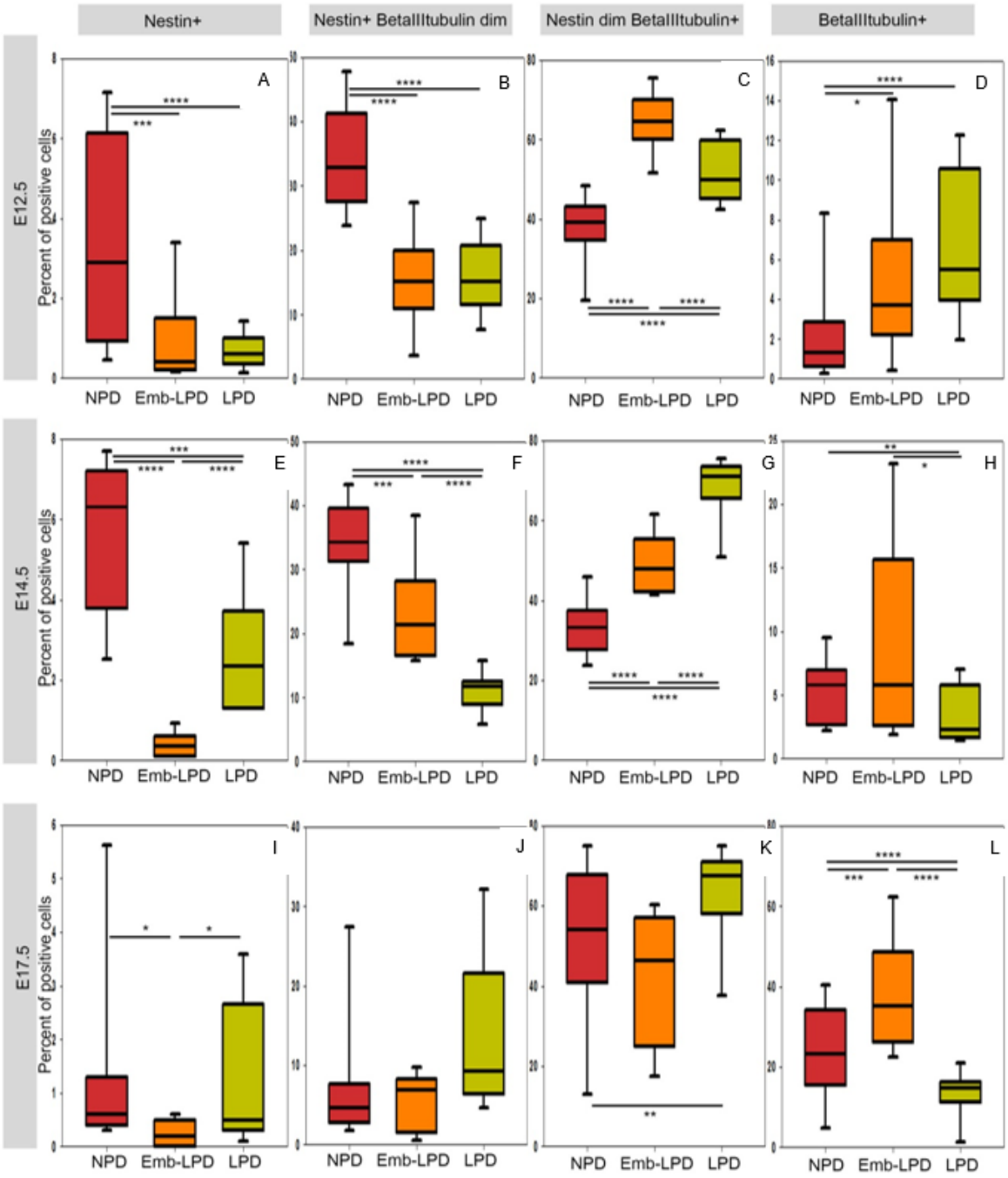


Figure 2.7 Maternal diet affects expression of neural stem cells and neuronal differentiation markers analyzed by flow cytometry in ganglionic eminences cells.

Quantification by flow cytometry of E12.5 (A-D) E14.5 (E-H) and E17.5 (I-L) ganglionic eminences cells stained for Nestin and Beta-III-tubulin.

Nestin only positive cells (A,E,I), double positive cells separated into Nestin+ Beta-III-tubulin dim (B,F,J), and Nestin dim Beta-III-tubulin+ (C,G,K), and Beta-III-tubulin only positive cells (D,H,L) were quantified.

E12.5 ganglionic eminences data represent n=24 (NPD), 18 (Emb-LPD), 21 (LPD) fetuses from 8 (NPD), 6 (Emb-LPD) or 7 (LPD) mothers.

E14.5 ganglionic eminences data represent n=131 (NPD), 125 (Emb-LPD), 124 (LPD) fetuses from 17 (NPD), 17 (Emb-LPD) or 18 (LPD) mothers.

E17.5 ganglionic eminences data represent n=18 (NPD), 18 (Emb-LPD), 18 (LPD) fetuses from 6 (NPD), 6 (Emb-LPD) or 6 (LPD) mothers.

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

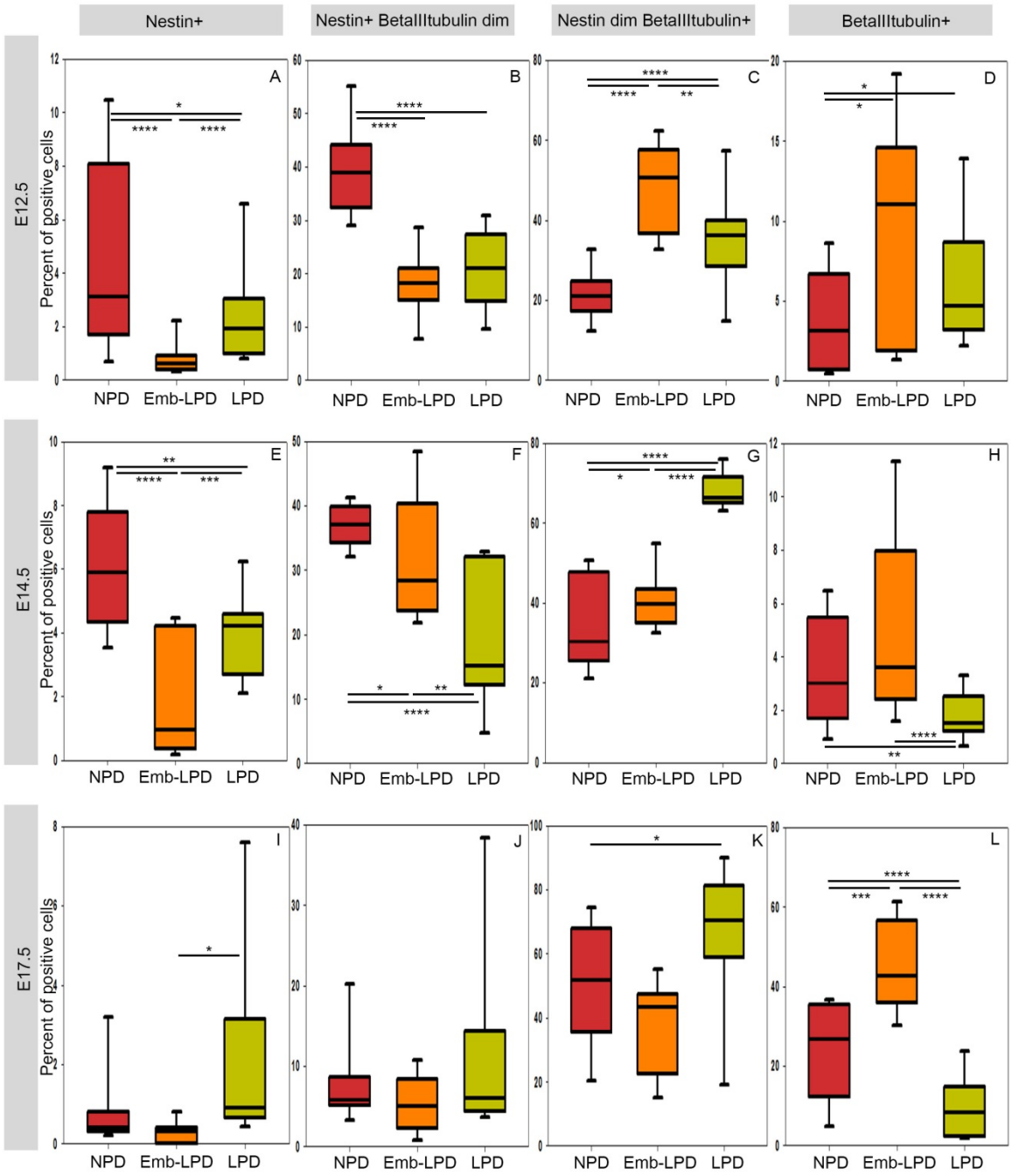


Figure 2.8 Maternal diet affects expression of neural stem cells and neuronal differentiation markers analyzed by flow cytometry in cortical cells.

Quantification by flow cytometry of E12.5 (A-D) E14.5 (E-H) and E17.5 (I-L) cortical cells stained for Nestin and Beta-III-tubulin.

Nestin only positive cells (A,E,I), double positive cells separated into Nestin+ Beta-III-tubulin dim (B,F,J), and Nestin dim Beta-III-tubulin+ (C,G,K), and Beta-III-tubulin only positive cells (D,H,L) were quantified.

E12.5 cortex data represent n=24 (NPD), 18 (Emb-LPD), 21 (LPD) fetuses from 8 (NPD), 6 (Emb-LPD) or 7 (LPD) mothers.

E14.5 cortex data represent n=18 (NPD), 18 (Emb-LPD), 19 (LPD) fetuses from 6 (NPD), 6 (Emb-LPD) or 6 (LPD) mothers.

E17.5 cortex data represent n=18 (NPD), 18 (Emb-LPD), 18 (LPD) fetuses from 6 (NPD), 6 (Emb-LPD) or 6 (LPD) mothers.

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Nestin+ Beta-III-tubulin+ cells represented the majority of cells and illustrated the fact that most of the cells were in transition between undifferentiated and neuronally-differentiated cells. When analyzed separately, the two neuronal progenitor populations showed very different results in both ganglionic eminences and cortex at E12.5 and E14.5: the early neuronal progenitors decreased significantly in both Emb-LPD and LPD compared to NPD (fig 2.8 B,F and fig2.9 B,F) whereas the late neuronal progenitors increased significantly in both Emb-LPD and LPD compared to NPD (fig 2.8 C,G and fig2.9 C,G). At E17.5, the proportion of both early and late neuronal progenitors in Emb-LPD and early progenitors in LPD was unchanged compared with NPD (fig 2.8 J,K and fig 2.9 J,K) whereas the LPD late progenitor proportion was increased (fig 2.8 K and fig 2.9K). Beta-III-tubulin only positive cells represent differentiated neurons and showed a significant increase in Emb-LPD E12.5 and E17.5 in both ganglionic eminences and cortex (fig 2.8 D,L and fig 2.9 D,L) with no difference at E14.5 (fig 2.8 H and fig 2.9 H). In LPD, the Beta-III-tubulin+ cell proportion was significantly increased at E12.5 whereas it was significantly decreased at E14.5 and E.17.5 in both ganglionic eminences and cortex (fig 2.8 D,H,L and fig 2.9 D,H,L).

These detailed FACS analyses are schematically summarized for ganglionic eminences primary cells (fig 2.10A), with Nestin+ cells data also only shown (fig 2.10 B). This shows clearly that the proportion of Nestin+ cells peaks at E14.5 compared to E12.5 and E17.5 in NPD as stated in the litreature. LPD Nestin+ cell proportions follow the same pattern across time although at reduced levels at E12.5 and E14.5, whereas the Emb-LPD Nestin+ cell proportions are low from E12.5 and continuously decrease until E17.5 (fig 2.10B). Early progenitor (purple, Nestin+ Beta-III-tubulin dim cells) proportions decrease at E12.5 and E14.5 in both LPD and Emb-LPD compared to NPD. Late progenitor (blue, Nestin dim Beta-III-tubulin+ cells) proportions increase in Emb-LPD and LPD at both E12.5 and E14.5 as well as in LPD at E17.5, compared to NPD. Differentiated neuron (light blue,

Beta-III-tubulin+ cells) proportions increase at E12.5 for both Emb-LPD and LPD, as well as at E17.5 for Emb-LPD, whereas they decrease in LPD at both E14.5 and E17.5, compared to NPD. Collectively, in Emb-LPD, there is a reduction in neural stem cells and an increase in differentiated neurons over time compared with NPD. In LPD, a reduction in neural stem cells is also evident, but differentiated neuron formation becomes stable over time, more similar to NPD.

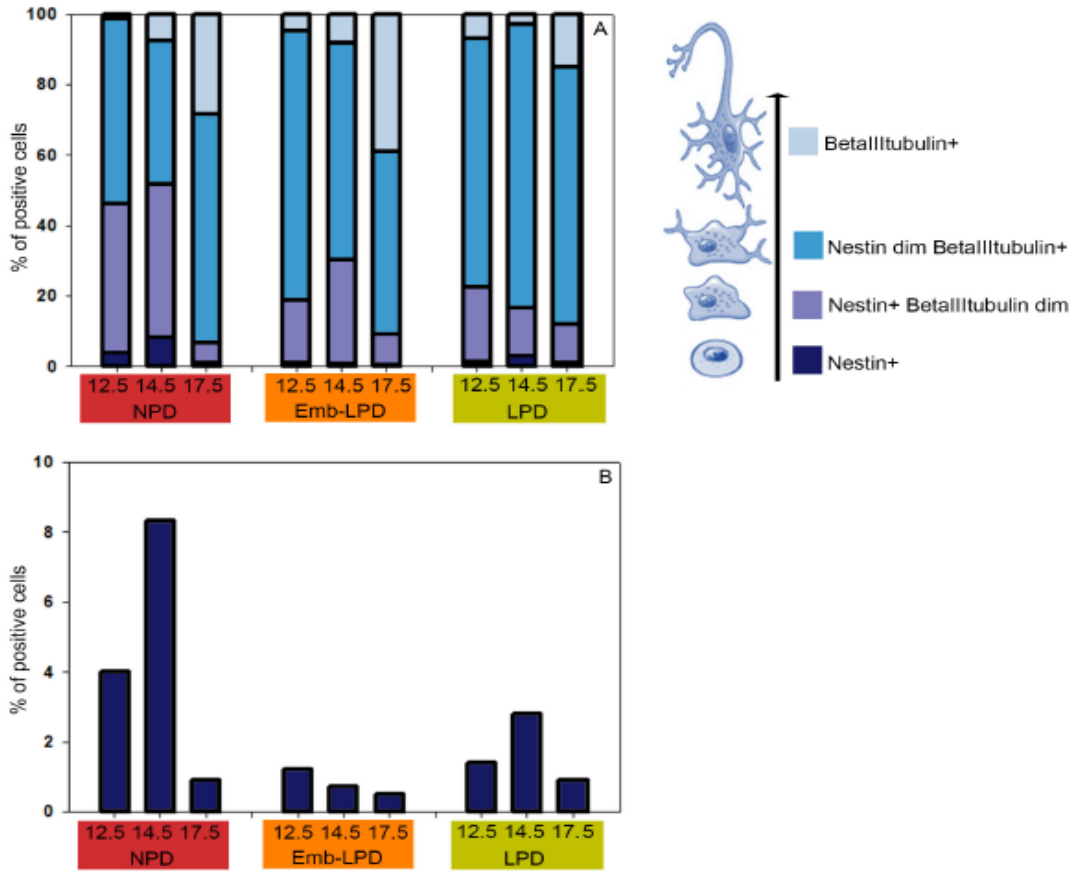


Figure 2.9 Summary of FACS data obtained for primary cells from ganglionic eminences

(A) Summary of the FACS data for ganglionic eminences primary cells from figure 2.7, with enlargement of the neural stem/progenitor cells data (Nestin+, dark blue, B). Early neuronal progenitors (Nestin+ Beta-III-tubulin dim, purple), late neuronal progenitors (Nestin dim Beta-III-tubulin+, royal blue) and neurons (Beta-III-tubulin+, light blue) are presented along the differentiation lineage (black arrow).

To confirm these results and to resolve any regional variation within ganglionic eminences and cortex, coronal brain sections were stained for markers of stem cells Sox2 and Nestin, and a marker of neural progenitors and young neurons, Beta-III-tubulin. Staining was quantified in ganglionic

eminences ventricular zone (VZ), subventricular zone (SVZ) and mantle zone (MZ), and in cortex in VZ, intermediate zone (IZ) and cortical plate (CP), when relevant. Sox2 staining was present in both the ganglionic eminences and cortex, mostly in the VZ and SVZ/IZ where stem cell niches reside. Quantification revealed a significant decrease of the number of positive cells in LPD compared to NPD, in the ganglionic eminences VZ at E12.5 (fig 2.10A) with a similar trend in the cortex VZ at the same age (fig 3.6B), as well as in both regions at E14.5 (fig 2.11 A,B). A similar decreasing trend is noticed in the Emb-LPD group at E12.5 (fig 2.10 A,B) and E14.5 (fig 2.11 A,B). No change is observed at E17.5 (fig 2.12 A,B) in both diet groups and regions, compared to NPD. The decrease in proportion of Sox2+ cells observed here confirms the decrease in neural stem/progenitor cells, seen in the sphere assay and FACS analysis.

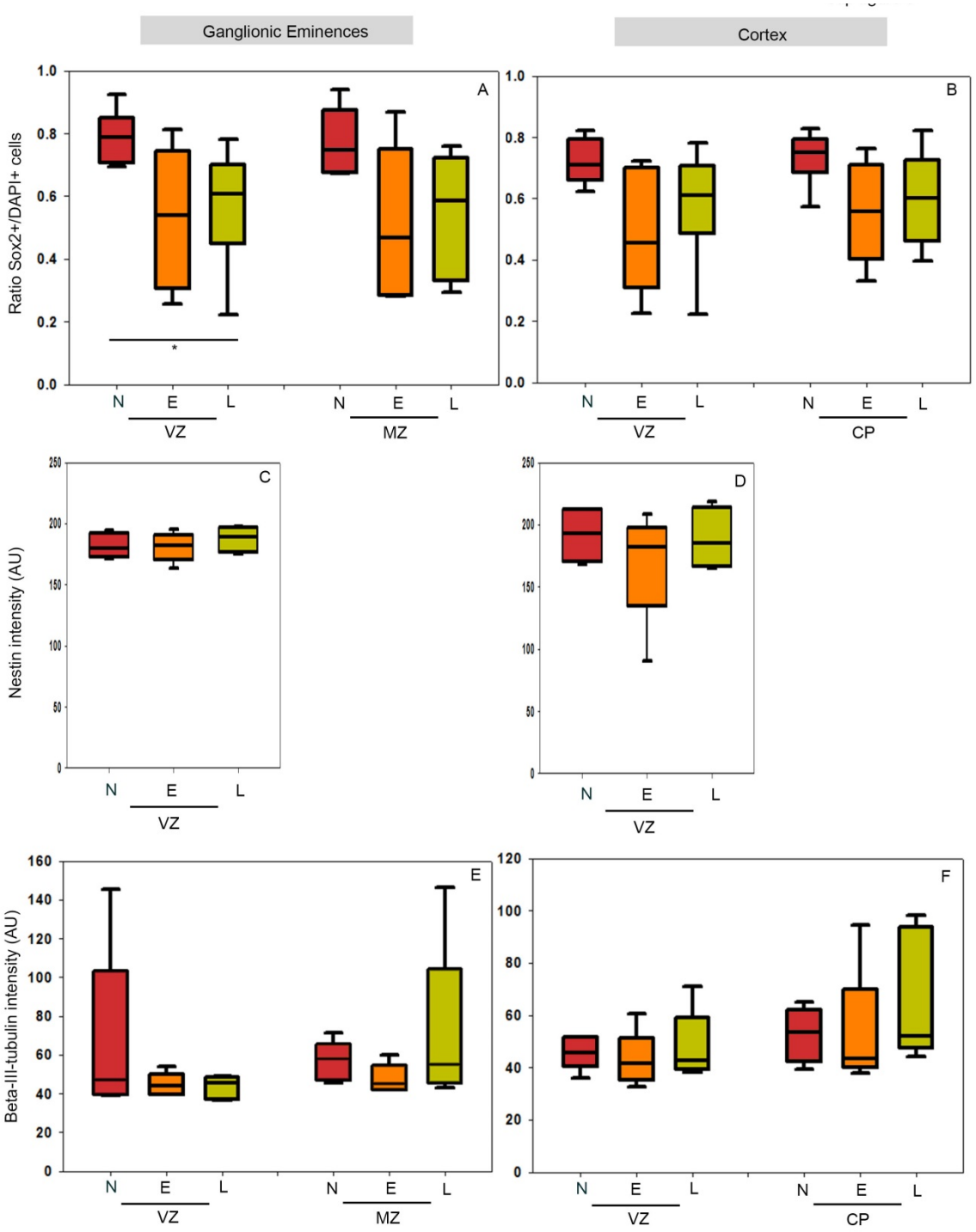


Figure 2.10. Maternal diet affects expression of neural stem cells and neuronal differentiation markers analysed by immunohistochemistry in E12.5 ganglionic eminences and cortex.

Quantification of Sox2 staining (% of Sox2+/DAPI+ cells) on E12.5 ganglionic eminence (A) and cortex (B) sections from different maternal diets. Data represent 3 quantifications/layer/section of 2 sections/brain from 6 fetal brains from 6 different mothers per diet.

Quantification of Nestin staining (% of positive pixels/area) on E12.5 ganglionic eminence (C) and cortex (D) sections from different maternal diets. Data represent 3 quantifications/layer/ section of 2 sections/brain from 6 (NPD), 5 (Emb-LPD) and 4(LPD) fetal brains from 6 (NPD), 5 (Emb-LPD) and 4(LPD) different mothers.

Quantification of Beta-III-tubulin staining (% of positive pixels/area) on E12.5 ganglionic eminence (E) and cortex (F) sections from different maternal diets. Data represent 3 quantifications/layer/section of 2 sections/brain from 5 fetal brains from 5 different mothers per diet.

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

N=NPD ; E=Emb=LPD ; L=LPD ; VZ=Ventricular Zone ; MZ=Mantle Zone ; CP=Cortical Plate

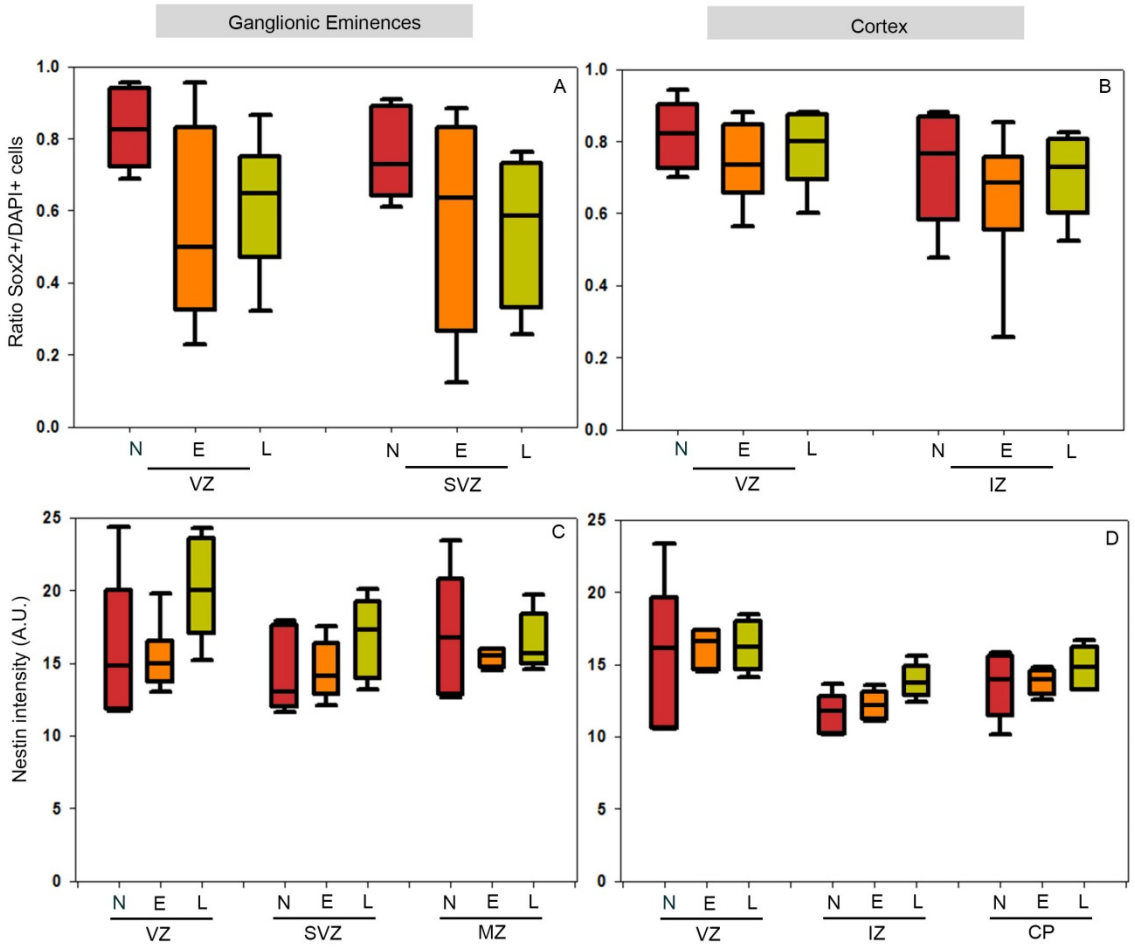


Figure 2.11. Maternal diet affects expression of neural stem cells markers analyzed by immunohistochemistry in E14.5 ganglionic eminences and cortex.

Quantification of Sox2 staining (% of Sox2+/DAPI+ cells) on E14.5 ganglionic eminence (A) and cortex (B) sections from different maternal diets. Data represent 3 quantifications/layer/ section of 2 sections/brain from 6 fetal brains from 6 different mothers per diet.

Quantification of Nestin staining (% of positive pixels/area) on E14.5 ganglionic eminence (C) and cortex (D) sections from different maternal diets. Data represent 3 quantifications/layer/ section of 2 sections/brain from 5 fetal brains from 5 different mothers per diet.

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

N=NPD ; E=Emb=LPD ; L=LPD ; VZ=Ventricular Zone ; SVZ=Subventricular Zone; MZ=Mantle Zone ; IZ=Intermediate Zone ; CP=Cortical Plate

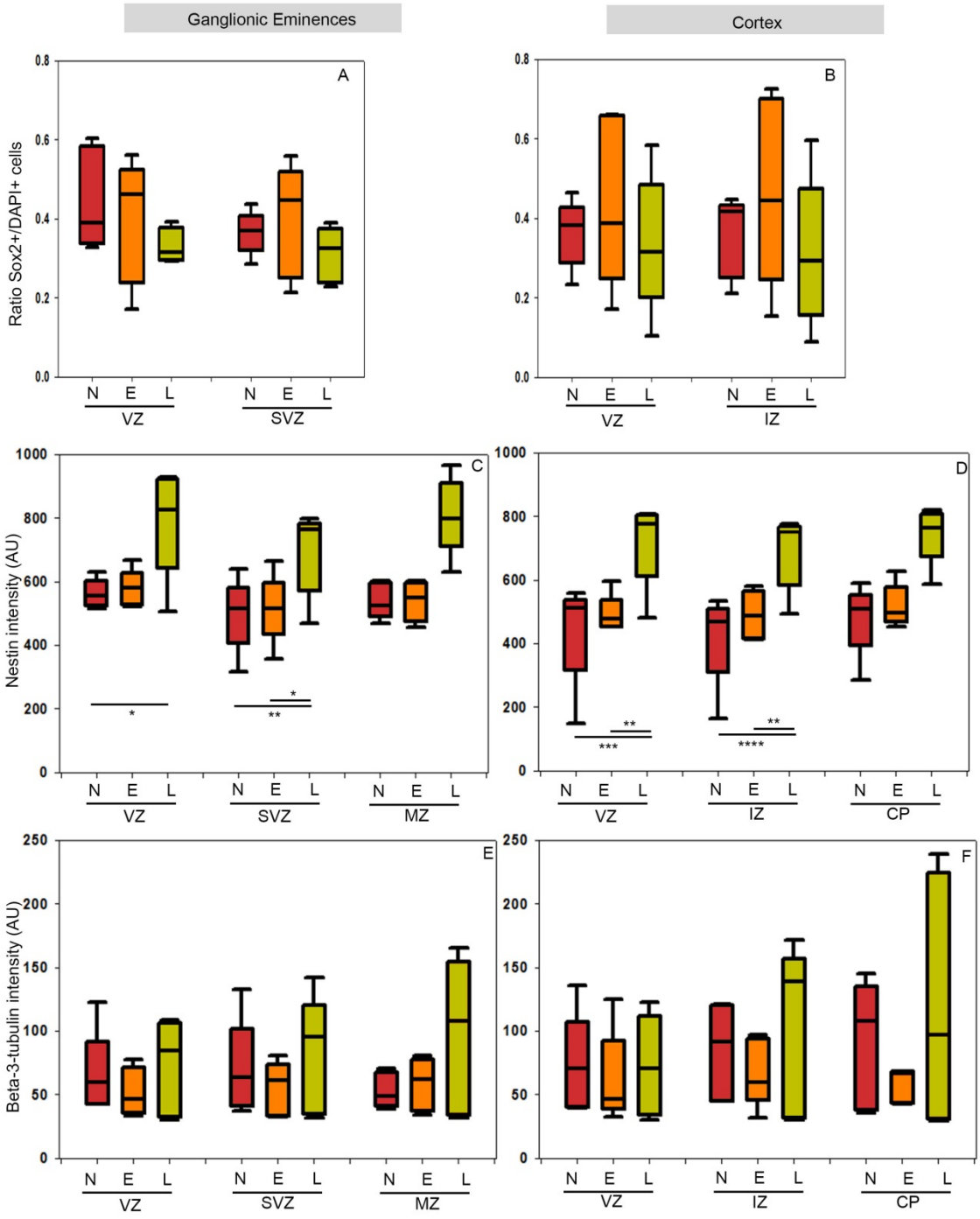


Figure 2.12. Maternal diet affects expression of neural stem cells and neuronal differentiation markers analyzed by immunohistochemistry in E17.5 ganglionic eminences and cortex.

Quantification of Sox2 staining (% of Sox2+/DAPI+ cells) on E17.5 ganglionic eminence (A) and cortex (B) sections from different maternal diets. Data represent 3 quantifications/layer/ section of 2 sections/brain from 5 fetal brains from 5 different mothers per diet.

Quantification of Nestin staining (% of positive pixels/area) on E17.5 ganglionic eminence (C) and cortex (D) sections from different maternal diets. Data represent 3 quantifications/layer/ section of 2 sections/brain from 5 fetal brains from 5 different mothers per diet.

Quantification of Beta-III-tubulin staining (% of positive pixels/area) on E17.5 ganglionic eminence (E) and cortex (F) sections from different maternal diets. Data represent 3 quantifications/layer/ section of 2 sections/brain from 5 fetal brains from 5 different mothers per diet.

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

N=NPD ; E=Emb=LPD ; L=LPD ; VZ=Ventricular Zone ; SVZ=Subventricular Zone; MZ=Mantle Zone ; IZ=Intermediate Zone ; CP=Cortical Plate

Nestin staining was present in both the ganglionic eminences and cortex in all layers analyzed, illustrating its broader expression compared to Sox2. Its quantification did not reveal any major effect of maternal diet at E12.5 (fig 2.10 C,D) and E14.5 (fig 2.11 C,D). However, Nestin staining increases significantly in LPD at E17.5 in the VZ and SVZ/IZ of the ganglionic eminences and cortex compared to NPD (fig 2.12 C,D). This immunofluorescence data confirms the FACS analysis showing increased Nestin staining (evident in all cell categories except differentiated neurons) in LPD at E17.5 (fig 2.8 & 2.9).

Beta-III-tubulin staining was analyzed in the ganglionic eminences, highest staining in SVZ and even more in the mantle zone (fig 2.13 A-C) and decrease in the VZ. Its quantification showed an increase in Emb-LPD SVZ and MZ in ganglionic eminences at E14.5 (fig 2.13D) and a comparable trend in the cortex at E14.5 (fig 2.13 E, p=0.0724 in IZ and p=0.0586 in cortical plate), compared to both NPD and LPD. This increase confirms the FACS analysis where collectively the three cell types positive for Beta-III-tubulin are increased in Emb-LPD at E14.5 (fig 2.9). Beta-III-tubulin staining was also performed on E12.5 and E17.5 cortex and ganglionic eminences sections, where no differences between diet groups were found (fig 2.10 E,F and fig 2.12 E,F), again confirming the collective data for Beta-III-tubulin from the FACS analysis (fig 2.9).

These results indicate that maternal diet not only affects the availability of neural stem cells but also affect their pattern of differentiation towards a neuronal fate. The Emb-LPD there is a decrease

in stem/progenitor cells and an increase in late neuronal progenitors and neurons. However, the LPD induces a decrease in stem/progenitor cells, an increase in late neuronal progenitors but this is not followed by an increase in neurons.

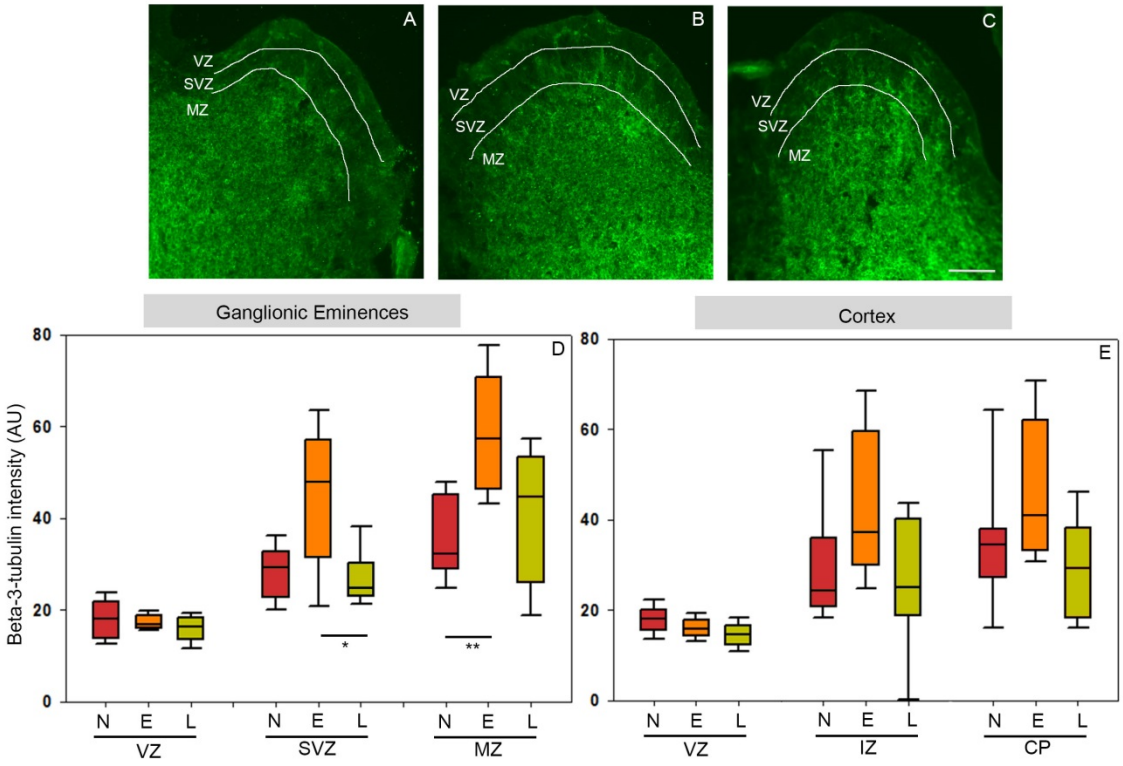


Figure 2.13 Maternal diet affects expression of neuronal differentiation markers analysed by immunohistochemistry in E14.5 ganglionic eminences and cortex.

Representative images illustrate the staining results quantified: Beta-III-tubulin (green, A-C) are stained on E14.5 ganglionic eminences sections from maternal NPD (A), Emb-LPD (B) and LPD (C). Scale bar=150µm.

Quantification of Beta-III-tubulin staining (% of positive pixels/area) on E14.5 ganglionic eminence (D) and cortex (E) sections from different maternal diets. Data represent 3 quantifications/layer/section of 2 sections/brain from 9 fetal brains from 9 different mothers per diet. Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

N=NPD ; E=Emb=LPD ; L=LPD ; VZ=Ventricular Zone ; SVZ=Subventricular Zone; MZ=Mantle Zone ; IZ=Intermediate Zone ; CP=Cortical Plate

2.4.3 Maternal protein restriction reduces proliferation of ganglionic eminences and cortex cells

Stem cells need to proliferate to maintain and/or increase their population *in vivo* and to form neurospheres *in vitro*. A deficit in proliferation rates maybe the reason for the apparent loss of neural stem cells seen following Emb-LPD and LPD in the neurosphere, FACS and immunofluorescence analyses. The growth fraction (Ki67+/DAPI+) was analyzed in the VZ of both cortex and ganglionic eminence coronal sections (fig 2.15 A-C). The growth fraction significantly decreased for both Emb-LPD and LPD compared to NPD, at E12.5, E14.5 and E17.5 (GE fig 2.15 D and cortex fig 2.15 A). Thus maternal Emb-LPD and LPD reduce the proliferation of ganglionic eminences and cortex cells in the VZ where most stem cells are located, thereby potentially contributing to the loss of neural stem cells reported above.

Cell death through apoptosis is another mechanism by which cell numbers might be regulated and we thus analyzed apoptosis by immunostaining for activated cleaved caspase-3 on coronal sections (fig 2.15 F-H). Quantification of the number of positive cells per area revealed that apoptotic cell numbers significantly increased in LPD in all three layers of the ganglionic eminences (fig 2.15 E) and cortex (fig 2.16 B) at E17.5. It was also significantly increased in Emb-LPD VZ and SVZ/IZ at E17.5 (fig 2.15 E and fig 2.16 B). However, there was no difference in both Emb-LPD and LPD at both E12.5 and E14.5, compared to NPD. This increase in apoptosis at E17.5 in LPD may further explain why the increased proportion of late neuronal progenitors do not lead to a proportionate increase in neurons formed.

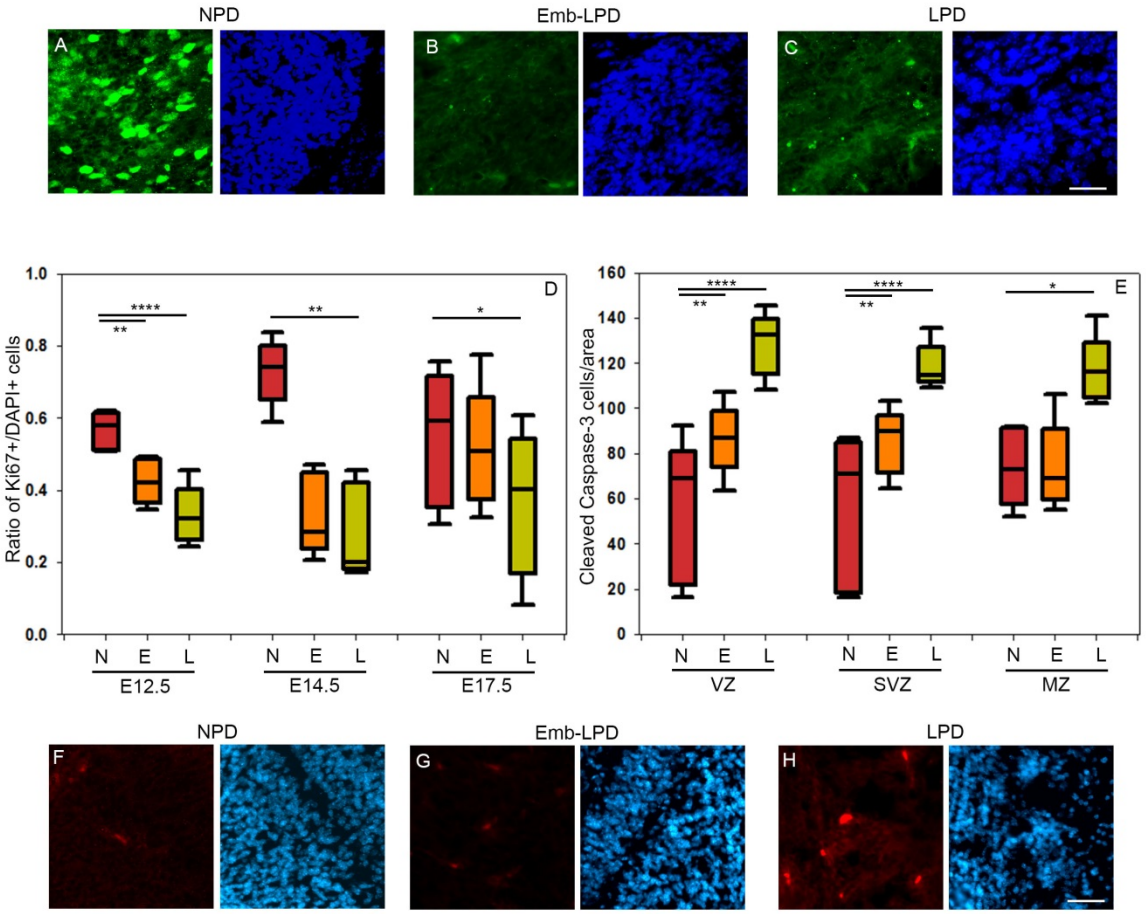


Figure 2.14. Maternal diet affects proliferation and apoptosis in ganglionic eminences

(A-C) Representative images illustrate the staining results quantified in D: Ki67 (green), DAPI (blue) are stained on E12.5 ganglionic eminences sections from maternal NPD (A), Emb-LPD (B) and LPD (C).

(D) The growth fraction quantifies the proportion of cells (DAPI+) which are proliferating (Ki67+) in E12.5, E14.5 and E17.5 ganglionic eminences ventricular zone sections. Data represent 3 quantifications/layer/brain of 5 fetal brains from 5 different mothers per diet.

(E) Quantification of Cleaved Caspase-3 staining (number of positive cells/area) on E17.5 ganglionic eminences sections. Data represent 3 quantifications/layer/brain of 5 fetal brains from 5 different mothers per diet.

(F-H) Representative images illustrate the staining results quantified in (E): Cleaved Caspase-3 (red) and DAPI (blue) staining of E17.5 ganglionic eminences subventricular zone sections from maternal NPD (F), Emb-LPD (G) and LPD (H).

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

N=NPD; E=Emb=LPD; L=LPD; VZ=Ventricular Zone; SVZ=Subventricular Zone; MZ=Mantle Zone; Scale bar=50µm.

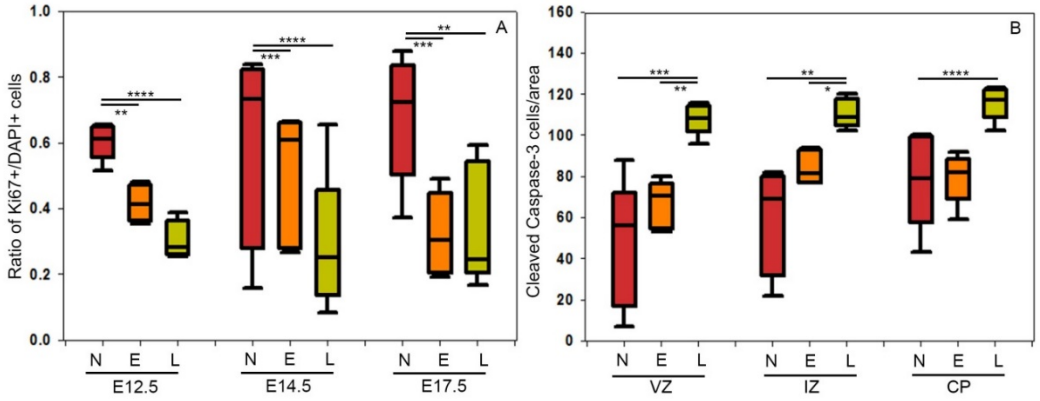


Figure 2.15. Maternal diet affects proliferation and apoptosis in cortex

(A) The growth fraction quantifies the proportion of cells (DAPI+) which are proliferating (Ki67+) in E12.5, E14.5 and E17.5 cortical ventricular zone sections. Data represent 3 quantifications/layer/ section of 2 sections/brain from 5 fetal brains from 5 different mothers per diet.

(B) Quantification of Cleaved Caspase-3 staining (number of positive cells/area) on E17.5 cortical sections. Data represent 3 quantifications/layer/ section of 2 sections/brain from 5 fetal brains from 5 different mothers per diet.

Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N=NPD; E=Emb=LPD; L=LPD; VZ=Ventricular Zone; SVZ=Subventricular Zone; MZ=Mantle Zone

2.5 Discussion

I have shown, using *in vivo* and *in vitro* techniques, that Emb-LPD and sustained LPD reduce neural stem and neural progenitor cell numbers through suppressed proliferation rates in both ganglionic eminences and cortex of the fetal brain at three different time points. Moreover, we find that the diminished stem cell pool after dietary treatment exhibits distinct differentiation dynamics with

Emb-LPD inducing an increase in late neuronal progenitors and young neurons while LPD induced an increase in late neuronal progenitors but not in young neurons. Our study therefore shows that maternal LPD affects stem/progenitor cells even during a very short and early period (Emb-LPD), possibly due to a decrease in proliferation and an increase in apoptosis. Our data further indicate that compensatory processes are put in place to alleviate the effects of the stem cell deficit and the excess of neurons, possibly due to activation of apoptosis (LPD). However, this compensation, activated before implantation, is present but redundant in Emb-LPD, thus leading to an overproduction of neurons.

2.5.1 A consistent low protein diet may induce a protective mechanism that rescues normal neurogenesis by E17.5.

Both the *in vitro* data and the *in vivo* data presented in this study indicates that there was no statistical difference in the proportion of NSCs, neural progenitors and neurones within the LPD cortex and ganglionic eminences at E17.5 (fig3.4A-F and fig3.5A-F). We interpret the initial reduction in neural stem cells found in both Emb-LPD and LPD samples as a consequence of adverse dietary programming of undifferentiated cells at E12.5 and E14.5. This data therefore indicates a possible scenario in which continuous protein restriction causes changes in foetal programming, allowing the developing foetus to compensate for maternal malnutrition, thus explaining why the initial detrimental effects observed at E12.5 and E14.5 are then lost by E17.5. However, the mechanism of induction is currently unknown. Subsequently, during fetal development, whilst both the Emb-LPD and LPD fetuses will be responsive to compensatory extra-embryonic systems, only the Emb-LPD fetuses will be in a 'catch-up' growth environment. Indeed, Emb-LPD offspring have increased mass during late gestation and postnatally compared with LPD and NPD offspring (6). Given these distinct characteristics, the Emb-LPD brain phenotype of reduced neural stem cells but leading to stimulated neurogenesis across the E12.5 to E17.5 time course compared to NPD, may reflect these systemic changes in programming environments. In contrast, the LPD brain, following neural stem cell loss, will be within a more restrained growth environment, limiting the rate of neurogenesis.

Evidence supporting this idea comes from observations from the Dutch Hunger Winter studies. It was shown that mothers subject to famine during mid and late gestation instilled an increased risk of cardiometabolic disease within their offspring, albeit to a lesser extent than early gestational exposure [27]. This indicates that maternal nutrition can impact foetal programming at multiple stages during gestation. Therefore as exposure within the LPD is continuous, subsequent effects after implantation may act to offset the initial changes. Hence in the Emb-LPD where the maternal protein restriction is inconsistent, the delayed protective role of a continuous LPD-induced

response will not be implemented, causing the negative consequences to be exacerbated and/or maintained. This possibly explains why changes in behaviour were only seen in the Emb-LPD mouse model and not the LPD group[12].

At E17.5 we have shown that there is not a statistical difference when comparing the NPD and the LPD for the neuron number with Beta-III-tubulin (fig 3.4L & fig 3.5L), neural progenitors and neural stem cells which is illustrated in both the flow cytometry and neurosphere data. This data is particularly exciting as it suggests at some point between E14.5 and E17.5 the rate at which the NSC and neural progenitor pool was being depleted is slowed or stopped, allowing normal proportions of the different cell types to be re-established. This supports further the idea that a protective mechanism employed by the LPD cohort had not been adopted by the Emb-LPD in this time period

2.5.2 Enhanced NSC differentiation may occur via changes in insulin signalling

Through a combination of our *in-vitro* and *in-vivo* results presented it has been suggested that an Emb-LPD is capable of inducing lasting changes within the NSC causing impaired neurogenesis. As the trainset protein restriction in the Emb-LPD occurs before both neurulation and gastrulation, the epigenetic changes, if any, must occur within the embryonic stem cells before the NSCs have been formed. Similarly to the TE and PE lineages, changes within the uterine fluid must be translated to embryonic stem cells, so as to modulate foetal development. One possible mechanism in which epigenetic changes could cause functional changes in gene expression, so as to lead to an enhanced neural differentiation, could be through aberrant signalling within the insulin receptor pathway. A rat model fed a LPD indicted that when combined with ageing a statistical difference in a variety of histone modification was observed. A significant increase in the expression of H3K9me2 and H3k27me3 and decrease in the expression of H3Ac and H3K4me1 was observed at the P2 promoter–enhancer region for the hepatocyte nuclear factor 4-α (HNF-4α)[156]. HNF-4α is a transcription factor that is required for pancreatic β-cell differentiation and glucose homeostasis. The post-translational histone modification seen in response to a LPD and ageing was shown to cause a general silencing of the Hnf4a locus[156]. Therefore, this indicates a scenario in which insulin secretion could become impaired [156].

Impaired insulin levels in the uterine fluid could lead to altered NSC differentiation. Both insulin-like growth factors 1 and 2 (IGF1 and IGF2) have been shown to act as major regulators of brain growth and differentiation [157]. Binding of IGF2 to the insulin receptor has been shown to maintain neural stem cells within the brain whereas binding of IGF1 and insulin to the insulin receptor was shown to induced NSC proliferation [158] [159]. A series of experiments investigating the effects of maternal LPD or Emb-LPD on the expression levels of insulin, IGF1/2 and insulin receptor have

indicated that in general a LPD is associated with decreased receptor and ligand expression. As previously mentioned exposure to a maternal Emb-LPD was associated with decreased levels of insulin within the maternal blood and uterine fluid during blastocyst formation. Investigation into the effects within the offspring additionally indicated that exposure to a maternal LPD in a rat model induced a decreased plasma insulin and insulin-like growth factor (IGF) expression within the foetus. This was then subsequently associated with a smaller body and brain size and reduced vasculature throughout the cerebral cortex at birth. The reduced vasculature was then also shown to remain throughout life, indicating long lasting morphological changes within the brain [160]. Similarly, exposure to maternal LPD in a separate rat model also indicated changes that persisted into birth. A 30% reduction in IGF-1 level in the neonatal plasma and 60% reduction within the liver were seen in LPD neonates compared to those fed a standard diet[161].More recently a study into the effect of protein restriction and methyl supplementation during gestation also indicated that a low protein diet induced a reduction in IGF2 and insulin receptor protein expression within the hippocampus at 21 days postpartum[127]. These effects were subsequently associated with reduced neural stem cell proliferation, reduced expression of neurones and increased expression of astrocyte progenitors in-vitro. These studies suggest that a maternal LPD diet during gestation can cause a decrease in the levels of insulin within the uterine fluid. This could then lead to altered levels of insulin, IGF1/2 and insulin receptors within the foetus that persists throughout birth and later life. The altered insulin signalling may therefore be sufficient to modulate neural stem cell differentiation and proliferation, impairing neurogenesis leading to altered behavioural phenotypes.

2.5.3 Enhanced NSC differentiation may occur via changes in fatty acid delivery across the placenta

Many factors may induce and contribute to the distinct embryonic programming mediated through maternal Emb-LPD and LPD, that may influence fetal brain development. Triglycerides cannot be transported across the placenta, therefore the essential fatty acids must be first formed in the maternal liver before being transported to the foetus, and fatty acids play a critical role in normal brain development. Whilst discussed earlier that an mTORC1-mediated pathway may induce embryonic programming mediated through maternal Emb-LPD and LPD. One candidate linking maternal LPD with offspring brain phenotype is docosahexaenoic acid (DHA). DHA concentration has been shown to be reduced in maternal liver and plasma after maternal LPD leading to a specific impaired accumulation of DHA in the offspring fetal brain [135 162]. DHA has been shown to increase neurosphere formation [163], which is consistent with the decrease in neurosphere formation shown here with LPD and Emb-LPD. As previously mentioned an Emb-LPD has been shown to effect the TE

and PE lineages. The PE cells are the progenitor of the visceral endoderm epithelial layer of the yolk sac [19] . Previous studies have indicated DHA is found within the visceral yolk sac therefore epigenetic changes within the PE cells may alter the transfer of DHA across into the foetal brain before choriollantoic placenta is formed[164].

An investigation into the epigenetic changes observed within the PE cells, in response to a maternal Emb-LPD, indicated a significant reduction in the acetylation of histone H3 and H4 within the Gata 6 promoter[165]. These changes were associated with decreased levels of both Gata 6 protein and gene expression, indicating a scenario where changes in the epigenome result in significantly altered protein levels. As Gata 6 has previously been associated with PE differentiation, this demonstrates a mechanism in which epigenetic modulation can lead to functional changes causing changes in endocytosis within the PE cells [165] .

DHA has also been shown to increase neuronal differentiation (beta-III-tubulin and MAP-2 positive cells) by decreasing Hes1 and increasing p27, thus leading to a cell cycle arrest of the NSCs [166]. Hes1 itself is important for NSC maintenance [167] [168]. This was confirmed by others showing increase in beta-III-tubulin and MAP2 via activation of the PKA and CREB pathway [169]. This might explain the effect of LPD on NSCs, but what about the Emb-LPD? In our Emb-LPD group, the LPD diet stops at E3.5, a few days before the NSC population is formed. However, the half-life of DHA in the rat brain (several weeks) [170] suggests that the effect of LPD may be long lasting and potentially retained in Emb-LPD up to the time of *in vivo* analysis. In previous data from our group we have shown interestingly that the LPD/Emb-LPD effect is rescued in secondary neurosphere formation. This might be due to the presence of linolenic acid, a precursor of DHA in the B27 supplement used in the cell culture medium. Moreover, the BCAAs are all present in the Neurobasal-A medium used. Thus, these nutrient supplements *in vitro* may not be available for sufficient time to rescue primary spheres (7 days) but able to rescue NSCs or their progeny in secondary spheres (14 days). A role for DHA in the propagation of LPD and Emb-LPD effects on fetal brain development would be a nice subject of a future study.

2.5.4 A mild maternal protein restriction during gestation may lead to aberrant positioning of GABAergic and glutamatergic neuronal cell bodies

At E14.5 the cortex and GE represents brain regions dominated by the presence of glutamatergic and GABAergic lineages respectively [171]. Therefore the early formation of neurones within the cortex may indicate a scenario in which the glutamatergic neurones reach their target neocortical layers before the tangentially migrating GABAergic neurones. Thus slightly offsetting the cell bodies, leading to different patterns of synaptogenesis resulting in aberrant neuronal wiring.

Synaptogenesis between the inhibitory and excitatory neuronal subtypes has been shown to be critical in ensuring normal cognitive function. Disruption of the excitatory pyramidal neurones and paravalbumin (PV)-positive GABAergic interneurons in the cortex and hippocampus have been shown to produce similar cellular, molecular and behavioural characteristics to that seen in schizophrenic patients [171 172]. Alternatively the enhanced production of late progenitors within the ganglionic eminences may also lead to early migration of the GABAergic neurones, meaning that formation of the different neocortical layers remains unimpaired. This misbalance of glutamatergic and GABAergic neurones could possibly be the reason for the behavioural phenotype we see in the females of the Emb-LPD[11].

Apart from its effect on NSCs, we show here that maternal LPD has an effect on neuronal differentiation. Indeed, LPD decreases the proportion of early progenitors while it increases the proportion of late progenitors. These results could be explained by a specific alteration of the markers Nestin and beta-III-tubulin expression. Indeed, higher expression of beta-III-tubulin in early progenitors or higher expression of Nestin in late progenitors would have led to higher proportion of late progenitors and lower proportion of neurons respectively. Such altered expression of Nestin has for example been observed in ischemic tissue damage [173], or following maternal restricted diet in the postnatal hippocampus [127]. Alternatively, maternal LPD during fetal development (as opposed to Emb-LPD) induces inhibition of differentiation of late progenitors into neurons. This disconnection between progenitor and neuron numbers has been described before in other contexts, where an increase in progenitor cells is not fully translated into the generation of mature neurons following a calorie-restricted diet [174] or in hippocampal adult neurogenesis [175]. In our model, the mechanism ensuring the elimination of excess neurons is conserved in LPD, whereas it is disturbed in Emb-LPD, suggesting that an early event in embryo development still affects neurogenesis days later E14.5 is the time in cortex development where 70% of the cells generated undergo programmed cell death [176] and our data suggest LPD and Emb-LPD could affect this process, as also identified in undifferentiated embryonic stem cell lines [154].

2.6 Conclusion

In this result chapter we have shown that maternal LPD has an effect on neuronal differentiation . Indeed, LPD decreases the proportion of NSCs and early progenitors at E12.5 and E14.5 while it increases the proportion of late progenitors and neurons.

The proposed maternal LPD induction of altered developmental programming through BCAAs [14 153] may also act via the growth factors BDNF and GDNF which protect against neuronal cell death and are regulated through BCAA availability [177]. We speculate that a deficit in these factors might be

responsible for the neurogenesis defect seen in our model. Moreover, loss of BDK (branched chain amino acid ketoacid dehydrogenase kinase), which decreases the BCAA concentration, has been shown to induce epileptic seizures and autistic features, which both could have developmental origins and be thus the consequences of the defects observed at E12.5, E14.5 and E17.5 [178] [179]. These symptoms were reversed by a BCAA supplementation of the diet [179]. Moreover, leucine-deficient diet has been shown to specifically affect the cortex with an upregulation of Tibr3 in the anterior piriform cortex, the region involved in the detection of essential amino acid intake imbalance [180].

In conclusion, we find that maternal protein restricted diet, even before embryo implantation, can alter the developmental programme and lead to deficits in foetal brain development, reducing NSCs and altering the dynamics of neuronal differentiation. As discussed, our data are consistent with early embryo sensing of dietary quality, which can have adverse consequences on foetal brain development and lead to adult offspring changes in behavior, suggesting why behavioural defects were only observed in the Emb-LPD cohort [12]. As the protein restriction was contained to the pre-implantation period, the mechanisms that alter the differentiation potential are likely to be due to a series of epigenetic changes induced within the ICM of the blastocyst. Therefore like the epigenetic modification suggested to lead to increased incidences of cardiometabolic disease, the changes in the epigenome will likely impact multiple organs[165] .

Overall this chapter has indicated that a mild protein restriction contained to the preimplantation phase is capable of significant impacts on the foetal brain.

2.7 Limitations of experimental design

I would possibly increase the n number to increase statistical power as there several strong trends within my data that may become significant.

Neurosphere assay could have been confirmed with other methods such as the neural colony forming assay but this could not be completed due to time constraints.

Additional flow cytometry markers could have been used in experimental design. The experimental design used, only highlighted the presence of neurones, neural progenitors and NSC. The remaining unstained cells indicated a large group of live cells that may influence neurosphere function and formation. Previous studies have indicated the presence of multiple glial lineages within the neurospheres [181]. One study has even shown that neurosphere formation caused the NSC to favour a glial linage when compared to the more traditional free-floating cellular aggregate method. This favouritism was then associated with an increase in migrational activity, possibly suggesting that

inductive signals released from glial lineages helps drive neurosphere formation and growth [182]. Additional experiments to analyse the contribution of glial lineages is therefore needed.

It would have also been interesting to look at the cell cycle with FACS, a more detailed analysis of the cell cycle would be interesting. To see if the diet had affected the cell cycle of the neural stem cells. Previous work has shown that cell cycle is altered in offspring of the LPD diet, however this has never been done in the Emb-LPD and see if it was also altered[113]. I would do this with Live/ Dead flow cytometry with propidium iodide this dye is stoichiometric, i.e. they bind in proportion to the amount of DNA present in the cell. In this way cells that are in S phase will have more DNA than cells in G1. They will take up proportionally more dye and will fluoresce more brightly until they have doubled their DNA content. The cells in G2 will be approximately twice as bright as cells in G1. This was we can identify if the proliferating neural stem cells had been affected by the diet.

Chapter 3 The effect of maternal low protein diet on the adult offspring behaviour.

3.1 Introduction

From the previous studies of Watkins et al, using the same maternal protein restriction diet model I use in this thesis, they foiund that adult female offspring of Emb-LPD had increased movement and rears in the repeated open field test[12]. This study highlights that an early dietary disruption during pregnancy can affect the offspring behaviour. One of the key epidemiological study that can link what we see in our mouse model is the Dutch hunger winter, people born after the famine found that the maternal malnutrition had a profound effect later in life [24]. The supplies of rations were 400-800 calories a day and food rations mainly consisted of bread and potatoes therefore not rich in protein. This was supplied to all residents including pregnant women regardless of social class. Detailed medical records of these people provided an excellent example to later support the DOHaD hypothesis. The post war period was prosperous after the famine. This study highlighted the importance of nutrition during critical periods of development that increased the risks of diseases in later life. Glucose intolerance was prevalent regardless of timing of the period of famine during pregnancy; however, those exposed to famine in early gestation had increased likelihood of obesity and neurodevelopmental disorders [26 183]. This would show similarities between the data we see in Chapter 2, in the our Emb-LPD has significant changes on the neurogenesis of the fetal brains.

Offspring of the Dutch hunger winter had an effect on neural development among the cohort conceived during the height of the famine; an increase in congenital neural defects, especially neural tube defects including spina bifida and anencephaly [27]. This early effect seen in the foetal neurodevelopment supported the hypothesis that prenatal famine could cause neurological problems. This led to a more long-term study of whether the famine caused a long term behaviour abnormality, and it was shown there was an increased risk of schizophrenia [24 28 29]. The exposed cohorts were chosen when the height of the famine corresponded to the periconceptional period or early gestation for this cohort. The Dutch psychiatric registry was used to compare psychiatric outcomes in adulthood for exposed and unexposed birth cohorts as showing the relative risk. The study found a 2- fold increase in the risk of schizophrenia in the exposed birth cohort[30]. Moreover, a subsequent study showed a 2-fold increased risk of schizoid personality disorder in the same exposed birth cohort[31]. Later analysis of the disease risks for successive birth

cohorts of 1944–1946 revealed striking peaks in the incidence of schizophrenia, schizoid personality, and congenital neural defects in this same birth cohort[30].

The Chinese Famine Study the relation of prenatal famine to risk of schizophrenia was successfully examined in a cohort in the Wuhu region of Anhui Province, China in the late 1950s [33]. A massive famine was precipitated in China by the marked social and economic change as the country farming communities radically changed and people went to work in the industrial cities. In the Chinese study, data on caloric rations were not available. However, they could examine whether the risk of schizophrenia was increased in the birth cohorts conceived during the height of this famine. The schizophrenia outcomes were obtained from systematic review of the health records and saw a marked increase in the incidence of schizophrenia[28]. Almond et al[186] also reported from a cohort of muslim women in Uganda, Iraq and the US, that the prevalence of disability in adulthood is increased by 20% in individuals whose mothers Ramadan fasted during early pregnancy, with an impaired cognitive function is increased by 50%.

These human study have lead to a number of different rodent model studies of specifically protein restriction during pregnancy and lactation. Recently a study by Reyes-Castro *et al,* demonstrated that a maternal LPD during pregnancy and lactation impaired offspring learning and motivationl behaviour [184]. Similar impairments in learning and memory were described after undernutrition [185].

As discussed in my previous chapter we have observed an enhanced neuronal differentiation in the Emb-LPD foetal brains. However, Watkin's previous behavioural analysis of the LPD model was very limited. The open field has many different interpretations; the open field activity can be used to assess locomotor activity levels of mice, which can be correlated with locomotive function, however it also quite regularly used to assess anxiety-like and exploratory behaviours [8]. However, the open field activity monitoring system also has significant challenges associated with it. The purpose of the experiments in this chapter is to further characterise using a range of behavioural methods throughout the life course to further identify the adverse behavioural phenotypes of both females and male offspring.

3.2 Aims

The aim of this study we will investigate the effect of maternal dietary restriction on adult offspring in terms of the following behaviours: neurological development; cognitive function in association with memory; coordination and anxiety-like behaviour

3.3 Methods

Eleven virgin female MF-1 were randomly allocated on to to one of three isocaloric dietary treatment groups upon positive plug, as detailed in section 2.3.2. A total of 72 MF1 mice were held from infancy to adulthood, up to and including post-natal day (PND) 105. Mother mice were housed singly for the duration of pregnancy. At term, each litter size was reduced to 4 females and 4 males respectively, where the day of birth was taken as post-natal day PND 0. Once weaned at PND 21, subjects were held per gender in groups 4 accordingly. All mice were held under standard laboratory conditions. Behavioural analyses were performed according to the timeline provided (Table 3.1).

PND	Behavioural Test
2-8	Righting reflex
6-11	Negative geotaxis
21	Weaning and litter size normalized
29	Open field test
35	Elevated plus maze – Trial 1
41-43	Novel object recognition – Trial 1
64-66	Novel object recognition – Trial 2
68	Rota rod
93-94	Open field test repeat
96	Novel object recognition – Trial 3
98	Elevated plus maze – Trial 2
100	T-maze
107	Social interaction
109	Perfusion and tissue collection

Table 3.1Timeline of behavioural analyses conducted from post-natal day 0 (PND0)

ne of behavioural analyses conducted from post-natal day 0 (PND0)

3.3.1 Righting Reflex Test

Righting reflex test was carried out consecutively between post-natal day (PND) 2 and 8, for all diet groups respectively. Each pup was placed in a supine position on a flat surface. The time was then recorded for the pup to turn over into a normal prone position, with all four paws placed flat on the ground. The latency time was recorded, up until 60 seconds inclusively. If the pup failed to reach the required prone position within the maximum 60 second limit, it was given a failed mark of 0. A scoring system was enforced as detailed in Table 3.2.

Score	Definition
0	Remains in dorsal position
1	Struggles to right itself
2	Slow righting reflex
3	Rights itself

Table 3.2Scoring system for righting reflex test.

3.3.2 Negative Geotaxis Test

Negative geotaxis was carried out between PND 6 and 11, for all diet groups respectively. Pups were placed facing downwards on non-slip board. The time was recorded for the pub to make a 180-degree rotation, to finish with the head facing upwards as detailed in Figure 3.3. The pup had to be vertical for the time to be recorded. The latency time was recorded to a maximum of 60 seconds, again if the pup failed a score of 0 seconds was given. Furthermore, a scoring system was enforced as detailed in Table 3.2. All mice were given 30 minutes' habituation time in the behaviour room before trials began. No habituation time was required in the apparatus the day before.

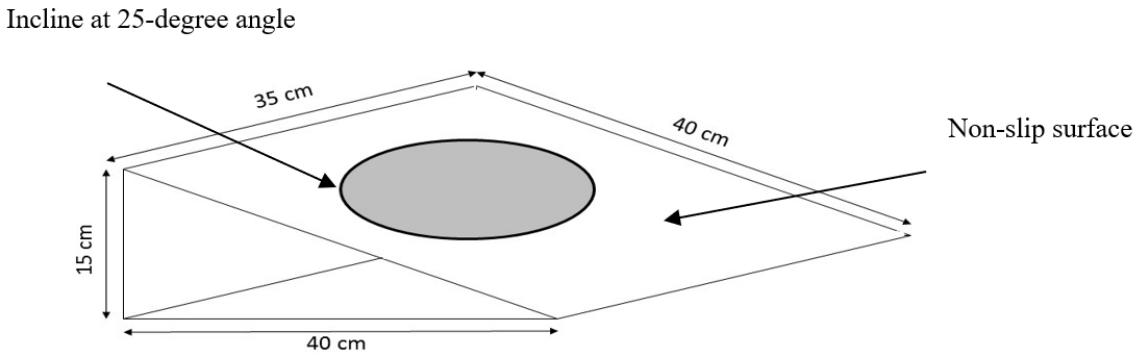


Figure 3.1. Schematic of apparatus for negative geotaxis test.

Score	Definition
0	Does not rotate
1	Struggles to rotate upwards
2	Slow rotation
3	Good rotation

Table 3.3. Scoring system for negative geotaxis test.

3.3.3 Open field test (OFT)

The open field apparatus, which is shown in Figure 3.2, is comprised of an open-topped box, of dimensions' length 28.5 cm x width 28.5 cm x 28.5 cm. The arena consisted of a white floor and glass walls. A video camera was fixed above the box and used to record all OFT trials. 70% ethanol

was used to clean the box between each trial. The day before the experiment was due to start, the mice were individually habituated in the arena for 5 minutes per individual, thus accounting for day 1 of the experiment time point. During trial days 2 and 3, the mice were habituated for 30 minutes in the behaviour room, in their home cages before the first trial began.

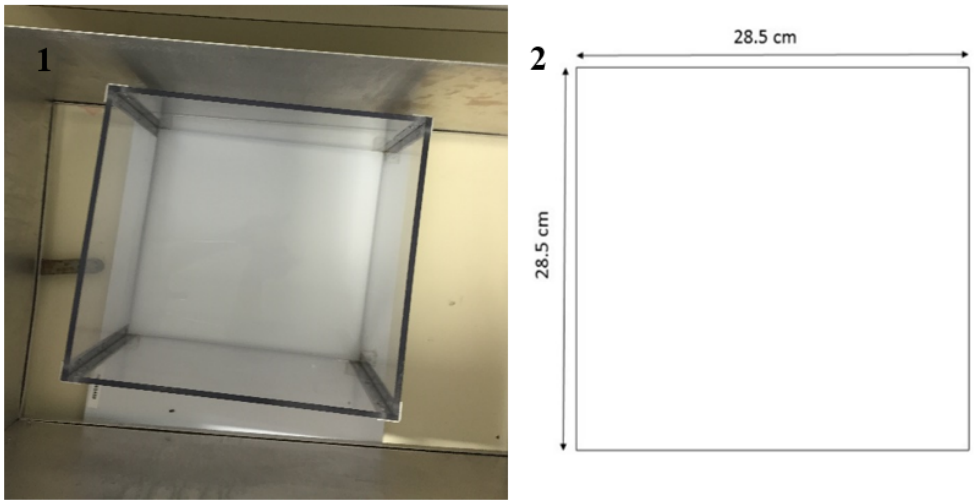
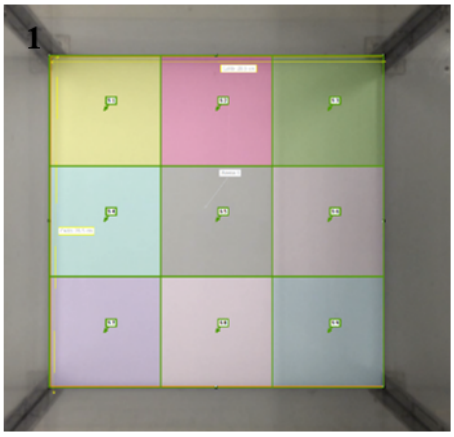


Figure 3.2 Photograph (1) and schematic representation (2) of the open field arena

OFT was carried out at postnatal days 29 and 93 for all three batches of mice. Prior to the trial, the mice were given a drop of non-toxic black paint, on their dorsal side from behind the head to the base of the tail. This was to provide contrast in colour between the subject and the arena background. To begin the trial, mice were placed individually in the arena into the central zone. The behaviour was then recorded via video for a total of 5 minutes. Mice were returned to their home cages upon the end of the trial.

Open field behaviour was collected using EthoVision XT 11.5, video recordings for all trials were blinded and scored manually. The arena was divided into 9 zones as demonstrated in Figure 3.3. where zone 5 represented the central zone. Acquisition began when the mouse was detected within the arena, and ran for 3 minutes.

1



2

1	2	3
4	5	6
7	8	9

Figure 3.3 Arena zoning system by photograph (1) and schematic representation (2)

The following behaviours were scored and defined as follows:

1. Total distance moved in 3 minutes (cm)
2. Average velocity in 3 minutes ($cm.sec^{-1}$)
3. Central zone frequency (zone 5), which is defined as the number of line crossings into the central zone.
4. Central zone cumulative duration, which is defined as the time in seconds the mouse spent in the central zone
5. Time spent in outer perimeter (zones 1,2,3,4,6,7,8,9).

Behaviours 1 and 2 were used as indicators of exploratory activity, where higher values indicate greater exploration and less anxiety. Behaviours 3 and 4 demonstrate the amount of time spent in an exposed central zone, where less time spent in the central zone is associated with higher anxiety.

3.3.4 Novel Object Recognition (NOR)

The NOR test is performed in the same arena as the open field test (see section 2.2.3). Two objects in different combinations were placed at equal distance from the top left corner and the bottom right corner respectively, as demonstrated in Figure 3.4. The two objects used were a can of 7 cm height and 3 cm diameter, and a cell culture flask of equal height and 3.5 cm width. A video camera was placed above the arena to record all trials. Both objects were too heavy to be moved by the mice during the trials. 70% ethanol solution was used to clean all apparatus, including objects between trials. All mice were given 30 minutes' habituation time in the behaviour room before trials began. No habituation time was required in the apparatus the day before.

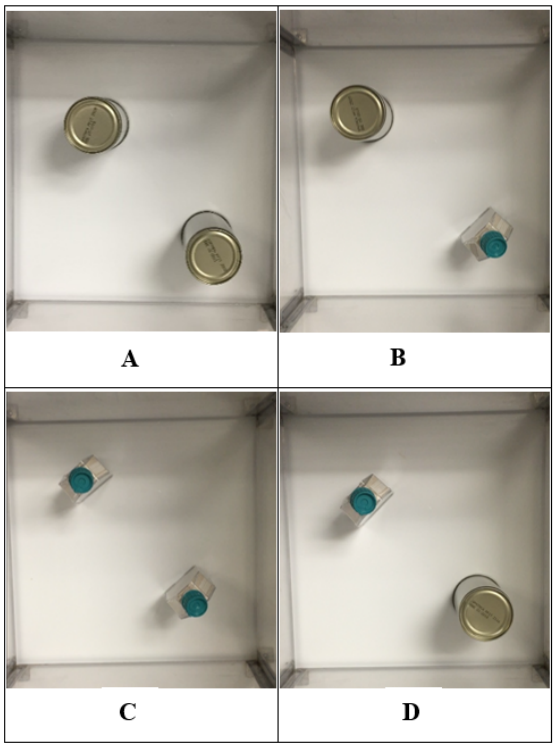


Figure 3.4 Novel object apparatus with different combinations of stimuli

A and C show 2 scenarios where mice are introduced with the same objects, while B and D show scenarios where the top left object remains the same, but a novel object is placed in the bottom right.

The NOR test was carried out at PND 41, 64 and 96, in order to have range of ages from juvenile to adult mice. Mice were selected randomly during the procedure. One complete NOR test consisted of 2 trials, one after the other. The first was the Acquisition Trial, and the second the Retention Trial, between which was an intermittent stage where the mice spent 1 minute in the home cage. The Acquisition Trial saw 2 identical objects be placed in the arena in their appropriate positions, and the mouse placed in the arena facing the top right corner, and trial was run for 4 minutes. During the interval the arena was cleaned using 70% ethanol, including all objects, in order to remove any olfactory trails. One object was replaced for a novel object, ensuring that the positioning of the object was also kept random. The second trial, the Retention Trial, began in the same manner as the first, where the subject was placed facing the top right wall. The mice were recorded for 4 minutes, and then placed back in their home cages.

Exploration behaviour was scored when the mice used their nose to smell or touch the object, no scoring was given when the mice sat on the object [186]. The time spent exploring each object in both the acquisition trial and the retention trial was recorded manually by a blinded individual. The Discrimination Index (DI) was used to quantify NOR, as detailed below:

$$DI = \frac{\text{exploration time of novel object} - \text{exploration time of familiar object}}{\text{Total exploration time of objects}}$$

3.3.5 Elevated Plus Maze (EPM)

The apparatus comprised of an elevated maze, where the dimensions are as detailed in Figure 3.5. All arms were of equal length, at 50 cm x 10 cm. Four arms in total were joined by a central box of 10 cm x 10 cm. Two opposite arms were open i.e. they had no side panels. While the other two arms were enclosed, this consisted of wall panels of height 50 cm, and lids to enclose the top. A video camera was placed above the maze, and recorded each trial respectively. A 70% ethanol solution was used to clean all apparatus between each trial. All mice were given 30 minutes' habituation time in the behaviour room before trials began. No habituation time was required in the apparatus the day before.

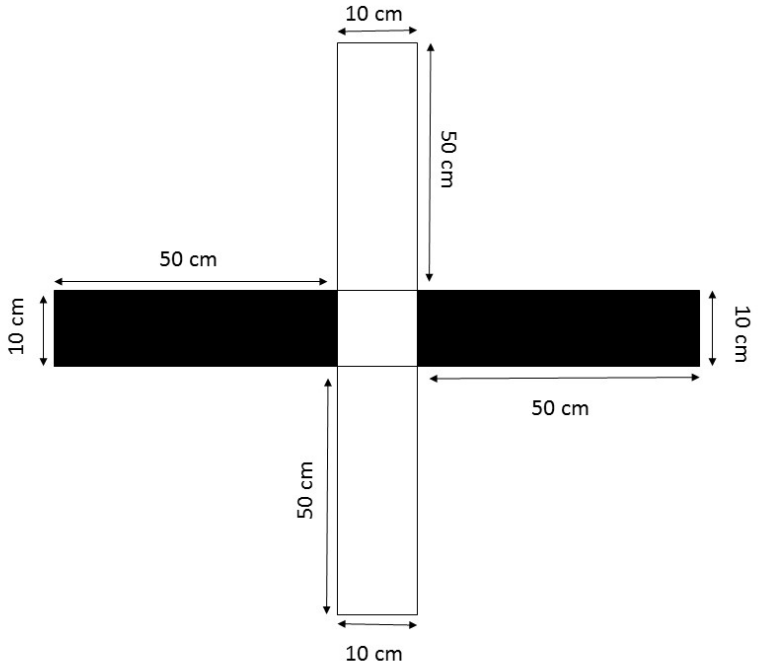


Figure 3.5 Schematic representation of EPM apparatus

The black and white aims represent closed and open arms respectively

EPM was run on two consecutive time points for all batches. These included PND 35 and 98. To begin the trial, each mouse was placed in the central box, facing an open arm as previously described [187]. Each trial was then recorded for 6 minutes respectively. Once the trial was finished, the mice were returned to their home cages. Mice were returned to their home cages upon the end of the trial.

Using EthoVision XT 11.5, video recordings for all trials were scored and analyzed blindly for 4.5 minutes. The cumulative duration (time spent in seconds) and the frequency (the number of times the subject entered a named zone) were recorded for all four arms. This was used to calculate the total time spend in closed arms in comparison to open arms in order to see a difference in behaviour across diet groups. If individuals spent more time on open arms they were less anxious than those that spent more time in closed arms [187].

We also investigated risk assessment behaviour [188] which was defined as how many times the mouse dipped head with a flattened body peered over the edge of an open arm with head, neck, and shoulders as shown in Figure 3.6.

Figure 3.6 Mouse performing risk assessment behaviour

The body is flattened with the head dipped over the edge of the maze.

3.3.6 Rota Rod

The apparatus consisted of a rotating, accelerating (4–40 rpm within 300 s) rod (diameter 3 cm) covered with a ribbed black rubbery surface (TSE, Bad Homburg, Germany). The accelerating rotarod test was performed to evaluate motor coordination, strength and balance of the animals. Animals had 1 trial attempt on the rota rod and tested at 68 PND, with an arbitrary maximum time of maintenance in the rotating rod of 300 s and mice unable to maintain 300s were made to undertake 3 trials separated by 2mins. Latency to fall off the rotating drum (in seconds) was recorded [189].

3.3.7 T-maze

Discrete trial spontaneous alternation in the T-maze was performed as previously described by Deacon and Rawlings[190]. The apparatus for this test consisted of a T-shaped maze with 30 ,10 and 29-cm arms, with a central partition extending 7 cm into the start arm from the back of the maze, and two guillotine doors each having the potential to block off the left or right goal arms. Mice were placed in the start arm of the maze (facing the wall) and allowed to make a choice to enter the left or right goal arm. Following their choice, they were then enclosed in that arm for 30 s to facilitate habituation by sliding down the appropriate guillotine door. Mice were then taken out of the maze, the central partition removed and guillotine doors reopened. Bedding and maze was rearranged to eliminate olfactory cues between all trials. Mice were once again placed in the start arm and allowed to make another free choice of either goal arm. Whether or not the mouse alternated was noted, with a score of 1 given if the mouse visited the other arm reflecting exploratory behaviour and memory of the first choice on its second trial, and a score of 0 given if the mouse went to the same arm on its second trial. The alternation ratio was obtained as the mean of the 20 scores. If animals did not move in 90 s or less these were considered failed trials and were not scored. Approximately 5% of trials were failures and there was no difference in proportion of failed trials between diet groups (P = 0.97). The test was performed four times a day for 5 days (a total of 20 trials) with an average spacing of 2hrs in between each trial at PND 100.

3.3.8 Social Interaction

Social interaction was tested in a clear Perspex open field chamber [28 × 28 × 16 cm] [191]. Each mouse was paired with an unfamiliar age-, weight- and sex-matched MF1 mouse. Both the test mouse and the unfamiliar MF1 conspecific were placed in the chamber simultaneously for a 10 min trial that was captured and recorded using a camcorder mounted above the chamber. Between each test, the chamber floor and walls were cleaned with 70% ethanol.

The diet group of each animal was blinding during both testing and subsequent coding of behaviours. All social and non-social exploratory behaviours were coded from the videos. Eight behaviours were coded and organised into the following behavioural domains: social investigation [anogenital sniffing: sniffing by test mouse of anogenital region of conspecific]; social dominance [walkover: test mouse places its front paws on head or back of conspecific; aggressive following: test mouse rapidly follows conspecific from behind, forcing it to retreat]; agonistic behaviours [pinning: test mouse pins conspecific to floor; clawing]; non-social exploration [rearing to wall; rearing free: test mouse is upright with front paws raised away from wall; sifting: test mouse sifts

through chamber bedding]. Total number of episodes, total duration, and latency to display were determined for each behaviour, initiated by the test mouse at PND107.

In order to minimise effect between mother variation in litter size influencing the data for any one group three to four litters were generated per treatment group, and the data was analysed by mulit level regression analysis showing the maternal litter size and maternal origin unless stated.

3.3.9 Statistical analysis

Data were analyzed using a multilevel linear regression model using PASW for Windows program version 21 (SPSS UK, Woking, Surrey, United Kingdom)**,** in which there was a random effect assigned to each litter. Thus we evaluated both between-litter and within-litter effects. We always included terms for the litter size and for the sex of the offspring. We used indicator variables to compare the Emb-LPD and the LPD with the NPD. This showed that differences identified between treatment groups are independent of maternal origin of litter and litter size. Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The open field test data was an exception and I used paired t-test to analyse this data (PASW for Windows program version 21), were individual animal data points were paired from day 2 and day 3 to see change in exploration of each individual mouse between days.

Data was represented throughout this thesis as a box and whisker diagram due to the data being non-parametric with the exception Negative geotaxis and Righting reflex where continuous histograms were used to represent the progression of mouse reactions over a number of days and genders represented separately to show differences.

3.4 Results

3.4.1 Maternal protein restriction impacts on developmental reflexes in offspring

The righting reflex and negative geotaxis tests were done to assess the basic early developmental reflexes of the developing pup and see if maternal diet had impacted them.

3.4.1.1 Righting reflex data

The results of righting reflex performance, as displayed in figure 3.7 indicate that offspring subject to maternal protein restriction during gestation (LPD), or confined to the preimplantation period (Emb-LPD), displayed no significant difference in righting reflex performance when compared to

the NPD controls (females: P=0.072, males; P=0.903, mothers n=11,). Irrespective of maternal diet, both females and males offspring exhibited the same reflex trajectory over time between PND 2 and PND 8.

Primarily, both females and males show a decreasing reflex time between PND 2 and PND 3, followed by a distinct peak at PND 4. Following this, the reflex time gradually decreases up to and including PND 8. A deviation in this pattern lies in the Emb-LPD male offspring, where the reflex time in fact increases between PND 2 and PND 3. These small observations aside, the results of the righting reflex test show uniformity between offspring of NPD controls and those of maternal protein restriction.

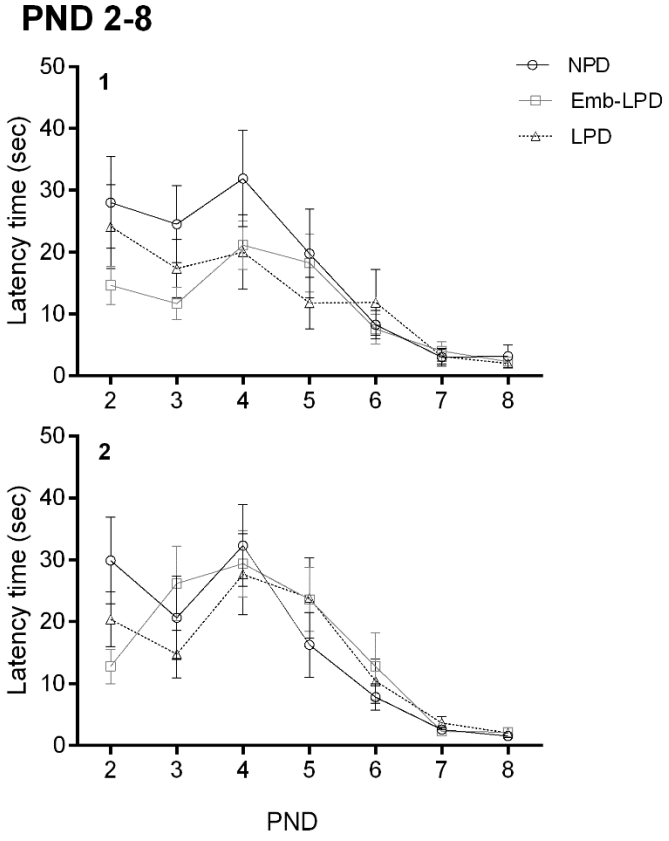


Figure 3.7. Maternal diet has no effect on righting reflex

Righting reflex time for females (1) and males (2), between PND 2-8 for the three diet groups NPD, EMB-LPD and LPD. Error bars showing the standard error of the mean. Latency time in seconds for the mouse to rotate from supine to a prone postion. Both sexes follow the same trend with a distinct increase in reflex time at PND4. Multilevel regression analysis shows no significant difference (females(1) p=0.072, mother n=11, pup n= 43; males(2) p=0.903, mother n=11, pup n= 45) and shows the 3 diets have no effect on rightining reflex.

3.4.1.2 Negative geotaxis test

The results of the negative geotaxis test, as illustrated in figure 3.8, demonstrate significant differences in reflex performance, in both sexes, between LPD/ Emb-LPD offspring and NPD controls (P=0.043; males: P=0.035). In particular, the LPD and Emb-LPD female offspring indicated a significantly greater reflex at PND 7, in comparison to the NPD controls (P=0.0003). Taking on average, 9.98 and 8.88 seconds longer to make the 180-degree rotation to an upwards position. While the LPD and Emb-LPD male offspring demonstrated slower reflex times between PND 8 and 10. In summary, offspring subject to maternal protein restriction, either LPD or Emb-LPD, show a slower reflex performance, in comparison to the NPD controls.

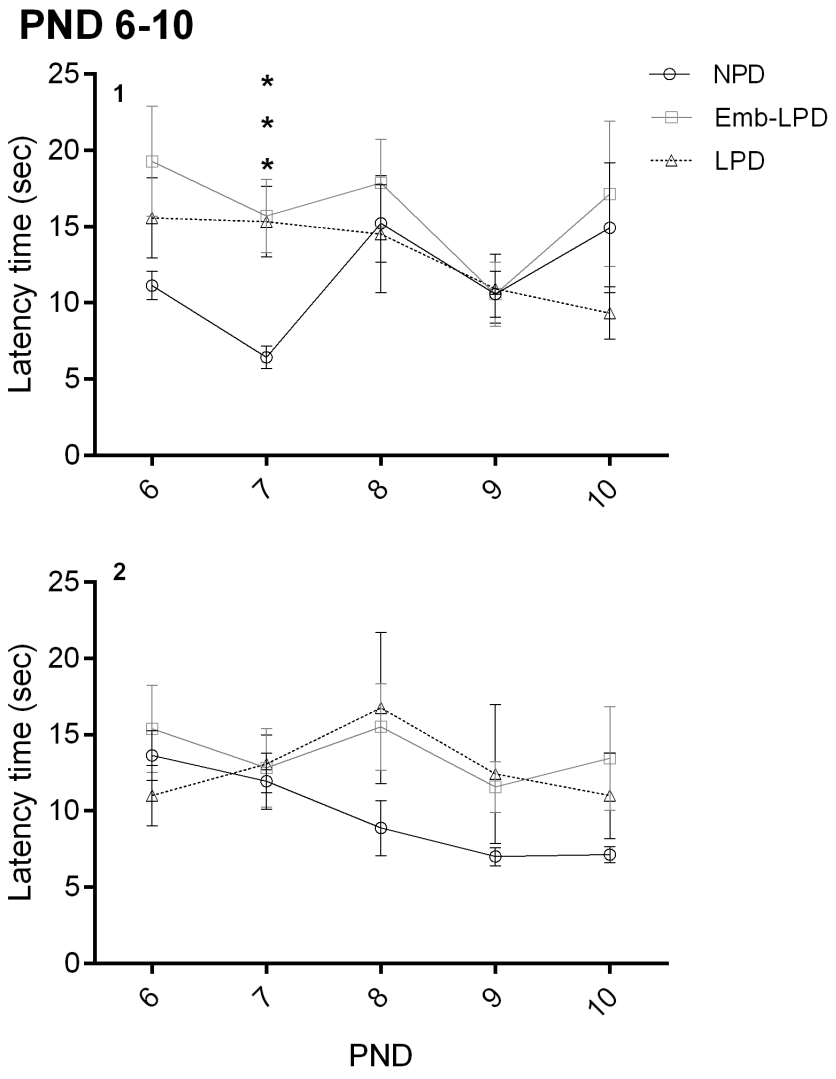


Figure 3.8. Maternal low protein diet slows negative geotaxis reflex

Negative geotaxis time course for females (1) and males (2), between PND 6-10 for the three diet groups NPD, EMB-LPD and LPD. Error bars showing the standard error of the mean. Latency time in seconds for the mouse to rotate 180 degrees. Both sexes show distinct differences, multi-level regression shows (females(1) p=0.043, mother n=11, pup n= 43; males(2) p=0.035, mother n=11, pup n= 45) for the 3 diet groups. Looking at PND 7 females shows a significant difference between NPD, Emb-LPD and LPD (P=0.0003)

3.4.2 Early maternal protein restriction alters cognitive function

Novel object recognition (NOR), T-maze and the open field test (OFT) are used to evaluate cognitive function. NOR is used to evaluate short-term, T-maze evaluates long term working memory and OFT is used to evaluate episodic memory, in which memory is assessed over the space of 2 days.

3.4.2.1 Emb-LPD decreases short-term memory

Figures 3.9, 3.10 and 3.11 illustrate the relative exploration between the acquisition trials and the retention trials, for TP1, TP2 and TP3 respectively. Irrespective of maternal diet, all offspring equally explored the two identical objects during the acquisition trial of TP1. This was true for the NPD offspring (Paired t-test; females: P=0.674, R^2=0.027, n=8 pairs, df=7; males: P=0.252, R^2=0.129, n=11 pairs, df=10), the LPD offspring (Paired t-test; females: P=0.151, R^2=0.311, n=7 pairs, df=6; males: P=0.161, R^2=0.352, n=6 pairs, df=5) and the Emb-LPD offspring (Paired t-test; females: P=0.204, R^2=0.173, n=10 pairs, df=9; males: P=0.144, R^2=0.222, n=10 pairs, df=9).

In comparison, differences become evident at the retention trial of TP1. Both female and male offspring of NPD controls showed a significant preference for the novel object over the familiar object (Paired t-test; females P=0.021, R^2=0.556, n=8 pairs, df=7; males: P=0.012, R^2=0.486, n=11 pairs, df=10). This was further demonstrated by the LPD males (Paired t-test; P=0.039, R^2=0.608, n=6 pairs, df=5) and LPD females (Paired t-test; P=0.0465, R^2=0.294, n=7 pairs, df=6). Emb-LPD offspring however, showed no significant preference for the novel object during the retention trial (Paired t-test; females: P=0.529, R^2=0.045, n=10 pairs, df=9; males: P=0.388, R^2=0.084, n=10 pairs, df=9).

TP2 Again, irrespective of maternal diet, all offspring equally explored the two identical objects during the acquisition trials. This was true for the NPD offspring (Paired t-test; females: P=0.621, R^2=0.037, n=8 pairs, df=7; males: P=0.128, R^2=0.238, n=10 pairs, df=9), the LPD offspring (Paired t-test; females: P=0.732, R^2=0.018, n=8 pairs, df=7; males: P=0.818, R^2=0.010, n=7 pairs, df=6) and

the Emb-LPD offspring (Paired t-test; females: P=0.802, R^2=0.008, n=9 pairs, df=8; males: P=0.413, R^2=0.076, n=10 pairs, df=9).

Again, differences became evident at the retention trial of TP2. Both female and male NPD offspring showed a significant preference for the novel object over the familiar object during the retention trial (Paired t-test; females P=0.005, R^2=0.759, n=7 pairs, df=6; males: P=0.035, R^2=0.407, n=10 pairs, df=9). This is further demonstrated by the LPD offspring of both sexes (Paired t-test; P=0.010, R^2=0.635, n=8 pairs, df=7; males: P=0.003, R^2=0.785, n=7 pairs, df=6). Again, the Emb-LPD offspring showed no significant preference for the novel object during the retention trial of TP2 (Paired t-test; females: P=0.907, R^2=0.002, n=9 pairs, df=8; males: P=0.475, R^2=0.066, n=9 pairs, df=8).

TP3 showed the same irrespective of maternal diet, all offspring equally explored the two identical objects during the acquisition trials. This was true for the NPD offspring (Paired t-test; females: P=0.721, R^2=0.039, n=8 pairs, df=7; males: P=0.228, R^2=0.258, n=10 pairs, df=9), the LPD offspring (Paired t-test; females: P=0.799, R^2=0.022, n=8 pairs, df=7; males: P=0.918, R^2=0.010, n=7 pairs, df=6) and the Emb-LPD offspring (Paired t-test; females: P=0.81, R^2=0.008, n=9 pairs, df=8; males: P=0.513, R^2=0.086, n=10 pairs, df=9).

Again, differences became evident at the retention trial of TP3. Both female and male NPD offspring showed a significant preference for the novel object over the familiar object during the retention trial (Paired t-test; females P=0.001, R^2=0.759, n=7 pairs, df=6; males: P=0.0012, R^2=0.407, n=10 pairs, df=9). This is further demonstrated by the LPD offspring of the males (Paired t-test; P=0.0390, R^2=0.635, n=8 pairs, df=7). However, LPD females no longer show a significant change between novel and same object (P=0.09, R^2=0.985, n=7 pairs, df=6) but the trend remains suggesting with age maybe LPD memory is decreasing. Again, the Emb-LPD offspring showed no significant preference for the novel object during the retention trial of TP2 (Paired t-test; females: P=0.99, R^2=0.002, n=9 pairs, df=8; males: P=0.902, R^2=0.066, n=9 pairs, df=8).

The ability of offspring to discriminate between a familiar object and a novel one, is evaluated by the 'Discrimination Index'. These results of NOR, as illustrated in figure 3.12, reinforce the findings of the retention trials previously described in figures 3.9-3.11. Beginning with TP1 (PND 41), there was a significant difference in DI between NPD offspring and Emb-LPD offspring, for both females and males (females: P>0.0001, mothers n=11, n=25,; males: P>0.0001, mothers n=11, n=27). Moreover, there was a significant difference in DI between NPD offspring and Emb-LPD offspring at TP2; (females: P>0.0001,, mothers n=11, n=25; males: P>0.0001,, mothers n=11, n=26). These differences were not evident for the LPD offspring. TP3 showed some changes.

In summary, these data of NOR indicate that Emb-LPD offspring have a reduced capacity for working memory, in comparison to NPD controls.

Summarizing the findings of the acquisition trials and the retention trials, These data indicate that Emb-LPD offspring have a reduced memory capacity, in comparison to NPD control and LPD offspring.

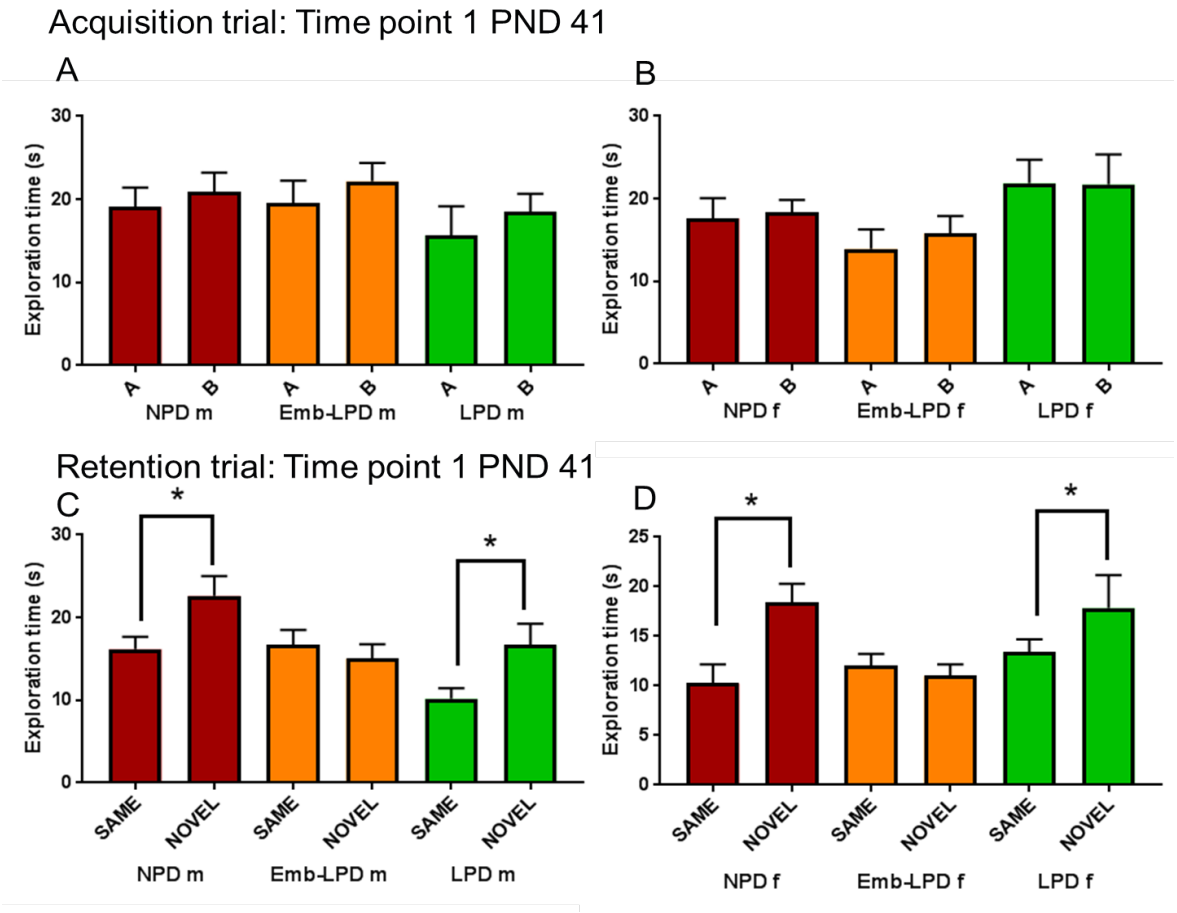


Figure 3.9. Acquisition and retention trials for novel object recognition at PND41.

(A-B) Acquisition trial of time point 1 PND 41 shows the amount of time exploring the identical objects A is male B is females. (C-D) is time exploring same object versus novel objects during the novel object recognition test at males (C), females (D). *$p<0.05$. Data represent 29 female and 30 male from 4 different mothers per diet.

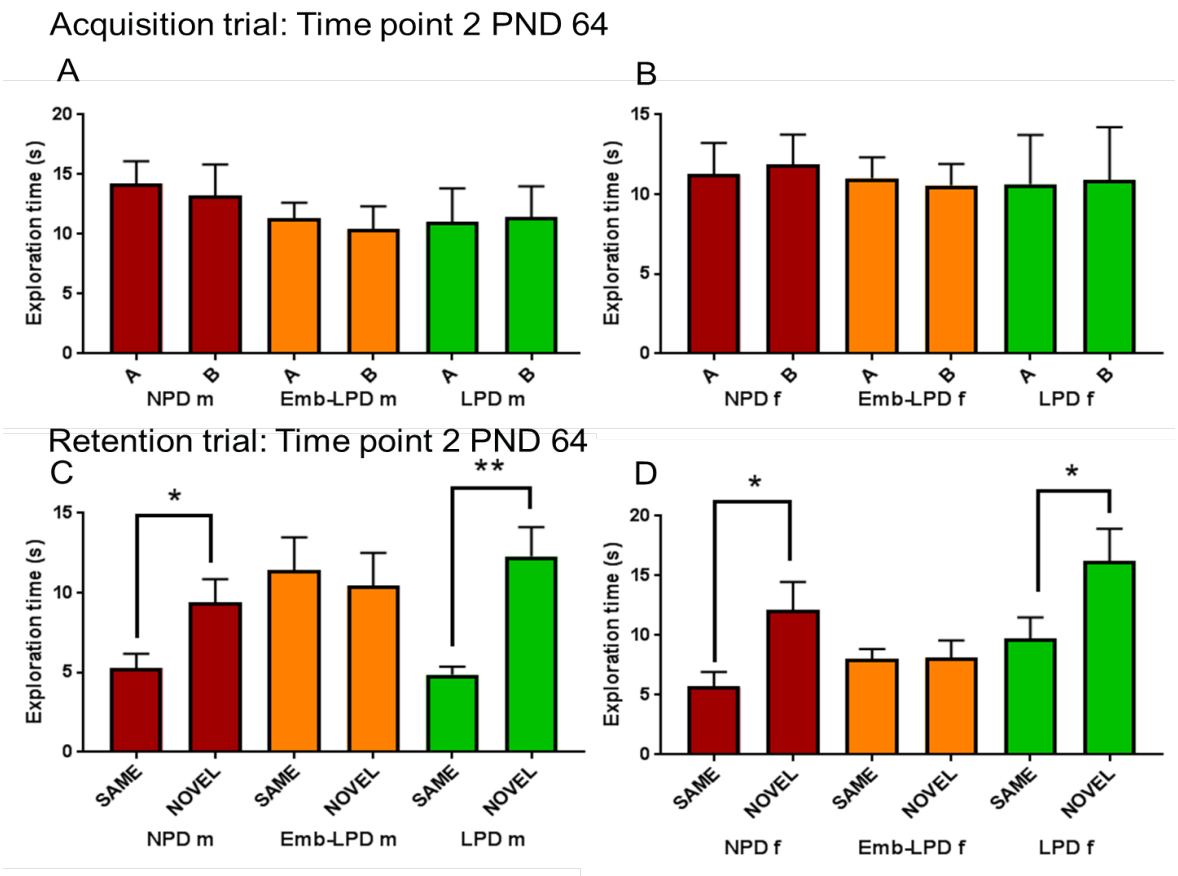


Figure 3.10. Acquisition and retention trials for novel objection recognition at PND64

(A-B) Acquisition trial of time point 2 PND 64 shows the amount of time exploring the identical objects A is male B is females. (C-D) is time exploring same object versus novel objects during the novel object recognition test at males (C), females (D). *p<0.05. Data represent 29 female and 30 male from 4 different mothers per diet.

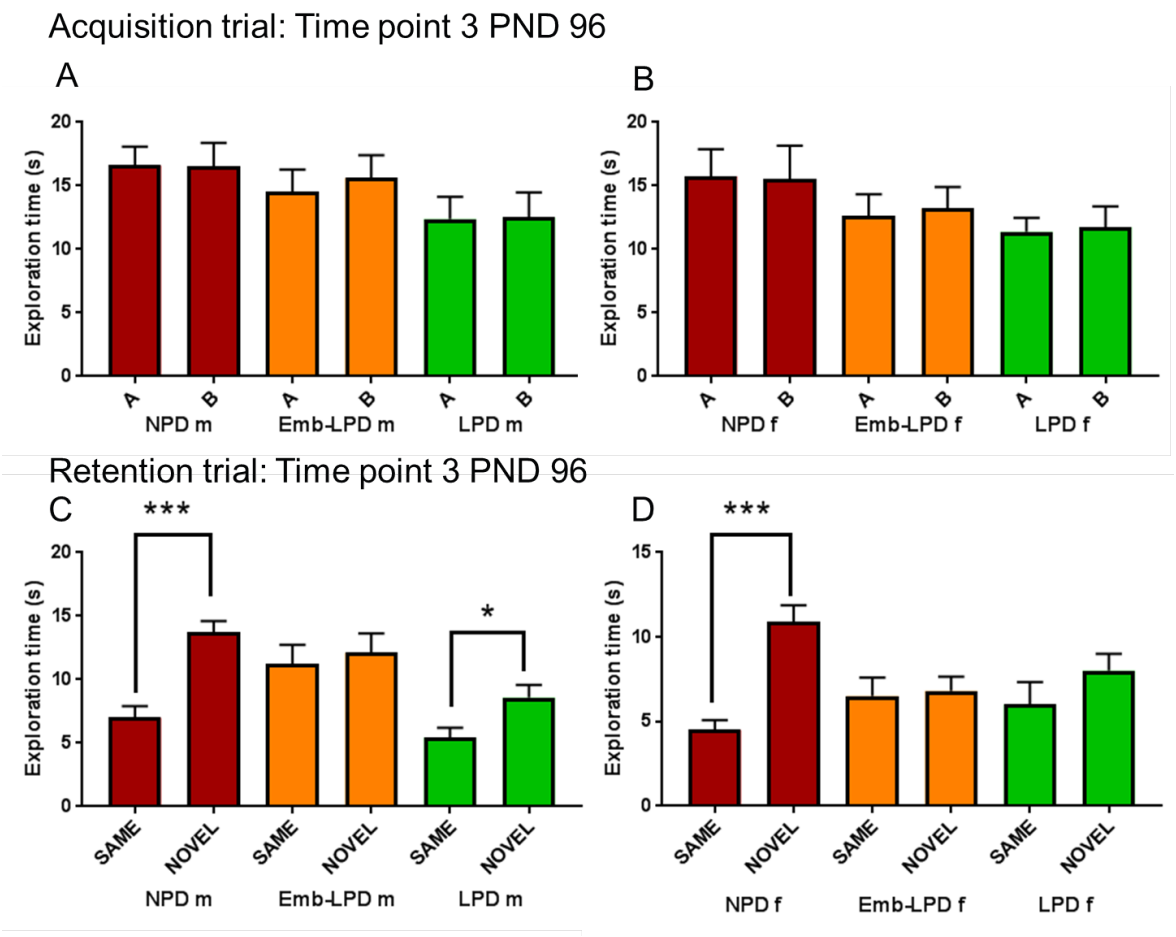


Figure 3.11. Acquisition and retention trials for novel object recognition at PND96

(A-B) Acquisition trial of time point 3 PND 96 shows the amount of time exploring the identical objects A is male B is females. (C-D) is time exploring same object versus novel objects during the novel object recognition test at males (C), females (D). *p<0.05 .Data represent 29 female and 30 male from 3-4 different mothers per diet.

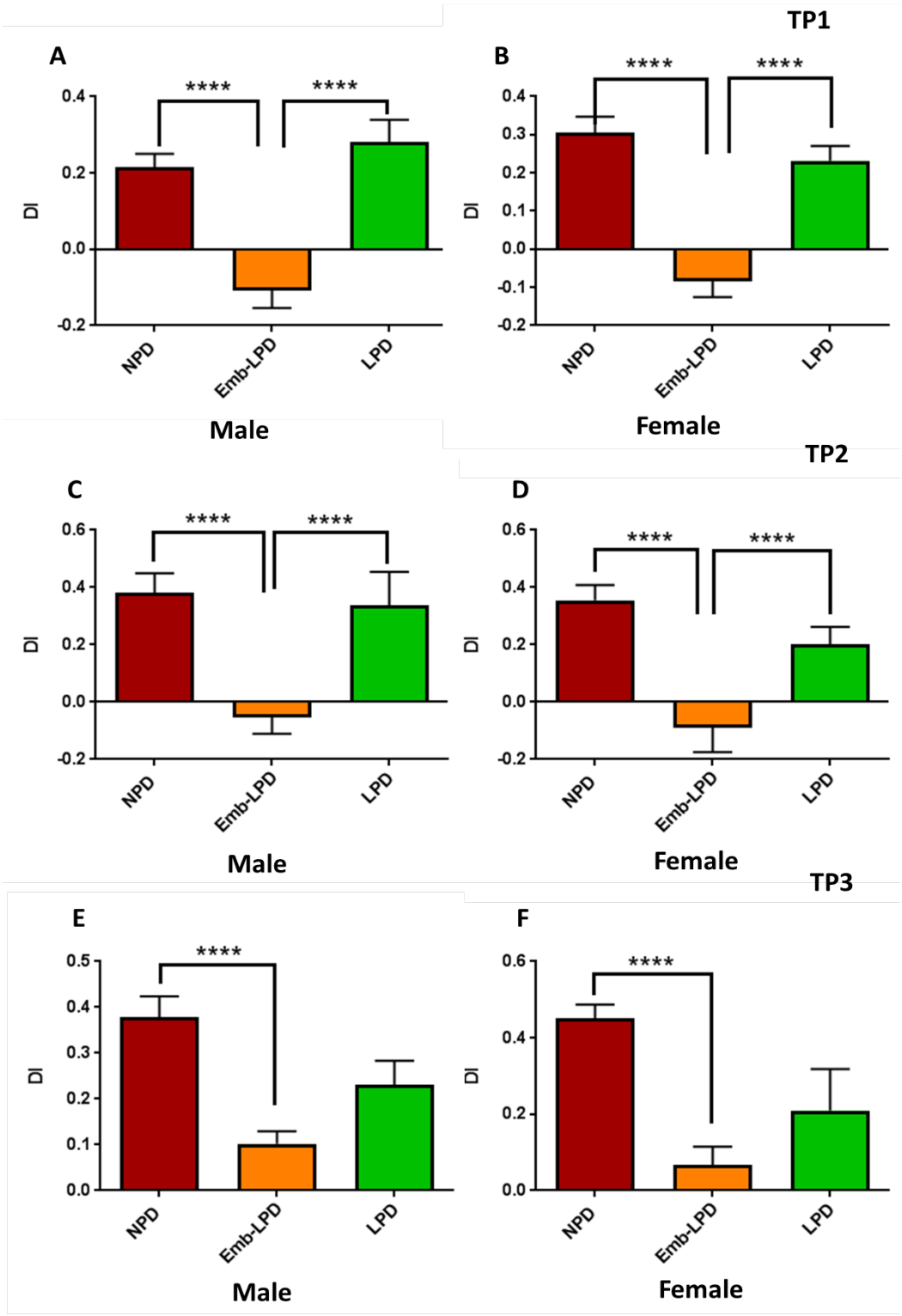


Figure 3.12 Discrimination index during novel object recognition over time

Discrimination index of familiar versus novel objects during the novel object recognition test at 41 days (A, D), 64 days (B, E) and 96 days (C, F). Data is separated by gender into male (A, C, E) and female (B, D, F). Data for exploration time for the novel object (Tn) versus the familiar object (Tf) are presented as the discrimination index DI = (Tn-Tf) / (Tn+Tf). **** p<0. 0001. Data represent 29 female and 30 male from 3-4 different mothers per diet.

3.4.2.2 Diet impairs working memory

The offspring working memory is tested by the spontaneous alternating T-Maze, is evaluated by the '% alternations'. These results of the T-maze, as illustrated in figure 3.13, the results reinforce the findings of the NOR, that the Emb-LPD mice have poor memory (females P=0.01 mothers n=11, n=27,; males: P=0.01, mothers n=11, n=23). However, additionally the LPD males show a significant decrease in their working memory. (females: P=0.465, mothers n=11, n=27; males: P=0.001, mothers n=11, n=23).

In summary, these data of T-maze indicate that Emb-LPD and LPD male offspring have a reduced capacity for working memory, in comparison to NPD controls.

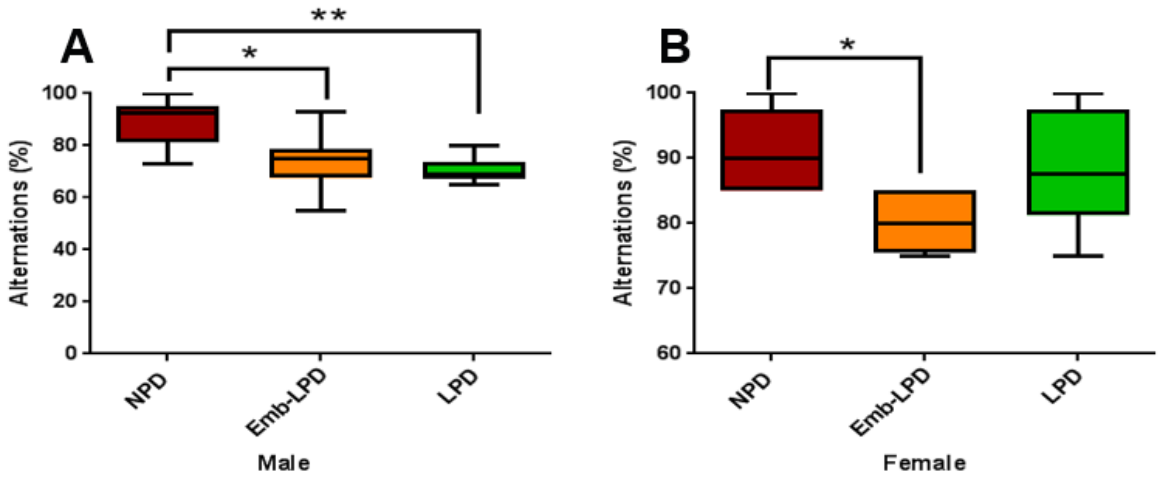


Figure 3.13. Spontaneous alteration in the T-maze at PND100 after maternal diets

Alternation was measured as % election of the alternative arm over 20 trials over 5 consecutive days. (A) represents males and (B) represents females. (females P=0.01 mothers n=11, n=27,; males: P=0.01, mothers n=11, n=23).

3.4.2.3 Open field test evaluation of episodic memory

Results of the open field test (OFT) are categorized into three different parameters. These include distance moved, center exploration and edge exploration. The three parameters are used to evaluate the episodic memory across two days, OFT day 2 and OFT day3 respectively. For the purpose of this study, time point 1 (TP1; PND 29) and time point 2 (TP2; PND 92) are compared. The rationale behind this test, is as follows. If an individual remembers the arena on OFT day 2, then it will explore less on OFT day 3 as a result. This reasoning can be applied to all three parameters

accordingly. Time spent in the centre examines if the mouse has an anxiety phenotype by avoiding the centre.

TP1, as displayed in figure 3.14, LPD and Emb- LPD male offspring showed no significant difference in the distance moved between OFT day 2 and OFT day 3 (Paired t-test; males: P=0.099, R^2=0.544, df=5). In contrast, the NPD male control offspring displayed a significant decrease in distance moved behaviour between OFT day 2 and OFT day 3 (Paired t-test; females: P=0.027, R^2=0.658, df=5; males: P=0.040, R^2=0.358, df=10). The Emb-LPD and LPD female offspring also displayed a significant decrease in the distance moved between OFT day 2 and OFT day 3 (Paired t-test; P=0.002, R^2=0.673, df=9). TP1 Emb-LPD females also showed a decreased distance moved on day 2 and 3 compared NPD (P=0.01).

Considering TP2, the same exploratory behaviour is presented for the NPD control offspring (Paired t-test; females: P=0.029, R^2=0.735, df=4; males: P=0.036, R^2=0.709, df=4) and the LPD offspring (Paired t-test; females: P=0.161, R^2=0.424, df=4; males: P=0.477, R^2=0.133, df=4) as TP1. While, both sexes of Emb-LPD offspring, present no significant difference in distance moved (Paired t-test; females: P=0.289, R^2=0.505, df=2; males: P=0.512, R^2=0.115, df=4). TP2 Emb-LPD and LPD males showed a decreased distance moved on day 2 compared to p=0.01 and p=0.001 respectively.

In summary, these results of OFT distance moved indicate that LPD and Emb-LPD offspring have episodic memoryreduced cognitive function, when compared to NPD controls.

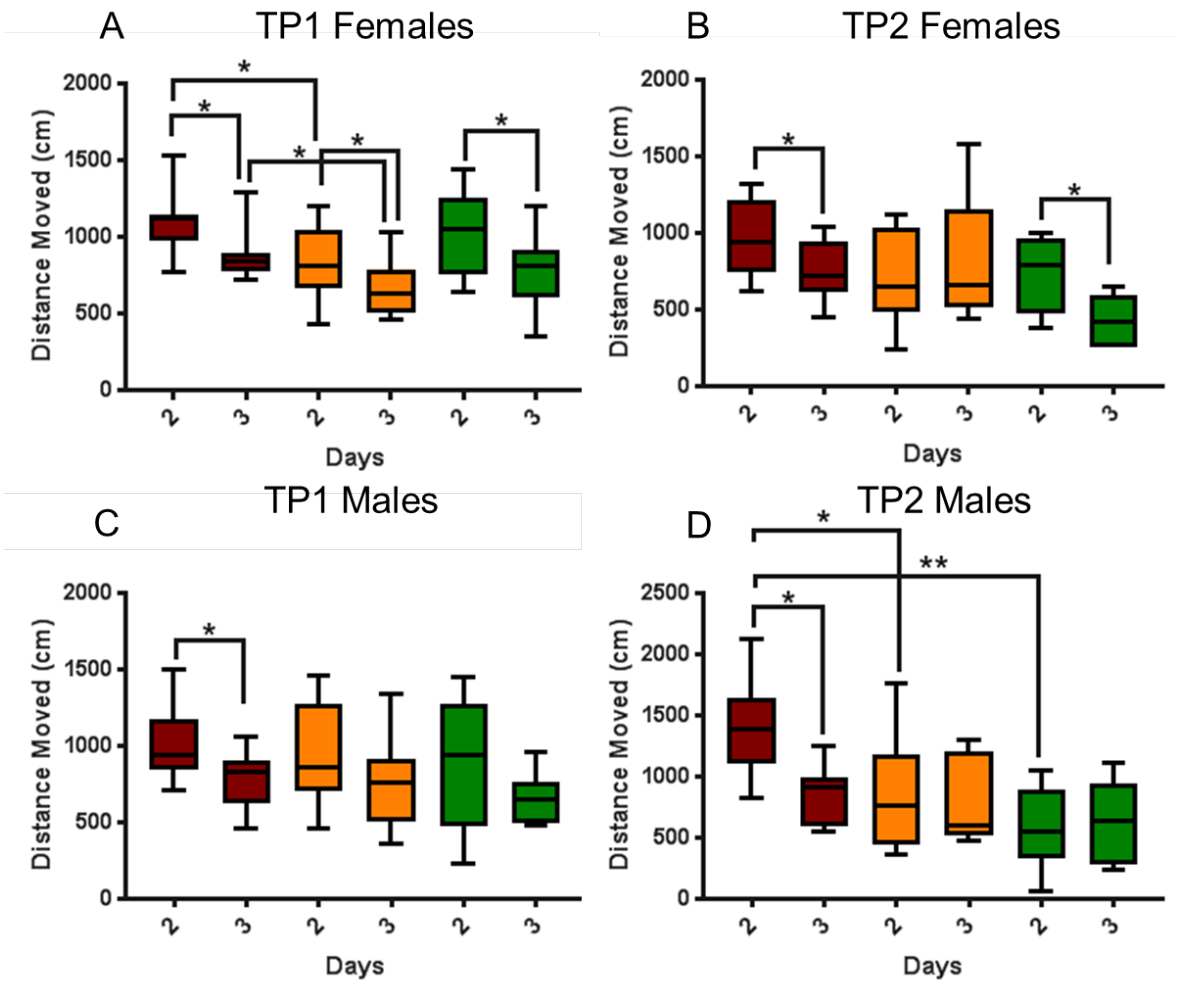


Figure 3.14 Analysis of distance moved in the open field test

A) TP1 PND 29 females, B) PND 92 TP2 females, C) PND 29 TP1 males and D) PND 92 TP2 Males. Distance moved (cm) was compared on days 2 and 3. 3 diet groups were analysed. Paired t- tests were conducted between day 2 and 3. TP1 females NPD=0.027 n=6, Emb-LPD=0.002 n=10, LPD p=0.05 n=8. TP1 Emb-LPD females also showed a decreased distance moved on day 2 and 3 compared NPD (P=0.01) multi-level regression analysis. TP1 MALES NPD p=0.04 n=11, Emb-LPD N=10, LPD n=6. TP2 Females NPD p=0.029 n=6, Emb-LPD n=6, LPD n=6). TP2 Males NPD=0.036, n=5, Emb-LPD n=5, LPD n=6 pairs. TP2 Emb-LPD and LPD males showed a decreased distance moved on day 2 compared to p=0.01 and p=0.001 respectively.

Centre exploration is the another parameter used to gauge the exploratory/ anxiety behaviour of mice during the OFT. Figure 3.15 illustrates the time spent in the center square of the arena**.** Firstly, looking at TP1 (PND 29), the NPD offspring (Paired t-test; female: P=0.831, R^2=0.008, df=6; males: P=0.569, R^2=0.034, df=10), the LPD offspring (Paired t-test; female: P=0.858, R^2=0.005, df=7; males: P=0.073, R^2=0.440, df=6), and Emb-LPD (Paired t-test; female: P=0.424, R^2=0.493, df=8; males:

P=0.423, R^2=0.494, df=8) showed no significant difference between the time spent in the center of the arena on OFT day 2 and OFT day 3. However, the Emb-LPD offspring showed a significant decrease in the time spent in the center between OFT day 2 and day 3. However, when we look at the males at TP1 figure 3.15, on both day 2 and 3 LPD males spend significantly less time in the centre compare to the NPD (P=0.01).

Considering TP2 (PND 92), the NPD offspring (Paired t-test; female: P=0.731, R^2=0.008, df=6; males: P=0.985, R^2=0.034, df=10), the LPD offspring (Paired t-test; female: P=0.899, R^2=0.005, df=7; males: P=0.83, R^2=0.440, df=6), and Emb-LPD (Paired t-test; female: P=0.624, R^2=0.493, df=8; males: P=0.923, R^2=0.494, df=8) showed no significant difference between the time spent in the center of the arena on OFT day 2 and OFT day 3. However, on day 2 LPD Males spend significantly less time in the centre (p=0.01).

This data suggests LPD males spend less time in the centre of the OFT and could therefore possibly be more anxious.

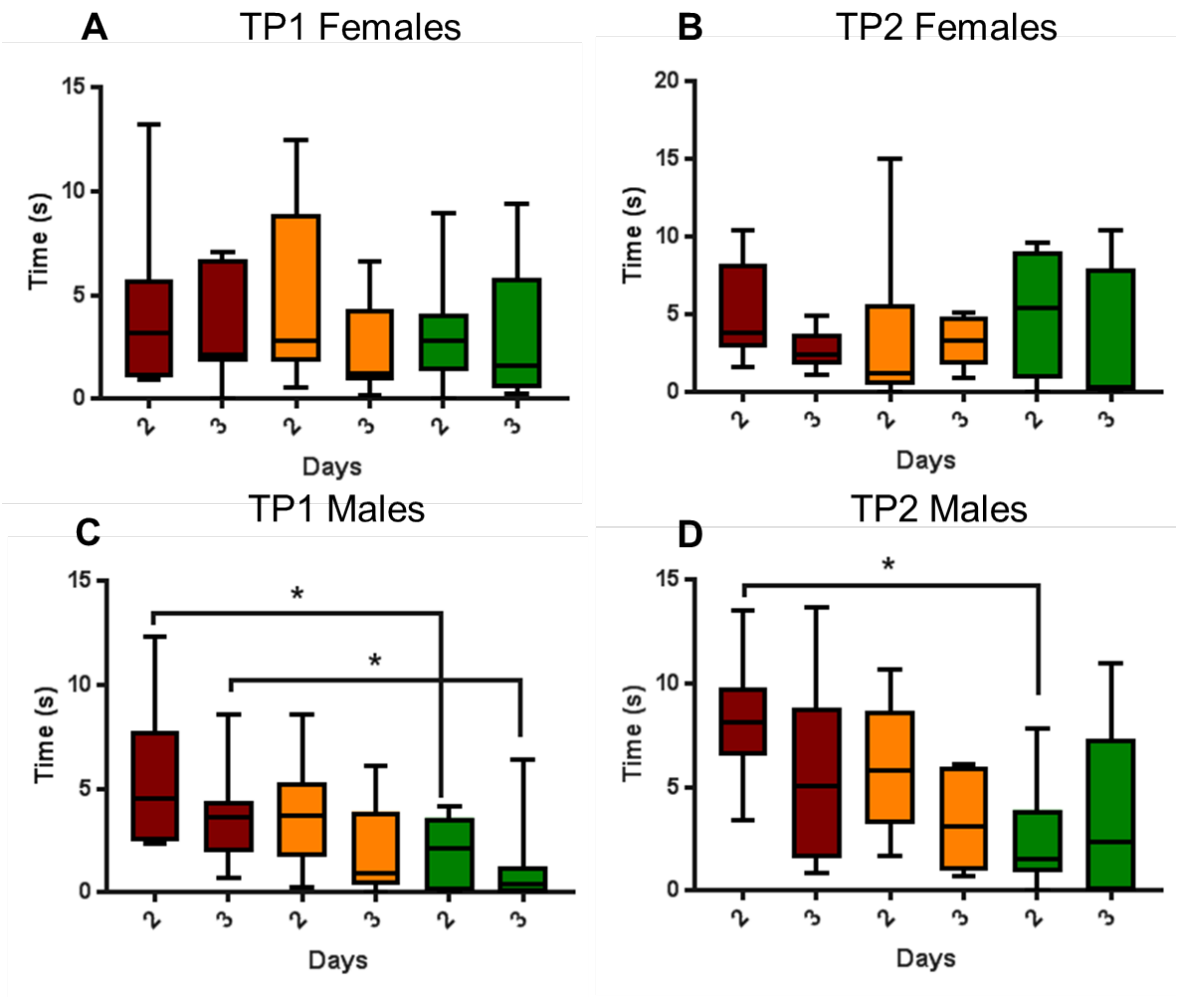


Figure 3.15. Analysis of time spent in the centre of the open field arena

A) PND 29 TP1 females, B) PND 92 TP2 females C) PND 29 TP1 males, and D) PND 92 TP2 Males. Time spent in the centre of the open field area was compared on days 2 and 3 after habituation. 3 diet groups were analysed. Paired t-tests were conducted between days 2 and 3 after habituation. TP1 and TP2 females NPD n=6, Emb-LPD n=10, LPD n=8 showed no difference time spent in the centre of the OFT. TP1 LPD Males day 2 and 3 s spend significantly less time in the centre (p=0.01), NPD n=11, Emb-LPD n=10, LPD n=6. TP2 Females NPD p=0.029 n=6, Emb-LPD n=6, LPD n=6). TP2 LPD males on day 2 spend significantly less time in the centre (p=0.01). NPD n=5, Emb-LPD n=5, LPD n=6 pairs.

Time spent in the edges of the OFT are another parameter used to gauge the exploratory/ anxiety behaviour of mice during the OFT. Figure 3.16 illustrates the time spent in the edges of the square of the arena**.** Firstly, looking at TP1 (PND 29), female offspring showed no significant difference between the time spent in the edges of the arena on OFT day 2 and OFT day 3. However, the LPD male offspring showed a significant increase in the time spent in the edge of the OFT on day 2 and day 3 (p=0.01). Confirming our previous data of time in the centre showing LPD males spend longer in the edges.

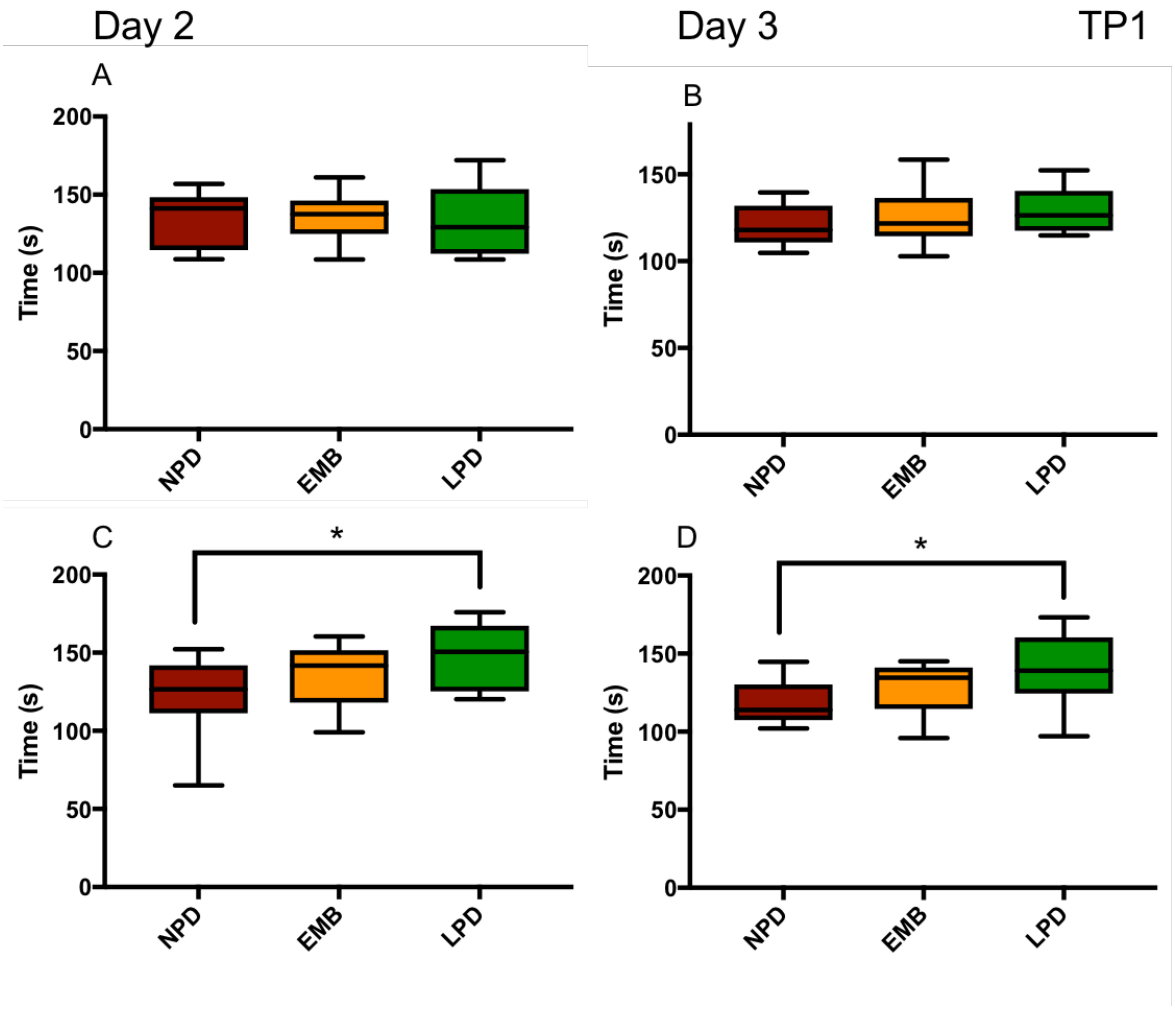


Figure 3.16. Analysis of time spent in the edges of the open field test at PND29

(A and B) TP1 PND 29, females showed no significant difference in time spent exploring the edges females NPD n=6, Emb-LPD n=10, LPD n=8. (C and D) LPD males show an increased time in spent the edges on both day 2 and 3 p=0.01, NPD n=11, Emb-LPD n=10, LPD n=6.

Time spent in the edges of the OFT are another parameter used to gauge the exploratory/ anxiety behaviour of mice during the OFT at TP2. Figure 3.17 illustrates the time spent in the edges of the square of the arena**.** Firstly, looking at TP2, female offspring showed no significant difference between the time spent in the edges of the arena on OFT day 2 and OFT day 3 as shown in TP1. However, the LPD male offspring showed a significant increase in the time spent in the edge of the OFT on day 2 (p=0.01) but not on day 3. Confirming our previous data of time in the centre showing LPD males spend longer in the edges.

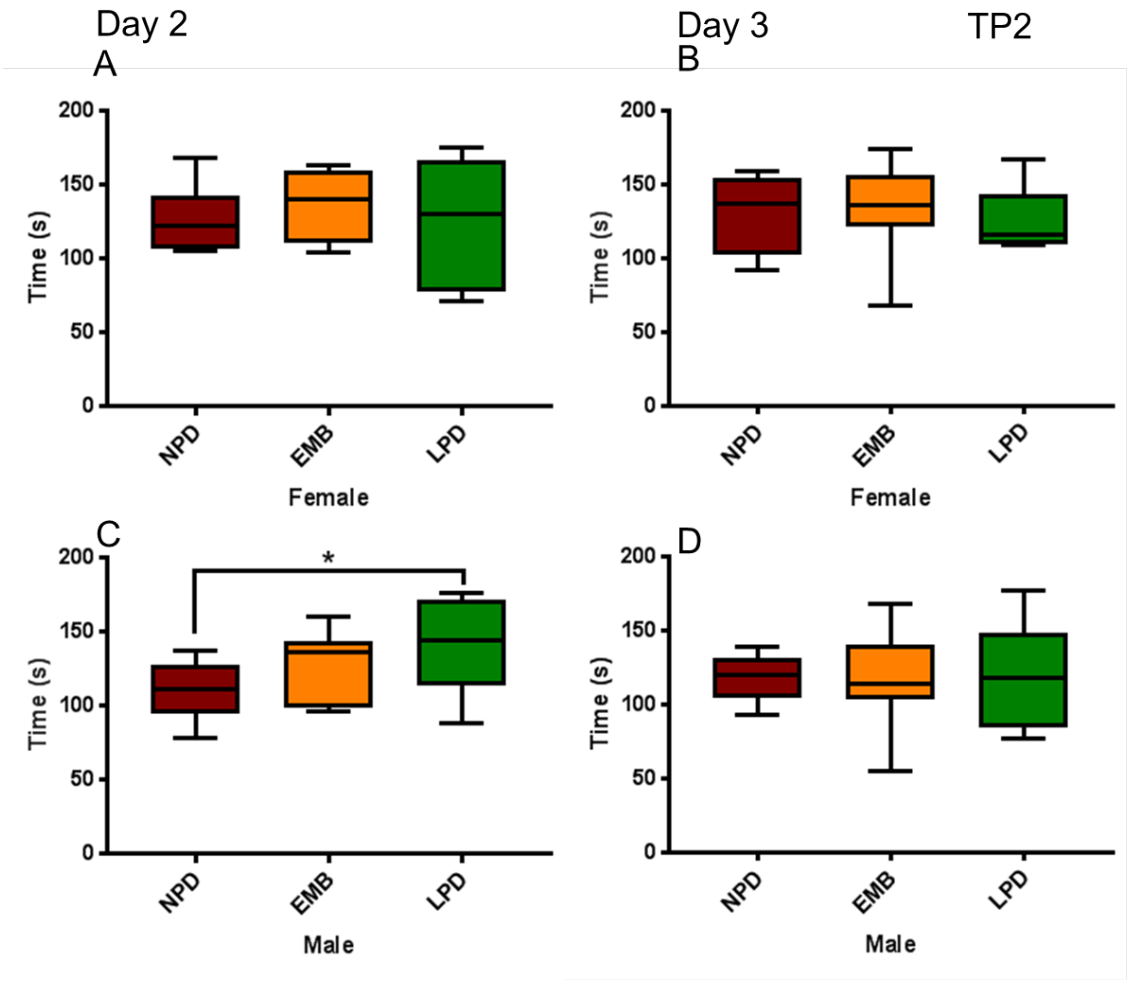


Figure 3.17. Analysis of time spent in the centre of the open field test

A)TP1 PND 29,females, C) PND 29, TP1 males, B)PND 92 TP2 females and D) PND 92 TP2 Males. Time spent (s) was compared on days 2 and 3. 3 diet groups were analysed. Paired t- tests were conducted between day 2 and 3. TP1 and TP2 females NPD n=6, Emb-LPD n=10, LPD n=8 showed no difference time spent in the centre of the OFT. TP1 LPD Males day 2 and 3 spend significantly less time in the centre (p=0.01), NPD n=11, Emb-LPD n=10, LPD n=6. TP2 Females NPD p=0.029 n=6, Emb-LPD n=6, LPD n=6). TP2 LPD males on day 2 spend significantly less time in the centre (p=0.01). NPD n=5, Emb-LPD n=5, LPD n=6 pairs.

3.4.3 Results of maternal protein restriction on anxiety behaviours – elevated plus maze

Figure 3.18 illustrates the results of the elevated plus maze (EPM) at TP1; PND 35, of which three parameters were taken into consideration, the time spent on the open arms, distance moved on the open arm and the number of line crossings into the open arms. Elevated plus maze is used to evaluate anxiety-like behaviour. There was no significant difference in the time spent on the open arms between the NPD control offspring and the LPD/ Emb-LPD offspring (p=0.966). In contrast, there was a significant decrease in the number of line crossings into the open arms between the female NPD control offspring and the Emb-LPD offspring (P=0.038), while no significant difference was evident between the NPD control males and those male offspring that were subject to maternal restriction (LPD/ Emb-LPD) (P=0.804). There was also no significant difference for distance moved on the open arm. In summary, these data indicate that the Emb-LPD females showed some aspect of anxiety-like behaviour in comparison to the NPD controls.

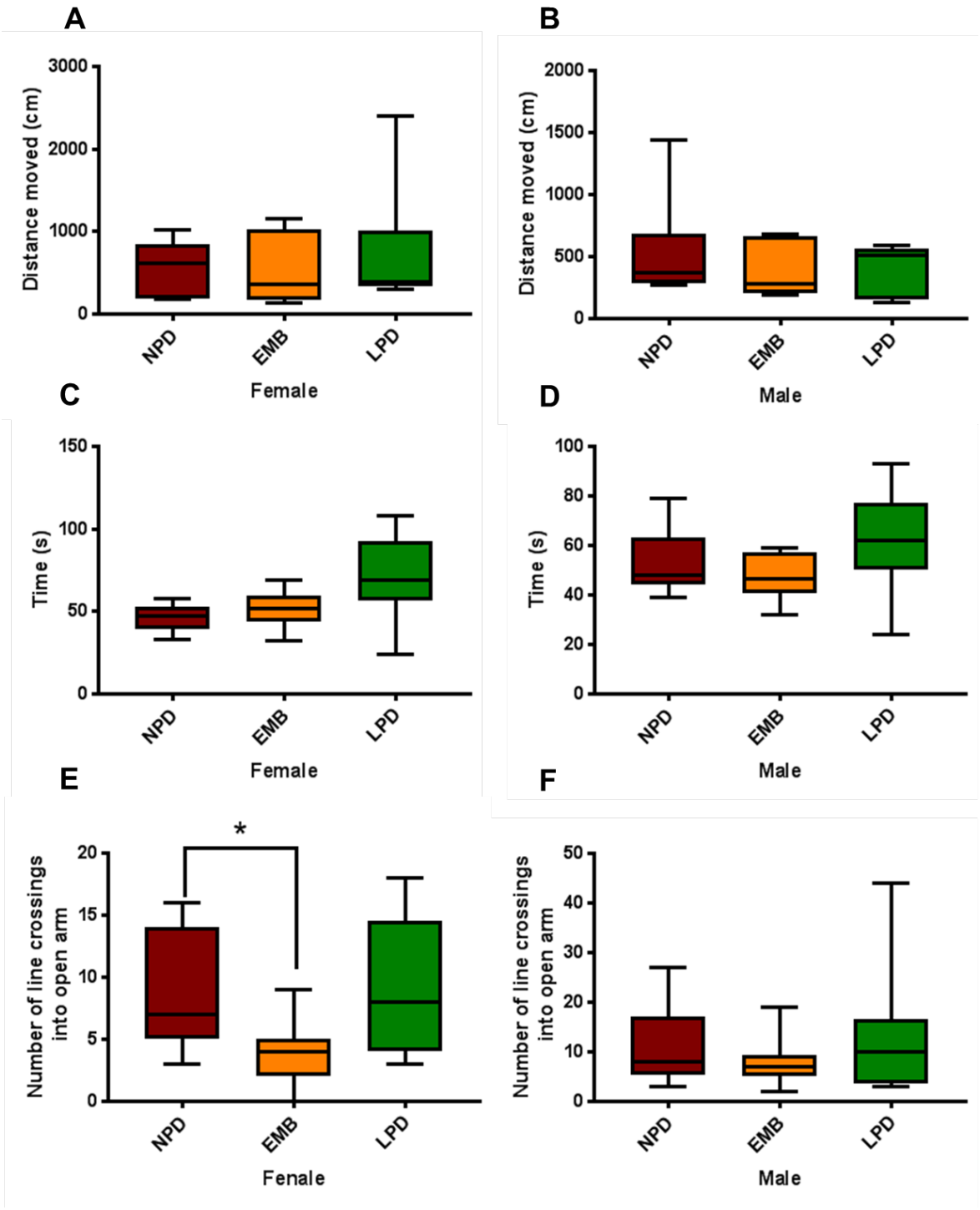


Figure 3.18. Behavioural analysis of the elevated plus maze at PND35

(A and B) demonstrate the distance moved in centimetres on the open arm. (C and D) demonstrates time spent on the open arm. (E and F) demonstrates the number of line crossings into the open arms. Time spend in the open arms and distance moved was no significant between groups or genders. Number of line crossings was significant for females ($p=0.038$) females n=27 males n=29.

Figure 3.19 illustrates the results of EPM at TP2; PND 98. Again, three parameters were taken into consideration, the time spent in the open arms, distance moved in the open arms and the number of line crossings into the open arms. There was no significant difference in the time spent on the open arms between the NPD control offspring and the LPD/ Emb-LPD offspring (P=0.53). Moreover, there was no significant difference in the number of line crossings onto the open arms between the NPD control offspring and the LPD/ Emb-LPD offspring (P=0.473; males: P=0.084, females). There was also no significance difference in distance moved (P=0.573; males: P=0.554, females). In summary, the only cohort to demonstrate different behaviour, when subject to the EPM, were the TP1 Emb-LPD female offspring.

Figure 3.20 illustrates the results of the number of risk assessments the mouse does on the EPM. Less risks assessments suggests the mouse would be more anxious. Considering Time point 1 PND 35 Emb-LPD females showed significant decrease in risk assessment behaviour (p=0.04), further supporting data from TP1 that Emb-LPD may have anxiety type phenotype. TP1 a when considering the males LPD males compared the NPD (p=0.028). When we look at TP2 there is no significant difference in risk assessment behaviour in either gender or diet group.

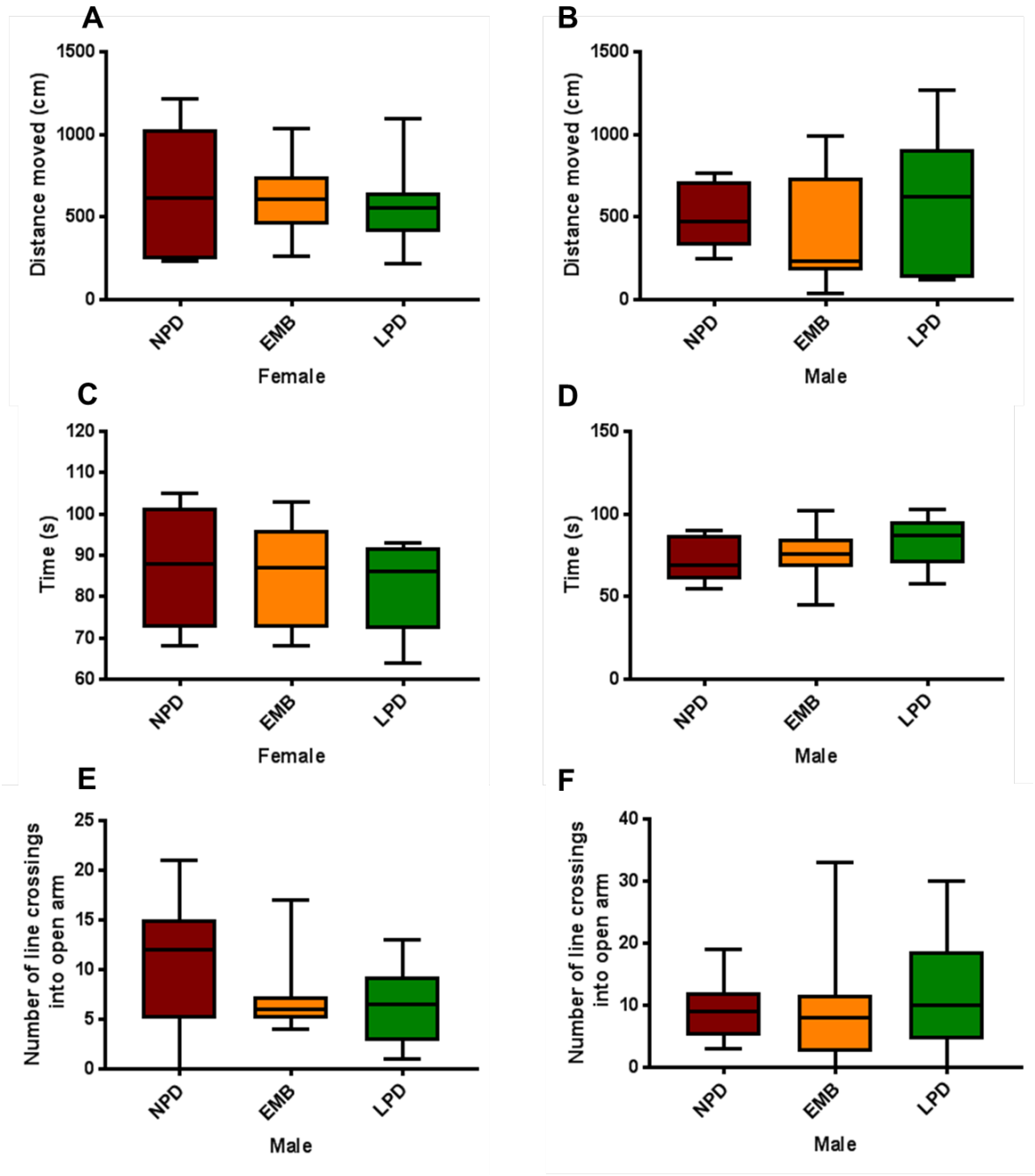


Figure 3.19. Behaviour analysis of the elevated plus maze at PND98.

(A and B) demonstrate the distance moved in centimetres on the open arm. (C and D) demonstrates time spent on the open arm. (E and F) demonstrates the number of line crossings into the open arms. Time spent in the open arms, distance moved and number of line crossings were all none significant between groups or genders females n=27 males n=29.

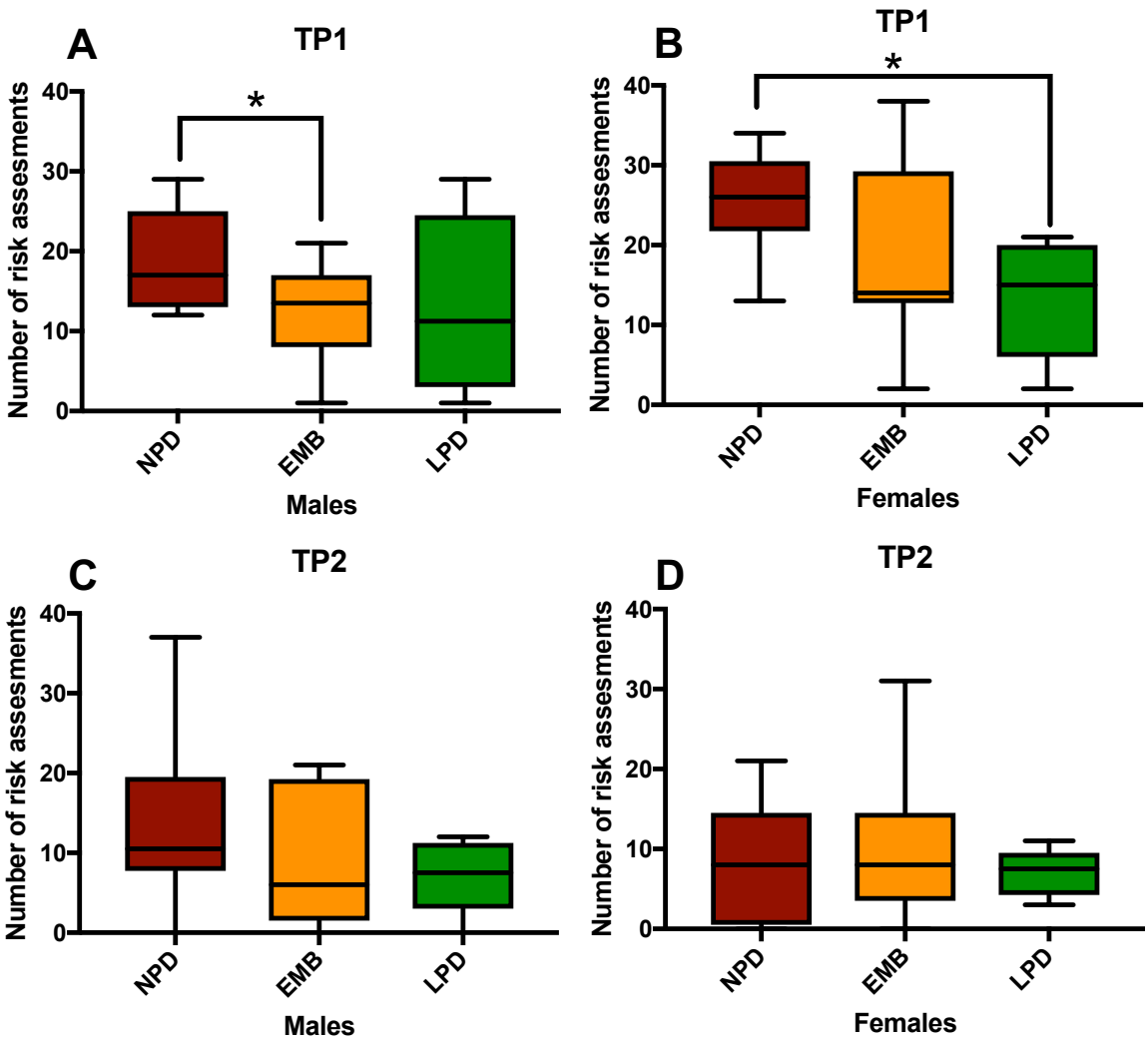


Figure 3.20. Risk assessment behaviour exhibited in the elevated plus maze

In TP1 females, risk assessment behaviour was significantly decreased in (A) Emb-LPD females (p=0.04) and in LPD males (B) (p=0.028). TP2 shows no significant difference in risk assessment between all diet groups and genders.

3.4.4 Results of social interaction

We used the social interaction sub behaviours to see if maternal diet had impacted social behaviour. In order to assess the sub behaviours were analysed using a social interaction test in figure 3.21. There were no significant differences for any parameter, with the only data suitable for analysis being collected for the non-social exploratory behaviours wall and free rearing. In figure 3.21, it does show a trend of decreasing incidence and duration with NPD displaying the highest frequency of the behaviour and EMB-LPD and LPD displaying less of the behaviour. This indicates the low protein groups didn't explore the environment as much as the control group but the reduction was

not large enough to yield any significance. During the test, there was only a low frequency of anogenital sniffing which is a truly social behaviour for all three diets with no significant difference detected for either male or female offspring. Analysis of the other behaviours was limited due to low frequency of behaviour displayed by mice of all three diets, the data for these behaviours is not shown. No difference was seen between diets for social dominance behaviours; for the score of paws on top of the other mouse there was a high amount of variation within each of the diets making the mean value similar across all groups. Only 12 of the 21 males displayed the paws behaviour while only 7 males displayed the aggressive following behaviour in the test, 3 NPD males, 3 LPD males and 1 EMB-LPD male. Like the social dominance behaviours, the two agonistic behaviours clawing and pinning were also unsuitable for analysis, with only three males and 1 female displaying any clawing behaviour, with each mouse only performing the behaviour once and a similar story for pinning with a low incidence in only 3 males and no female mouse displaying the behaviour for any of the diets.

Statistical analysis between male and female didn't show any significant.

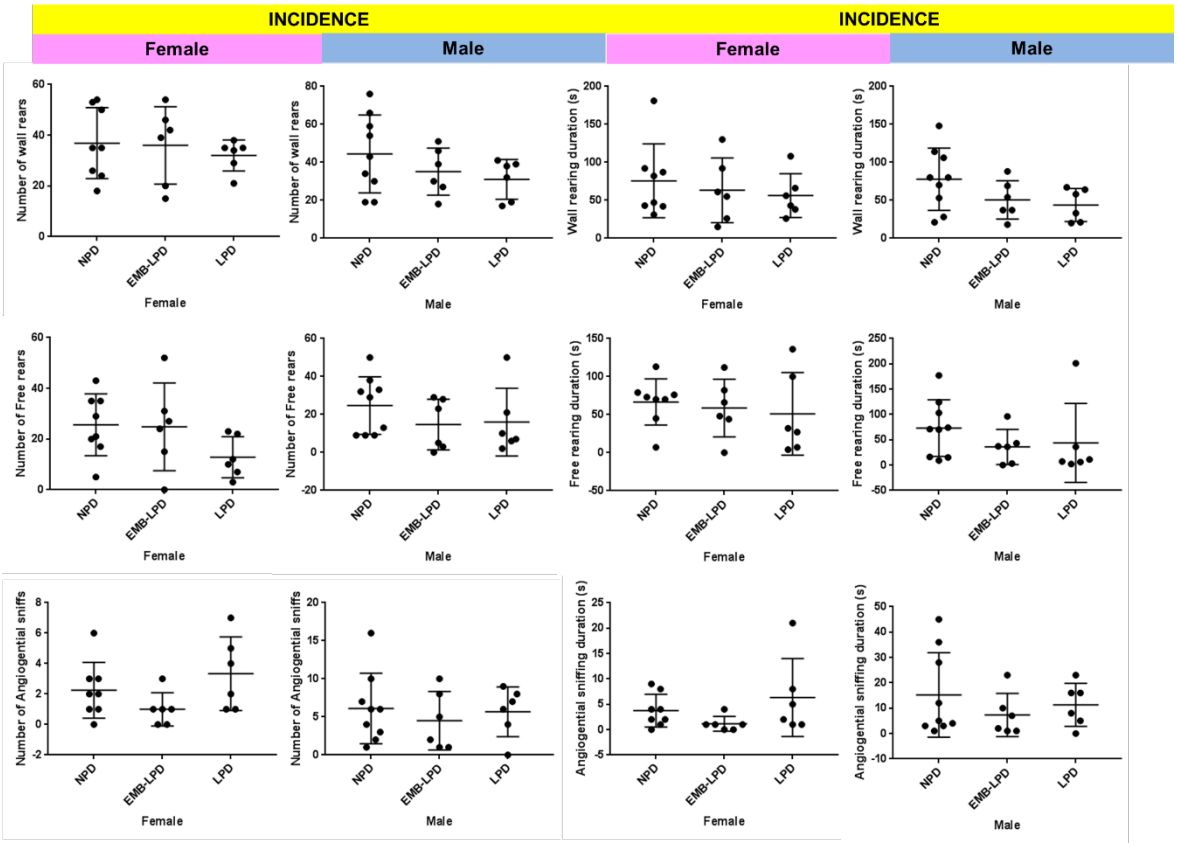


Figure 3.21. Incidence and duration of three behaviours analysed in the social interaction test.

Wall Rearing, Free Rearing and Anogenital Sniffing for male and female offspring. Mean value and standard deviation shown. Analysed by Multi Level Regression. Male NPD n = 9, EMB-LPD n = 6 and LPD n = 6. Female NPD n = 8, EMB-LPD n = 6 and LPD n = 6. No significant differences were identified.

3.4.5 Results of rota rod test for motor co-ordination and motor learning

The offspring motor coordination and learning is tested by the accelerating rota rod, is evaluates the amount of time they remain on the rota rod before falling. These results of the rota rod, as illustrated in figure 3.22, (females P=0.01 mothers n=11, n=27,; males: P=0.048, mothers n=11, n=23).

In summary, these rota rod data indicate that Emb-LPD offspring have a reduced motor coordination and learning.

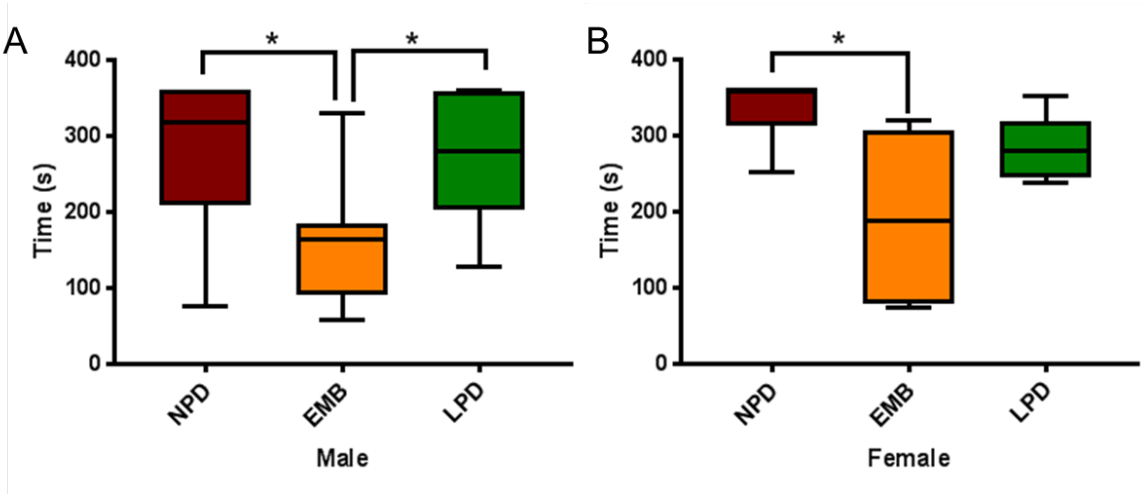


Figure 3.22. Rota rod data from mice at PND68 after different maternal diets

Mice were time to assess how long they could stay on the rota rod in seconds. (A) Emb-LPD males and (B) females both showed a significant decrease in time spent on the rota rod. (P<0.01 females, n=27,; males n=23).

3.5 Discussion

Using a range of behavioural tests, we showed that Emb-LPD and sustained LPD have some differing behaviours compared to the NPD. We find that the Emb-LPD have significantly poorer short term and long-term memory based on the NOR, T-maze tests and OFT, with the LPD males only show poor long-term memory in the T-Maze. Our study therefore shows that maternal LPD affects behavioural outcomes for the offspring.

3.5.1 Maternal protein restriction and infancy neurological development

We measured early neural development, by using the righting reflex test and the negative geotaxis test [192 193], was evaluated for mice pups at PND's 2-8 and PND's 6-10 respectively. Righting reflex and negative geotaxis relate to vestibular function and locomotory function[194] permitting the

investigation of sensory system development. The results of the righting reflex test indicate that the righting reflex performance of pup infants is not impacted by a maternal protein restriction, either in the LPD or Emb-LPD. This is in line with other studies from Akitake *et al* that investigated the neurological development of an Intrauterine Growth restricted (IUGR) (protein restricted), phenotype in a mouse model. IUGR (introduced by a 70% restriction between E 8 and day 7 of lactation inclusive) did not impact the righting reflex performance in comparison to controls. However, a sluggish reflex performance was evident PND's 4-6 [129], which coincides with the results of our study figure 4.2. Together, these results indicate that a maternal low protein restriction does not affect the overall early development of vestibular function.

In contrast, Zhang *et al.* provided opposing results in the application of a 50% food restriction diet regime on a rat model. The 50% food restriction introduced between ED 0 and lactation (PND 21), gave rise to a significant reduction in righting reflex performance at PND 3 (males and females) and PND 4 (males only)[195] . By comparison, a moderate protein restriction applied in this study has less impact on vestibular development when compared to a severe 50% food restriction. This would suggest impairing of the vestibular system a larger nutritional impact must occur and this could be why we so no change in our model. The effect of maternal protein restriction confined to the preimplantation period only is widely unknown, our righting reflex results of the study are the first to confine moderate maternal protein restriction to the preimplantation period only and we see no effect of either protein restricted diet on this neurological outcome.

We also carried out the negative geotaxis test. Offspring subject to a maternal protein restriction displayed a reduction in negative geotaxis performance when compared to NPD controls. Gender-specific susceptibility is evident with the effect of maternal protein restriction most prominent in females at PND 6-8 while in the males demonstrated similar effects at PND 8-10. These windows of rodent reflex performance decline translate to the human neurodevelopmental time course [196 197]. The results of this study agree with Akitake and a study by Bellusci, where the negative geotaxis in IUGR phenotype pups was significantly slower compared to controls. Moreover, similar to this study, the general reflex performance improved over the negative geotaxis time course[129]. The results of Belluscio study also showed a significantly higher negative geotaxis reflex by the LPD offspring on PND 7, for both genders of pup. Our study demonstrates sex-specific differences with only female offspring effected. One reason this could be is due to the fact we observed between days a change in limb morphology in offspring of both genders also indicated sex-specific anatomical differences with some pups on certain days maybe being able to succeed in this test due to their limbs forming at a better rate. The study by Wang found significant muscle impairments in male offspring of a maternal LPD origin[198], which might support my observations. Future study

of muscle development alongside neurological development is required to gain more conclusive results.

3.5.2 Maternal protein restriction and memory function

Analysis of the NOR test, T-Maze and the open field test (OFT) indicated major changes in cognitive capacity induced by a maternal LPD or an Emb-LPD treatment. Firstly, I will consider the results of the NOR test, which was evaluated for PND 41, PND 64 and PND 96 respectively. Short-term memory, allows us to maintain an active representation of information for a brief period[199 200]. In particular, NOR permits disturbances in non-spatial memory to be indicative of cognitive impairment [201 202]. The inter trial interval of 60 seconds between the acquisition trial and the retention trial of the NOR test provides a suitable period to test short term memory. The short term memory of LPD offspring and Emb-LPD offspring were compared to NPD control offspring using the discrimination index (DI)[203]. The DI provides a measure of discrimination between the novel object and the familiar object[203], where those with a cognitive impairment will not remember the familiar object from the acquisition trial, resulting in loss of preference for the novel object during the retention trial[203].

The results indicate that maternal protein restriction throughout gestation (LPD) does not affect the immediate short-term memory of mice. In contrast, a maternal diet confined to the preimplantation period (Emb-LPD) strongly impacts the short-term memory function of mice, showing no sign of gender-specific susceptibility or difference over time. These results strongly agree with previous studies that adverse behavioural traits may be linked to abnormal brain function in the limbic region of the brain associated with emotion and memory [108 204].

I will now consider the results of the open field test and the T-maze. Long-term memory is required when we wish to memorise a piece of information for a substantial period of time. A sub-system of long-term memory called episodic memory is evaluated by the OFT and the T-maze tests the working memory. Exposing mice to the OFT arena exploits the natural aversion mice have for open spaces[205]. The OFT was evaluated between two time points, PND 29 and PND 92. By carrying out multiple open field trials over the course of 2 days, the mice should become habituated to the arena on OFT day 2 and subsequently show less exploratory behaviour on OFT day 3. Application of this principle to this study explains the changes observed in exploratory behaviour, using parameters such as distance moved, and time spent in edge and centre. Work by Cohen has also demonstrated that a reduced habituation response during open field studies using mice can be related to impaired attention in humans[206].

The results of distance moved in the OFT indicate that offspring of maternal LPD at PND 29 (time point 1) and Emb-LPD at PND 92 (time point 2) have a reduced cognitive capacity when compared to NPD controls. Emb-LPD and LPD female offspring at PND 29 being an exception. The LPD and Emb-LPD male offspring demonstrated no reduction in exploration behaviour between OFT day 2 and OFT day 3 compared to the NPD control offspring, representative of a lack of habituation response. Emb-LPD and LPD female offspring at PND 29 were the exception, showing normal cognitive function at a younger age (PND 29). However, when we consider TP2 the story changes with Emb-LPD females showing the same exploration between day 2 and 3, suggesting Emb-LPD females are initially resistant to maternal protein restriction effects, but still develop a cognitive decline at a later age (PND 92). At TP2 both Emb-LPD and LPD males still show no change in exploratory behaviour between day 2 and 3. What is most interesting about the TP2 open field data is that is it supports the T-Maze result on PND 100 which shows a decreased working memory in the Emb-LPD male and female offspring as well as the LPD male offspring. Together these results indicate gender-specific susceptibility during the early time point (PND 29), where the males show cognitive decline, but the females do not in the LPD.

In contrast to the distance moved, centre exploration and edge exploration we only saw a decrease in centre exploration and increase in the LPD males. This some what supports recent study by Akitake, which found distinct decreases in centre exploration in an IUGR (protein restricted) phenotype in comparison to controls. The difference exists in the period of maternal protein restriction exposure, where the exposure used by Akitake, extends to lactation. I propose that centre exploration may lend to more anxiety-like traits, to coincide with mice aversion to exposed well-lit spaces, which is programmed during postnatal malnutrition. From review of the literature; the open field test is normally used to assess anxiety related or exploratory behaviour in a single episode[205]. However, we wanted a shorter method to replicate Watkins et al [12]. who undertook OFT trials on offspring in an Emb-LPD mouse model. The assessment of the parameters distance moved, velocity and resting found evidence for female susceptibility to maternal protein restriction with no postnatal effect on the behavioural phenotype of males [12]. In contrast, my work is the first to indicate clear cognitive deficits in offspring of both genders as a result of maternal Emb-LPD and that the increased distance moved, rears and jumps reported by Watkins could be due to their poor memory and therefore doing more exploratory behaviours.

We also need to consider our rotarod data, rotarod tasks are routinely used to assess motor coordination and motor learning in rodents, especially when deficits in the midbrain dopaminergic system are expected. What is rather interesting is we have a decrease in ability to stay on the rotarod in the Emb-LPD male and female offspring, which further supports memory and learning of these offspring has been impaired.

The most critical period that leads to adverse behavioural phenotypes is one of current debate. Work by Alamy & Bengelloun, proposes that postnatal malnutrition induces more direct behavioural and cognitive effects [107]. However, my results indicate that prenatal exposure to protein restricted diets, have an adverse effect on adult offspring. The findings of the Helsinki human epidemiological study, investigating the Dutch Hunger Winter, are also support this study in mice[26]. People conceived during the famine demonstrated development of adverse behavioural traits during their adult life, including an increased risk of schizoaffective disorders[26].

These results of the novel object recognition test, the repeated open field test, T-maze and rota rod support the DOHaD hypothesis. Maternal protein restriction in critical periods of cellular change, i.e. during fertilisation, leads to programming of adverse cognitive effects, including decline in working memory and episodic memory.

3.5.3 Maternal protein restriction and anxiety-like behaviour

The elevated plus maze (EPM) test was used to evaluate anxiety-like behaviour in LPD and Emb-LPD offspring compared to NPD control offspring at PND 35 and PND 98. All LPD offspring and Emb-LPD male offspring did not demonstrate anxiety-like behaviour. In contrast, Emb-LPD female offspring indicated potential development of anxiety-like behaviour at time point 1 (PND 35) (shown through a reduction in the number of line crossings onto open arms, even though there was no significant difference in the duration of time spend in the open arms). We also looked at risk assessment behaviour which was only significantly decreased in Emb-LPD females the TP1 and LPD males at TP1. With this data is could suggest the Emb-LPD females with their reduced risk assessment and line crossing may have an anxiety phenotype at TP1. However it is reported as mice age, they risk assess less[38]. This is why there is a decrease in risk assessment from TP1 to TP2.

These findings show some discrepancies compared to rat model studies carried out by Almeida indicated that malnutrition in the elevated T-maze test, lead to a reduction in anxiety-like behaviour rather than an increase, although gender-specific susceptibility remained evident[207].

3.5.4 Social interaction

The Social Interaction test was performed to discern if there is any detectable difference in the social ability between the three maternal diets for the offspring or if there was any indication of aggressive or agonistic behaviour in social situations. Again as we discussed earlier, evidence from the Dutch Hunger Winter showed mothers who experienced the famine in early pregnancy had children with a higher incidence of schizophrenia, autism and depression [24] compared to children whose mothers experienced the famine in late pregnancy. Several low protein diet models have

also reported changes in social behaviour and depressive like behaviour after prenatal exposure to maternal protein restriction[130 208]. Analysis of changes in social interaction after protein restriction exclusively in the pre-implantation period is novel and had not been tested before, as evidence in the literature supports the view that prenatal protein restriction can alter offspring social behaviours this test was carried out to see if the Emb-LPD or the LPD diet resulted in altered social interactions, adding to existing literature.

However, my work found no change in social behaviour between the three diets groups but this result may be in part due to limitations of the test design. Another study of prenatal and postnatal low protein diet showed mice also displayed a significant decrease in amicable behaviour and a large increase in aggressive behaviour in a social competition test very similar to the social interaction test carried out in this current study[117]. Evidence like this from the literature suggested the mice in this model may have exhibited some deficit in social interactions. For this current study this test was severely limited in its ability to measure any differences in behaviour between the three diet groups due to many of the behaviours either not being displayed by the mice or having a very low frequency of incidence.

The only measure gained from this study was of non-social exploratory behaviour, how many times the mice reared up against a wall or free reared away from the wall to look around and search the environment. Although there was no significant result the trends suggested the Emb-LPD and LPD displayed these behaviours less and for a shorter amount of time compared to the control mice. When rearing the mice are looking up and out of the closed environment or through the clear plastic of the arena. This behaviour is consistent with the mice trying to find a way out of the arena, it is hard to interpret a behavioural meaning for this difference as it can be interpreted in several different ways. The first interpretation is that the mice from the two low protein diets are not worried[209] about the novel mouse and therefore are not as eager to escape the box as they do not perceive a threat from the other mouse. The second interpretation suggests the complete opposite in which the low protein diet mice are displaying reduced rearing behaviour as they are scared or anxious of the other mouse and therefore are not moving around as much and so do not actively explore the environment, being more focused on the other mouse's movements. This means a conclusion on this behaviour is hard to draw and with the behaviour not being a true social behaviour it doesn't answer the question this behavioural test set out to investigate, whether or not the low protein mice have a social deficit compared to the control mice.

The protocol used and the behaviours scored for the social interaction test were taken from a schizophrenic mouse model study [191] and involved placing two mice which had not met each other before, one of which is of interest, in an open field and video recording their interactions for a period of time, after which a score of the behaviours is made from the videos. This paper had more success within their model and could determine differences within the amount of time spent aggressive following between different animal groups as well as establish aggressive behaviour. This is probably due to the type of model they were using; a mouse model at high risk for schizophrenia which is a mental disorder with well-defined behavioural phenotypes and will display stronger behavioural phenotypes than a low protein diet model where the behavioural difference could be subtler. They did however encounter the same problem as this low protein diet model did in regard to the low frequency of agnostic behaviour displayed by the mice. To get around this problem and gain a measure they pooled the two behaviours, clawing and pinning, together. Using this method for this low protein diet study still didn't get a high enough frequency of behaviour to detect differences, with a total of 6 males and 1 female displaying agonistic behaviour after clawing and pinning measures are combined. Many other studies have successfully used the social interaction tests to display behavioural differences in animal models. This test may have been more suitable for a rat model; rats display more social behaviour than mice and actively play with each other. The use of this test in a rat model may have resulted in a wider range of social behaviours being observed than is seen with a mouse model so using rats may be more suitable for the analysis of potential social deficits after a maternal low protein diet. Mice do display social behaviour however in the form of sniffing the head, body and anogenital region of another mouse, pushing past each other with physical contact and crawling under or over each other[210]. Out of all these social behaviours only anogenital sniffing was measured, a better measure of social interaction ability would have included these other behaviours as well which were noticed in the test videos. Measuring all of these would have given a complete picture of social interaction and a deficit could have indicated autism like phenotypes which would have linked back to the Dutch Hunger Famine epidemiological work mentioned above[26]. In this study only incidence and duration of behaviours were analysed which doesn't give temporal information about the behaviours throughout the duration of the test. Plotting the incidence of behaviours over time would provide more information on the social interaction, i.e. latency to approach the other mouse, a brief interaction at the beginning of test and then losing interest quickly or prolonged investigation and interaction with the stranger mouse throughout the test.

There are different methods on how to set up a social interaction test, with many studies opting to have the control mouse inside of a chamber within the arena e.g. a wire mesh cylinder or box, with

the subject mouse given free reign of the arena and the number of approaches to the chamber and contact with the container being recorded and regarded as social interaction or interest in the other mouse[211] (73). This is a different technique to the one used in this study where both mice were free to move about the arena at will. While this test aimed to be able to specific behaviours shown by mice which may have changed, this simpler approach of just distinguish quantifying approaches and contacts may have been more suitable and allowed collection of more usable data. Further attempts at this social interaction test using alternative protocols and counting methods may give a more definitive answer to whether the social ability of these mice is compromised, which this test failed to answer adequately.

3.6 Conclusions

In conclusion, maternal protein restriction either confined to the preimplantation period or throughout gestation leads to adverse behavioural phenotypes. Furthermore, these results indicate a reduction in learning capacity within LPD offspring and Emb-LPD offspring, as a result of maternal protein restriction. However, I do not think that Emb-LPD and LPD diets had as a significant effect on anxiety and social behaviour. Gender differences and susceptibility has been apparent throughout this chapter, showing for the first time that males have cognitive impairment as a result of maternal protein restriction. The resistance in the Emb-LPD females at time point 1 during the open field studies could be due to oestrogen effects, that protect in some cases and worsen in others.

	Emb-LPD Females	Emb-LPD Males	LPD Females	LPD Males
Developmental Reflexes	No change	No change	No change	No change
Short term memory	↓	↓	No change	No change
Working/ Episodic memory	↓	↓	No change	↓
Motor memory	↓	↓	No change	No change
Anxiety	No change	No change	No change	No change
Social interaction	No change	No change	No change	No change

Table 3.4 Summary of behavioural adult offspring tests.

Chapter 4 The effect of maternal low protein diet on adult brain morphology and gene expression.

4.1 Introduction

Many studies have highlighted the role of under-nutrition during gestation and early postnatal life, revealing an important link to many neurological conditions and altered behavioural phenotypes. Even deficiencies in certain micronutrients such as vitamin D have been associated to psychopathologies including autism, schizophrenia and ADHD [140 141].

Many studies have investigated the effect of dietary restriction on cytoarchitecture of both foetal brain and adult brain, giving rise to many differing theories. These differing theories may be due to the design of the study in regard to length of time the diet is implemented for, and age of the animals tested. We have shown in chapter 3 that the Emb-LPD produces significant changes on the foetal brain by causing an increase in neuronal differentiation. In addition, we have has also seen significant behavioural differences in the Emb-LPD and the LPD offspring in chapter 4.

Restriction of major macronutrient groups is known to lead to alterations in neurogenesis. Dietary restriction in adult animals has been shown to stimulate neurogenesis which is thought to be in part stimulated by increased expression of BDNF [212], while also making the neurons more resistant to dysfunction and damage. There is also some epidemiological evidence from longitudinal studies showing that a low-calorie diet throughout a person's lifetime reduces the chance of Parkinson's disease and Alzheimer's disease[137] and rats fed a restricted diet perform better in learning and memory tasks[138]. However, maternal dietary restriction has also been shown to impact the offspring neurogenic potential. One particular study showed that a 50% reduction in food intake during pregnancy led to higher neurogenesis and decreased proliferation in the dentate gyrus of the offspring at different time points in adulthood [81].

Another rat model investigated the effect of maternal malnutrition on the development of dentate gyrus in the foetal and adult offspring mice, with the diet consisting of protein restriction through a 6% casein diet implemented 5 weeks before conception and throughout gestation, compared to the control 25% casein diet [139]. No change in the numbers of pyramidal neurons on E12, E16 or E20 was found in the protein-restricted offspring but the number of granule neurons was significantly decreased in these rats on E20 [139]. However, the highest proportion of neurogenesis occurs postnatally with only 15% of the adult population generated before birth [139] so the impact of this maternal malnutrition diet was then investigated in the early postnatal period. On postnatal day 8

(PND8) a significant decrease in the neurogenesis of granule cells was seen in the protein-restricted rats. This suggests a compensatory mechanism whereby the initially reduced pool of neurons is increased by PND 30 and shows maternal prenatal protein restriction alters the pattern of neurogenesis in the early life of the offspring [139]. Other studies have limited the protein restriction to the gestation and weaning periods to investigate the in-utero programming effects[79] . A rat study also demonstrated that mothers who were fed a diet of 8% protein during gestation showed a reduction in cell proliferation and numbers of hippocampal progenitors in adult offspring and a reduction in number of proliferating cells in the lateral wall of lateral ventricle[79]. Despite the difference in the outcomes of these two studies, it is clear that maternal malnutrition and particularly protein restriction impacts the functionality and supply of NSC in the offspring, leading to changes in neurogenesis which persist into adulthood. However, it remains unknown whether protein restriction during the periconceptional period leads to altered morphology.

4.2 Aims

While a body of evidence has been generated to understand the impact of maternal low protein diet to the offspring brain development and behaviour, the consequences of protein restriction exclusively in the pre-implantation period has not been elucidated. This chapter aimed to investigate whether protein restriction in the Emb-LPD and LPD diets had an impact on adult offspring brain gross morphology and gene expression and could we find reasons for behavioural deficits documented in the previous chapter. Neural and glial populations were analysed using immunohistochemical techniques with particular interest for consequences to cortical and hippocampal development and RNA seq was carried out on the hippocampus.

4.3 Methods

The following methods for adult brain morphology and quantification are described in section 2.6. QPCR analysis is described in section 2.7.

4.3.1 Adult brains

Experimental mice were terminally anaesthetised with pentoject (Pentobarbitone sodium 20% w/v solution Animal care, UK) and perfused with 0.9% NaCl containing 5 units/ml heparin sodium (CP Pharmaceuticals, UK). Brains were then extracted and weighed, one hemisphere was fixed in 4% PFA overnight for immunohistochemistry and the cortex and hippocampus were dissected from the other hemisphere and snap-frozen in liquid nitrogen for qPCR analysis. Fixed hemispheres were then left in 70% sucrose (Sigma-Aldrich, UK), embedded in optimal cutting temperature medium

(OCT; VWR, UK) and frozen on isopentane (ThermoFisher Scientific, UK) stored on dry ice and samples were kept at -20°C until use in immunohistochemistry.

4.3.2 Tissue selection

Depending on the marker of choice different regions were chosen to investigate the regions of choice. (see Figure 4.1).

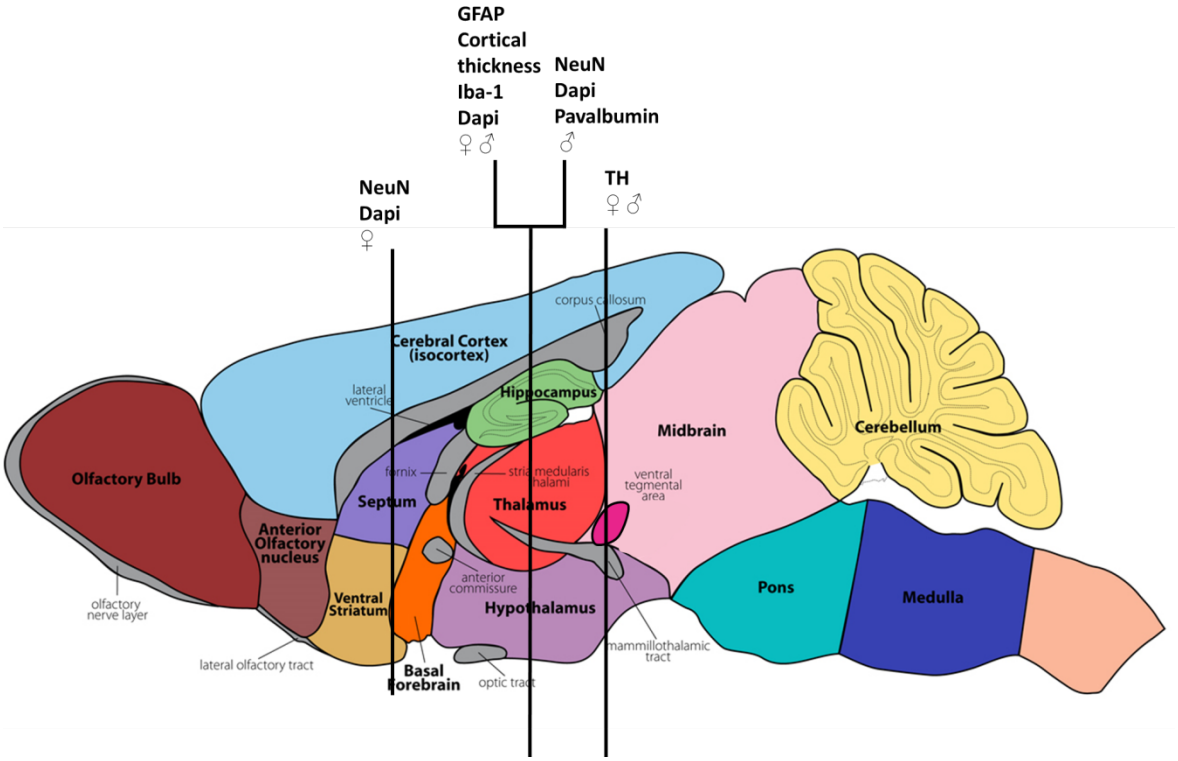


Figure 4.1. Sagittal view of the mouse brain

The three regions of interest for this project, cerebral cortex, hippocampus and ventral tegmental area (VTA) are highlighted. Position along the anterior to posterior axis for sections chosen for immunohistochemical analysis is anotated on the diagram. Important to note is female NeuN and DAPI data was collected more anterior to the rest of data set meaning results cannot be directly compared to the male NeuN and DAPI due to different positioning. Adapted from www.gensat.org (The Gene Expression Nervous System Atlas at Rockefeller Univesity).

4.3.3 Immunofluorescence staining of adult and foetal brains

Slides were thawed for 45mins at 37°C, adult brain sections were fixed in 4% PFA at room temperature for 20 mins, while foetal brain sections skipped this step. Citrate buffer was used for heat shock antigen retrieval using an 800w microwave at half power for 25mins (ThermoFisher

Scientific, UK). Slides were then placed under cold running water for 3 minutes, tapped off onto a piece of tissue and washed in PBS 3 x 2 minutes. Slides were then washed in 1X PBS-T for 15 mins and sections were drawn around with a wax pen to allow staining of different sections with different primary antibodies. Sections were blocked for 30mins using PBS containing 5% BSA and 10% DS (Donkey Serum; Sigma-Aldrich, UK) and were then washed 3x5mins in PBS-T. Sections were stained with primary antibodies in PBS-T at 4°C for 16-24 hours (Table 2.5). Slides were washed 3x5 mins in PBS-T and then stained with secondary antibodies in PBS-T for 60mins (Table 2.6). Slides were submerged in 1µg/ml DAPI solution for 10mins and were then washed for 3x10 mins in PBS-T and coverslipped with Mowiol mounting medium (Biomedical imaging unit, SGH).

4.3.4 Morphological analysis of adult cortex and hippocampus

All quantitative methods for cell counting, cortical thickness and thresholding techniques were performed using FIJI software. The same region of the cortex was used in each assay to quantify the different neural populations and take measurements of the cortical thickness. The primary somatosensory cortex was selected for quantification due to the ability to easily distinguish cortical layers in this region as well as ease of identification by using the hippocampus as a landmark and overall shape of the coronal sections of brain. Layer 1, also called the molecular layer was not counted for cortical analysis. This layer only contains a small number of neurons spread throughout the layer and mostly consists of glial cells including astrocytes and microglia as well as dendritic tuffs from pyramidal neurons and horizontal axons[213]. It was discounted due to damage to this layer in some of the brains due to processing and sectioning, meaning some were not fit for quantification.

4.3.5 Quantification of adult NeuN, DAPI and parvalbumin immunofluorescence staining

Coronal sections were imaged using a DM5000 (Leica Microsystems) 3 channel fluorescence camera and images of the cortex where taken at x10 magnification. Images were quantified by placing 3 boxes of 275µm x 150µm in each of the layers 2-6 of the cortex. Positive cell number was determined by colocalization of positive discrete green fluorescence staining and DAPI nuclear staining. Total cell number was determined by the DAPI positive cells within the box. Ratios of different cell types i.e. NeuN to DAPI were also taken to examine differences in the proportions of different cell types within the brain cells crossing 2 sides of the box while the other two sides of the box were ignored and not included in the quantification illustrated in Fig 4.2.

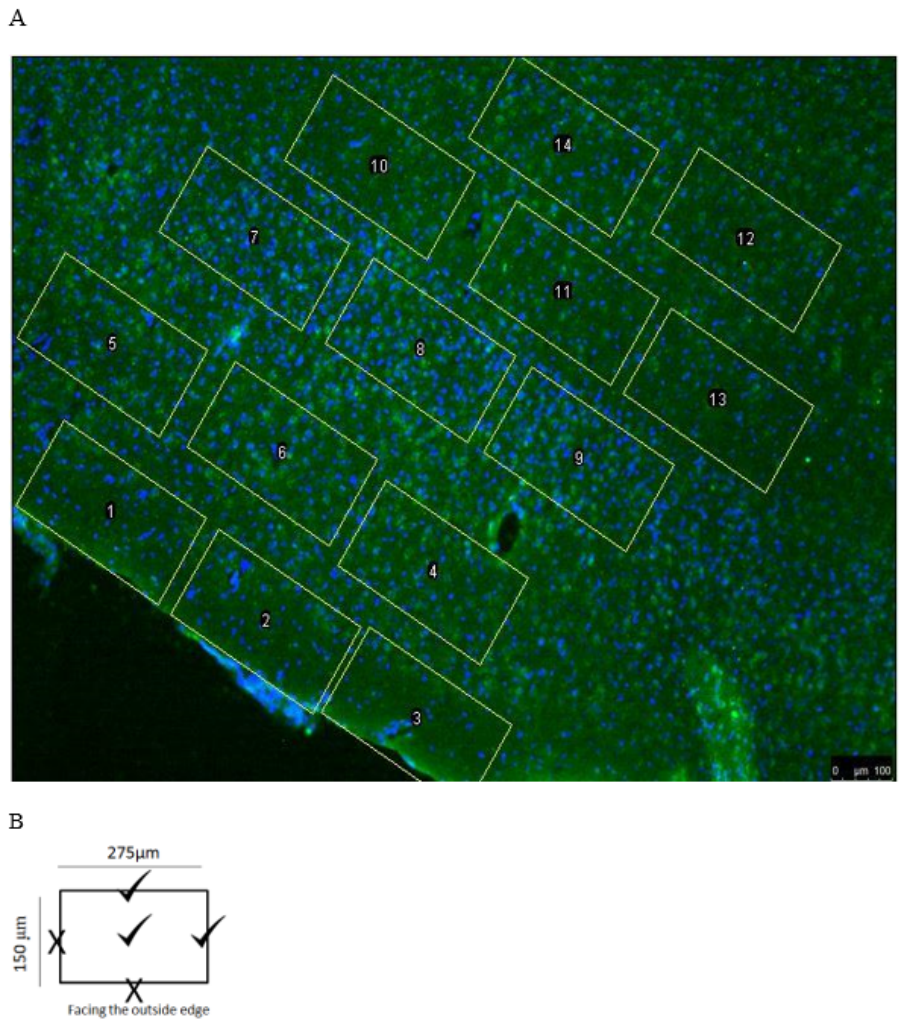


Figure 4.2. Representative quantification of NeuN staining in adult cortex

A) Male cortex stained for NeuN in green with DAPI counterstain in blue, this displays the method of placement of boxes for quantification of neurons within the offspring cortex. B) Dimensions of the box used as the quantification field, with ticks representing areas where cells are included in the count.

4.3.6 Quantification of adult GFAP and SOX2 immunofluorescence staining

GFAP and Sox2 staining confocal microscopy was used to allow clear determination of which cells were co-expressing both markers and identification of radial glia like cells. Z stacks were taken at x20 magnification in three regions of the hippocampus; the dentate gyrus (DG), CA1 and CA3. Imaging of the cortex was also taken at x20 for quantification of GFAP and Sox2 cells within the cortical layers. A representative slice was taken from the Z stack and used for quantification of GFAP and Sox2 positive cells within FIJI. The three regions of the hippocampus were quantified separately; within the dentate gyrus one box with the dimensions of 450µm x 450µm was used as the area of quantification. Within the CA3 region two boxes of 450µm x 300µm were used, one placed on top

of the dense Dapi layer and one placed outside of the dense Dapi layer so it is encompassed by the layer. The CA1 region also had two boxes, the dimensions in this region being 450µm x 200µm, again one placed on top of the dense Dapi layer and one further into the hippocampus, placed where connections between the CA3 and the CA1 are located. Examples of these placements are displayed in figure 4.3. Consistent with the quantification of NeuN, Parvalbumin and Dapi, GFAP and SOX2 positive cells were counted within the boxes and those who touched either of two chosen sides were included in the count. Radial Glia Like (RGL) cells were classified on their morphology; cell body within the sub granular zone and radial process extending through the granule cell layer as well as being co-stained with GFAP and SOX2. Only cells where the cell body could be seen and Dapi costain present were included in the count, cells where only the processes were visible where not counted.

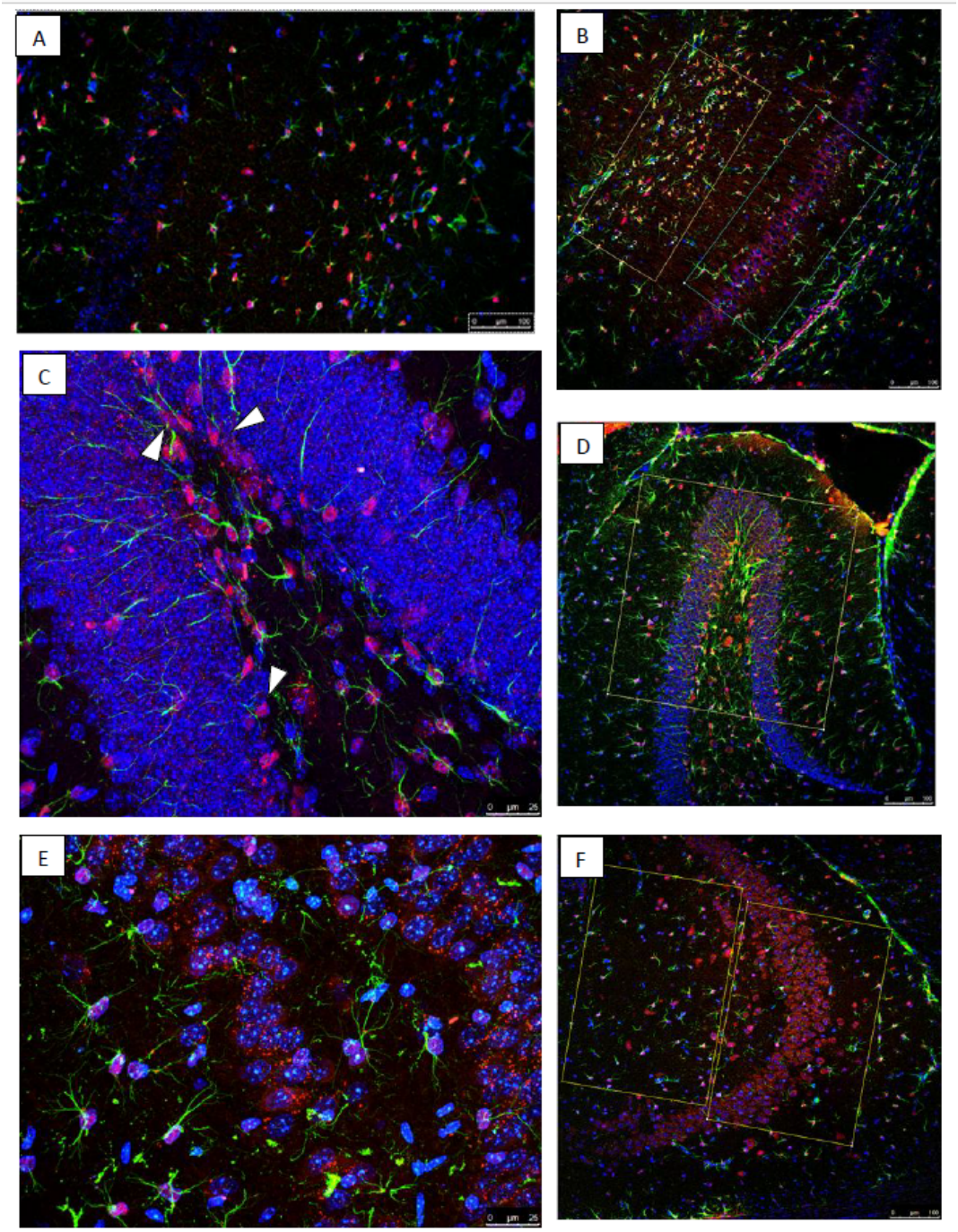


Figure 4.3. Representative quantification of GFAP and SOX2 in adult hippocampus

Coronal sections of hippocampus stained for GFAP in green, SOX2 in red and DAPI co-stain in blue by fluorescent immunohistochemistry imaged by confocal microscopy. A) Representative stain taken at x20 of the CA1 region. B) CA1 region showing placement and size of the two boxes used for quantification, image taken at x20 magnification. C) Dentate gyrus imaged at x63 magnification with arrows indicating example RGL cells. D) Dentate gyrus imaged at x20 with positions of boxes shown. E) Area of CA3 region imaged at x63 36 magnification showing representative stain. F) CA3 region imaged at x20 with box placement shown.

4.3.7 Analysis of cortical thickness

The widths of individual cortical layers were measured by identifying the edges of each layer, with the layers being defined by the changes in cell density visible between the layers as shown in figure 4.4. The end of the cortex was identified when the corpus calosum becomes visible. Straight lines are drawn at the beginning and end of each layer with perpendicular line created throughout all of the layers, with the length between each start and end point of the layer used as a measure of that layer's width.

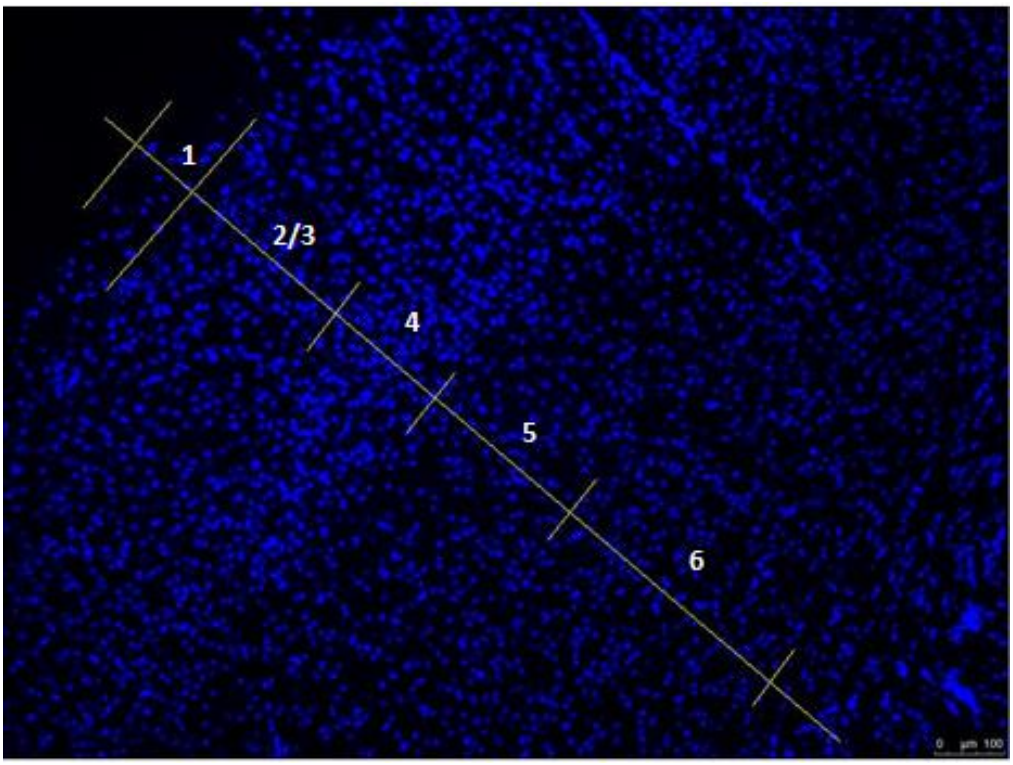


Figure 4.4. Representative cortical nuclear staining in adult male offspring

DAPI staining reveals that distinctly layered architecture of the cortex and the thickness of layers were quantified according to the stratification shown.

4.3.8 3,3'-Diaminobenzidine DAB immunohistochemistry for adult offspring for Tyrosine Hydroxylase and Iba-1

Slides were thawed for 45mins at 37°C, adult brain sections were fixed in 4% PFA at room temperature for 20 mins. Sections were quenched with 0.3% H_2O_2 (ThermoFisher Scientific, UK) in PBS-T for 10 mins and then washed in PBS-T for 15 mins. Citrate buffer was used for heat shock antigen retrieval using an 800w microwave at half power for 25mins (ThermoFisher Scientific, UK).

Slides were then placed under cold running water for 3 minutes, tapped off onto a piece of tissue and washed in PBS 3 x 2 minutes.

Sections were blocked for 30mins using PBS containing 5% BSA and 10% DS (Donkey Serum; Sigma-Aldrich, UK) and were then washed 3x5mins in PBS-T. Sections were incubated with primary antibodies in TBS (or TBS only for controls) at 4°C 16-24 hrs. Slides were washed 3x5mins in PBS-T and then incubated for 1hr at RT with appropriate secondary antibodies in TBS (Table 2.6). Sections were washed 3x5 mins in TBS and then incubated with avidin-biotin complex (Vectastain ABC Kit; Vector Labs, UK) as per the manufacturer's protocol for 30 mins. Sections were washed 3x10mins in TBS. Slides were placed in 0.1M phosphate buffer containing 0.05% 3,3'-Diaminobenzidine (DAB, Sigma-Aldrich, UK) and 0.015% H_2O_2. Sections were counterstained in haematoxylin (BDH Laboratory supplies, UK) and destained in acid alcohol (70% ethanol and 1% HCl). Slides were dehydrated by placing them in increasing ethanol concentrations and xylene (ThermoFisher Scientific, UK) and then coverslipped (VWR, UK) using DPX mounting medium (ThermoFisher Scientific, UK).

4.3.9 Imaging and quantification of tyrosine hydroxylase, collagen IV and Iba1

Overlapping 20x maximal projected images were taken of each brain section and stitched together into a composite .tif image using the VS110 Virtual Slide Microscopy Systems (Olympus). Sections stained with the same marker were blinded prior to quantification. For percentage area stained quantification for Tyrosine hydroxylase, used to stain dopaminergic neurones in the Ventral tegmental Area (VTA) and collagen IV used to stain blood vessels in the cortex. Areas of major damage were excluded and in ImageJ and the colour deconvolution plugin (FIJI) was used to produce an image of DAB staining or fluorescence without haematoxylin, dapi or background colouring. The image was then converted into a black/white binary image where any level of staining above a defined threshold was converted into black, while anything below the threshold was converted into white. The threshold for each stain was manually defined based on stain intensity and background while blinded. The percentage area in black within the selected in the area of interest and then was t calculated to determine percentage area stained.

Iba-1 our microglia stain we counted the number of positive cells in region were manually counted within the hippocampus the dentate gyrus, CA3 and CA1 with the placements shown in figure 4.5. The areas of boxes placed on image for quantification are the same as used in GFAP and SOX2 quantification for both the dentate gyrus and the CA1 region, with the CA3 region in this quantification using a slightly smaller box of 120,000 μm^2, 400 µm x 300 µm (Figure 4.5).

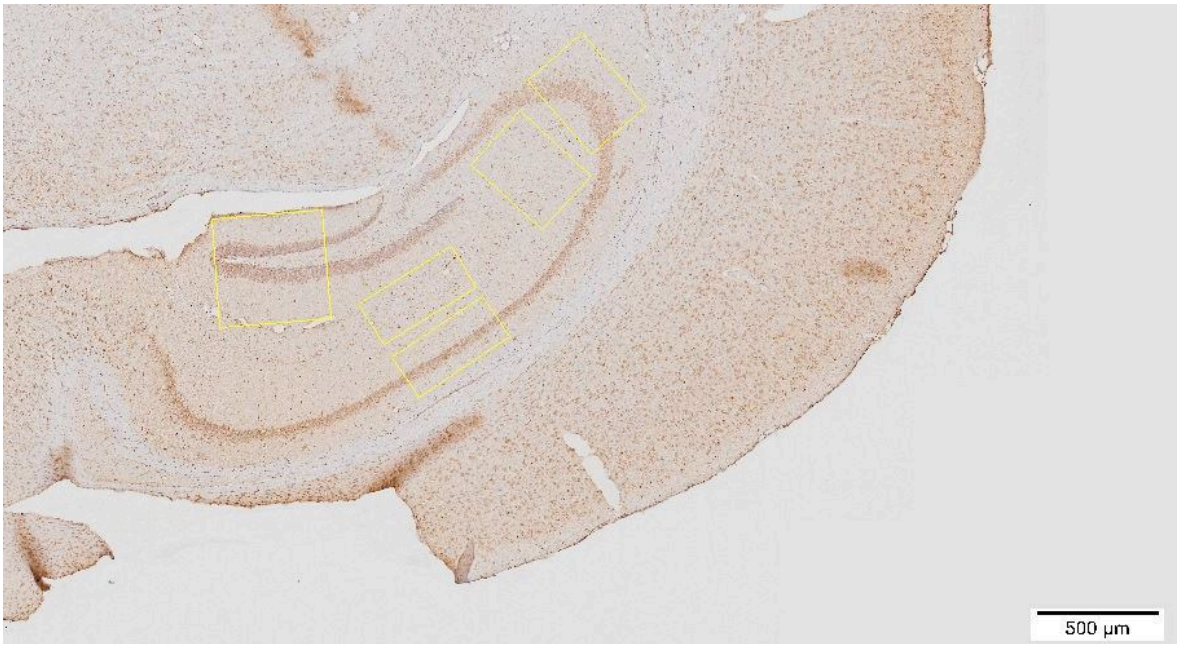


Figure 4.5. Representative staining of microglia in the adult offspring brain

Coronal section of adult mouse brain showing the hippocampal formation stained for IBA1. Image taken during quantification using Fiji with yellow boxes displaying the placement of regions used for analysis.

4.3.10 GFAP and SOX2 imaging in the adult offspring.

Confocal images were taken for GFAP and SOX-2. Sections were imaged with a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, UK). DAPI was excited with a 405nm laser, AF488 was excited with the 488 line of an argon laser and AF568 was excited with a 561nm solid state laser. Detection bandwidths were placed under peaks of the emission spectrum for each dye. Sequential imaging of DAPI and AF568 followed by AF488 was performed to avoid spectral bleed-through of DAPI into the AF488 channel. Secondary antibody only controls were used to set the maximum sensitivity of each detector, thereby controlling for autofluorescence and non-specific binding. A Z-stack through the whole section was taken and a maximum intensity projection of each stack was generated in FIJI (Schindelin et al. 2012). NeuN and pavalbumin was taken on coronal sections were imaged using Leica Fluorescent microscope DM5000, images of the cortex and hippocampus where taken at x10 magnification to ensure all six of the desired layers where present in the image using secondary antibody only controls to set the sensitivity.

Target	Antibody type	Distributor	Product ID (Clone)	Concentration (Dilution)
Tyrosine Hydroxylase (TH)	Rat anti-mouse mAb	Abcam UK	Ab75875	5µg/ml (1:200)
Glial Fibrillary Acidic Protein (GFAP)	Hamster anti-mouse mAb	Dako	Z0334	2µg/ml (1:500)
Sex Determining Region Y-Box 2 (SOX2)	Rabbit anti-mouse mAb	Santa Cruz	Sc-17320	(1:50)
Ionized Calcium Binding Adapter molecule 1 (Iba1)	Rat anti-mouse mAb	Wako	ncnp24	5µg/ml (1:200)
Neuron- Specific Nuclear Protein(NeuN)	Rat anti-mouse mAb	Millipore	MAB377	(1:250)
DAPI		Calbiochem	CAS 28718-90-3	(1:2000)
Collagen IV	Rabbit anti-mouse pAb	Abcam, UK	ab6586	2µg/ml (1:500)
Parvalbmin	Rat anti-mouse mAb	Swant, UK#	PV235	5µg/ml (1:200)
Tomato Lectin		Vector, UK#	DL-11178	5µg/ml (1:200)
Nestin	Rat anti-Rabbit mAb	Millipore, UK*	MAB353	(1:100)
Beta-III-tubulin	Rabbit anti-mouse pAb	Biolegend, USA	801202	(1:500)
Cleaved caspase3'	Rat anti-Rabbit pAb	Cell signalling, UK	Asp 175	(1:100)
Ki67	Donkey anti-rabbit pAb	Novusbio, UK	F2266	(1:400)

Table 4.1. Primary antibodies used in immunohistochemical techniques

Target (Conjugate)	Antibody type	Dilution	Product ID	Distributor
Rabbit IgG (Biotin)	Goat anti-rabbit pAb	1:200	BA-1000	Vector Labs, UK
Mouse IgG (Alexa Fluor 488)	Donkey anti-mouse mAb	1:200	A-21202	ThermoFisher Scientific, UK
Rabbit IgG (Alexa Fluor 568)	Goat anti-rabbit pAb	1:200	A11036	ThermoFisher Scientific, UK
Rabbit IgG (Alexa Fluor 568)	Donkey anti-rabbit mAb	1:200	A-21206	ThermoFisher Scientific, UK
Goat IgG (Alexa Fluor 568)	Donkey anti-goat mAb	1:200	A-11057	ThermoFisher Scientific, UK

Table 4.2. Secondary antibodies used in immunohistochemical techniques

4.4 RNA Seq and qPCR analysis

4.4.1 RNA isolation & RNA Seq

RNA was isolated using the lipid easy tissue RNA isolation kit from Qiagen, Inc. (Valencia, CA). The snap frozen cortex and hippocampus were immediately placed in QIAzol reagent and homogenized using a handheld electric homogeniser. RNA was eluted from the midi columns with using 250 µL each of RNase free water. Concentration and RNA integrity was analysed using the bioanalyzer nanochips 8000 (Agilent, UK). An RNA integrity score above 8 confirmed the quality of the sample was sufficient for RNA Seq. RNA samples were stored at -80°C until use. For RNA Seq, RNA from the female hippocampi was delivered to University of Huddersfield were Patrick McHugh conducted stranded RNA seq library preparation. The libraries for this project were constructed using the NEXTflex™ Rapid Directional RNA-Seq Kit (PN: 5138-08) with the NEXTflex™ DNA Barcodes – 48 (PN: 514104) diluted to 6uM. The library preparation involved an initial QC of the RNA using Qubit DNA (Life technologies Q32854) and RNA (Life technologies Q32852) assays as well as a quality check using the PerkinElmer GX with the RNA assay (PN:CLS960010)

1ug of RNA was purified to extract mRNA with a poly- A pull down using biotin beads, fragmented and first strand cDNA was synthesised. This process reverse transcribes the cleaved RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase and random primers. The second strand synthesis process removes the RNA template and synthesizes a replacement strand to generate cDNA.

The ends of the samples were repaired using the 3' to 5' exonuclease activity to remove the 3' overhangs and the polymerase activity to fill in the 5' overhangs creating blunt ends.

A single 'A' nucleotide was added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction.

A corresponding single 'T' nucleotide on the 3' end of the adapter provided a complementary overhang for ligating the adapter to the fragment. This strategy ensured a low rate of chimera formation.

The ligation of a number indexing adapters to the ends of the DNA fragments prepared them for hybridisation onto a flow cell.

The ligated products were subjected to a bead based size selection using Beckman Coulter XP beads (PN: A63880). As well as performing a size selection this process removed the majority of un-ligated adapters.

Prior to hybridisation to the flow cell the samples were PCR'd to enrich for DNA fragments with adapter molecules on both ends and to amplify the amount of DNA in the library.

Directionality is retained by adding dUTP during the second strand synthesis step and subsequent cleavage of the uridine containing strand using Uracil DNA Glycosylase. The strand that was sequenced is the cDNA strand.

The insert size of the libraries was verified by running an aliquot of the DNA library on a PerkinElmer GX using the High Sensitivity DNA chip (PerkinElmer CLS760672) and the concentration was determined by using a High Sensitivity Qubit assay and q-PCR.

The constructed stranded RNA libraries were normalised and equimolar pooled into one final pool of ~ 9.86 nM using elution buffer (Qiagen). The library pool was diluted to 2 nM with NaOH and 5µL transferred into 995µL HT1 (Illumina) to give a final concentration of 10pM. 120 µL of the diluted library pool was then transferred into a 200 µL strip tube, spiked with 1% PhiX Control v3 and placed on ice before loading onto the Illumina cBot. The flow cell was clustered using HiSeq PE Cluster Kit v4, utilising the Illumina PE_HiSeq_Cluster_Kit_V4_cBot_recipe_V9.0 method on the Illumina cBot. Following the clustering procedure, the flow cell was loaded onto the Illumina HiSeq2500 instrument following the manufacturer's instructions. The sequencing chemistry used was HiSeq SBS Kit v4 with HiSeq Control Software 2.2.58 and RTA 1.18.64. The library pool was run in a single lane for 125cycles of each paired end read. Reads in bcl format were demultiplexed based on the 6bp Illumina index by CASAVA 1.8, allowing for a one base-pair mismatch per library, and converted to FASTQ format by bcl2fastq.q

4.4.2 Bioinformatics of RNA seq data

Illumina data for 24 hippocampi RNA samples the underwent quality control using FastQC (fastqc-0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to check for the basic metric of quality control in the raw data. An in-house contamination-screening pipeline called Kontaminant (http://www.tgac.ac.uk/kontaminant/) was used to check for any obvious contamination in the raw reads.
The Kallisto program is then used for quantifying abundances of transcripts from RNA-Seq data, or more generally of target sequences using high-throughput sequencing reads.

Kallisto quant mode then produces output files by default:

- abundances.h5 is a HDF5 binary file containing run info, abundance estimates, bootstrap estimates, and transcript length information length. This file can be read in by sleuth.

- abundances.tsv is a plaintext file of the abundance estimates for transcripts. It does not contains bootstrap estimates. The first line contains a header for each column, including estimated counts, TPM, effective length.

Differential Expression analysis is carried out using DESeq2 v1.14.0. The DESeq2 package provides methods to test for differential expression by use of negative binomial generalized linear models. The standard differential expression analysis steps are wrapped into a single function. The counts were filtered to keep genes with a total count across all 24 samples of > 5 (27556 genes).

Differential expression analysis was then carried out between:

NPD vs Emb-LPD

NPD vs LPD

Emb-LPD vs LPD

4.4.3 Analysis and statistics for RNA seq

baseMean	mean of normalized counts for all samples
log2FoldChange	log2 fold change (MAP): condition Normal vs Low
lfcSE	standard error: condition Normal vs Low
stat	Wald statistic: condition Normal vs Low
pvalue	Wald test p-value: condition Normal vs Low
padj	BH adjusted p-values

Table 4.3 Explanatory table of RNA seq data.

RNA-seq raw counts, the variance grows with the mean. For example, if one performs PCA directly on a matrix of size-factor- normalized read counts, the result typically depends only on the few most strongly expressed genes because they show the largest absolute differences between samples. However, our solution to this is using the program *DESeq2,* which offers transformations for count data that stabilize the variance across the mean. One such transformation is the *regularized-logarithm transformation* or *rlog*. For genes with high counts, the rlog transformation will give similar result to the ordinary log2 transformation of normalized counts. For genes with lower counts, however, the values are shrunken towards the genes' averages across all samples. Using an empirical Bayesian prior on inter-sample differences in the form of a *ridge penalty*, the rlog-transformed data then becomes approximately *homoskedastic*, and can be used directly for computing distances between samples and making PCA plots. This data is then used to quantify the differential expression analysis which is done using the appropriate *mus musculus* model in sleuth to form final list of genes and padj values. A systematic review was done on the top 80 genes with papers collected on them for the last 10 years if they had a neuroscience basis and the top 22 genes are then.

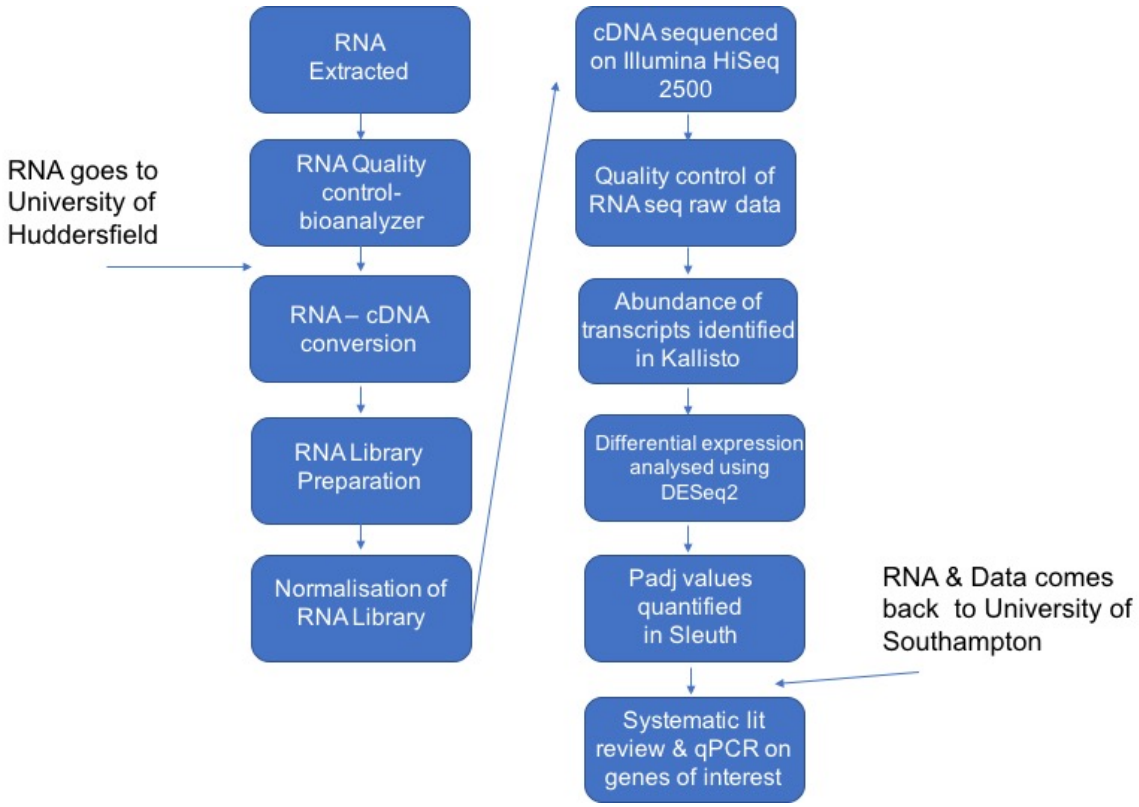


Figure 4.6 RNA Work Flow.

RNA was extracted and RNA quality was analysed using the bioanalyzer. RNA was then delivered to the University of Huddersfield were RNA was converted into cDNA, RNA library was prepared and normalised. The cDNA was then ran on the Illumina HiSeq 2500. Quality control of the RNA seq data was done and further bioinformatics techniques were carried to look for the abundance of transcripts using Kaillisto software and differential expression analysed using DESeq2 software and padj values were determined using models in the sleuth software. The data then underwent a systematic literature review where genes were ordered by padj vaule and key target genes were picked for further qPCR analysis. Data displayed in the thesis is top 22 genes for NPD vs Emb-LPD and NPD vs LPD with a p value less than $p<0.0005$.

4.4.4 RNA to cDNA conversion

1ug RNA was transcribed into cDNA using the iScript cDNA synthesis kit (Biorad, UK) as per the manufacturer's protocol for a 20µl reaction. Briefly, 1ug of RNA was added to 4ul of 5x iScript reaction mix and 1ul of iScript reverse transcriptase and RNA free water to make the volume to 20ul. The 20µl solutions were placed in a PTC240 tetrad 2 peltier thermal cycler at 25°C for 5mins, 48°C for 20mins, 95°C for 1mins and then kept at 4°C until cDNA was diluted 1 in 5 in RNAse free water and stored at -20°C.

4.4.5 Quantitative real-time PCR

Primers (Sigma-Aldrich or PrimerDesign, UK) were reconstituted to a 100µM stock solution in water and were stored at -20°C until use. Primers were designed according to the parameters shown in Table 4.3.

	Minimum	Optimum	Maximum
Product length (bp)	70	-	300
Melting temperatures (°C)	57	60	63
Primer size (bp)	15	20	25
Max self complementarity	0	0	7
Max 3' self complementarity	0	0	3
Primer GC content%	35	50	65

Table 4.4. Criteria used during primer design by Primer-Blast.

Where possible one primer of each pair recognised an exon-exon boundary to prevent the amplification of genomic DNA.

15µl of 1.2 µM of forward and reverse primers (Sigma-Aldrich or Primer deisign, UK) (Table 2.5) and 10ul Precision plus SYBR (Primer design, UK) in 3.8 ul RNAse free water was added to wells of a 96 well plates (Starlab, UK). 5µl of diluted cDNA was added to each well in duplicate. As a negative control, RNAse free water without cDNA was added to mastermix. Plates were covered with optical caps and placed in a C1000 Thermal Cycler with a CFX96 detection module (Bio-Rad, UK). qPCR was carried out using the following protocol: 95°C 10mins; 95°C 15s, 60°C 1min x 50; 72°C 10mins; 95°C 10mins; 4°C forever. MJ Opticon Monitor Software (Bio-Rad, UK was used to plot fluorescent intensity over time and C(t) values were calculated for each marker at the autocalculated threshold. Melting curves in 0.2°C intervals between 55°C to 90°C were compiled to ensure that the template was amplified specifically.

C(t) values for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and Adaptor Related Protein Complex 3 Delta 1 Subunit (*Ap3d1*) levels were measured for each cDNA sample as a reference C(t) value. Reference genes were selected using the 12 Genenorm kit (Primer design, UK) and the most stable housekeeping gene was selected. C(t) values for other genes were obtained and relative gene of interest (GOI) expression levels were calculated by normalising the GOI C(t) values to the GADPH C(t) values using the $2^{-\Delta\Delta C(t)}$ method such that relative expression = $2^{(C(t)\ GADPH\ -\ C(t)\ GOI)}$

Gene name	NCBI Gene ID	Common protein names	Strand	Primer Sequence (5'-3')
Fxr2	23879	FMR1 Autosomal homolog 2	Forward Reverse	TCAGGACAGAAGGGTGACTC GAAAGGAGGGATGTGGACCG
Fmrp	14265	Fragile X mental retardation 1	Forward Reverse	TCAGGCGCTCAGCTCCGTTTCGGTTTCA AAGCGCCATTGGAGCCCCGCACTTCC
Ap3d1	11776	Adaptor Related Protein Complex 2 Delata subunit 1	Forward Reverse	*
Gapdh	14433	GAPDH-	Forward Reverse	*

Table 2.4 – List of primer sequences used for qPCR.

*Primers Ap3d1 and *Gapdh* sequences form part of the geNormPlus kit, and are proprietary property of PrimerDesign.

4.4.6 Statistical analysis

Data were analyzed using a multilevel linear regression model using PASW for Windows program version 21 (SPSS UK, Woking, Surrey, United Kingdom)**,** in which there was a random effect assigned to each litter. Thus, we evaluated both between-litter and within-litter effects. We always included terms for the litter size and for the sex of the offspring. We used indicator variables to compare the Emb-LPD and the LPD with the NPD. This showed that differences identified between treatment groups are independent of maternal origin of litter and litter size. Boxes represent interquartile ranges with middle lines representing the medians, whiskers (error bars) above and below the box indicate the 90th and 10th percentiles because the data was non-parametric. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.5 Results

4.5.1 Cortical thickness of adult offspring

Exposure to the Emb-LPD diet during gestation was shown to change the gross morphology in relation to width of cortical layers for both female and male offspring. In female offspring an increase in width of the cortical layer was seen for layers 2/3, 5 and 6 in the EMB-LPD group when comparing to the NPD and the LPD groups. This increase in width was also seen in total thickness of the cortex, a measure taking into account all the layers and combining them into a global

measure of cortical thickness. In male offspring this increase in width was only significant for layer 6 and total thickness although a trend of EMB-LPD offspring having thicker cortical layers is seen in the remaining layers. For both sexes LPD offspring did not display any measurable difference in cortical thickness when compared to the NPD control group.

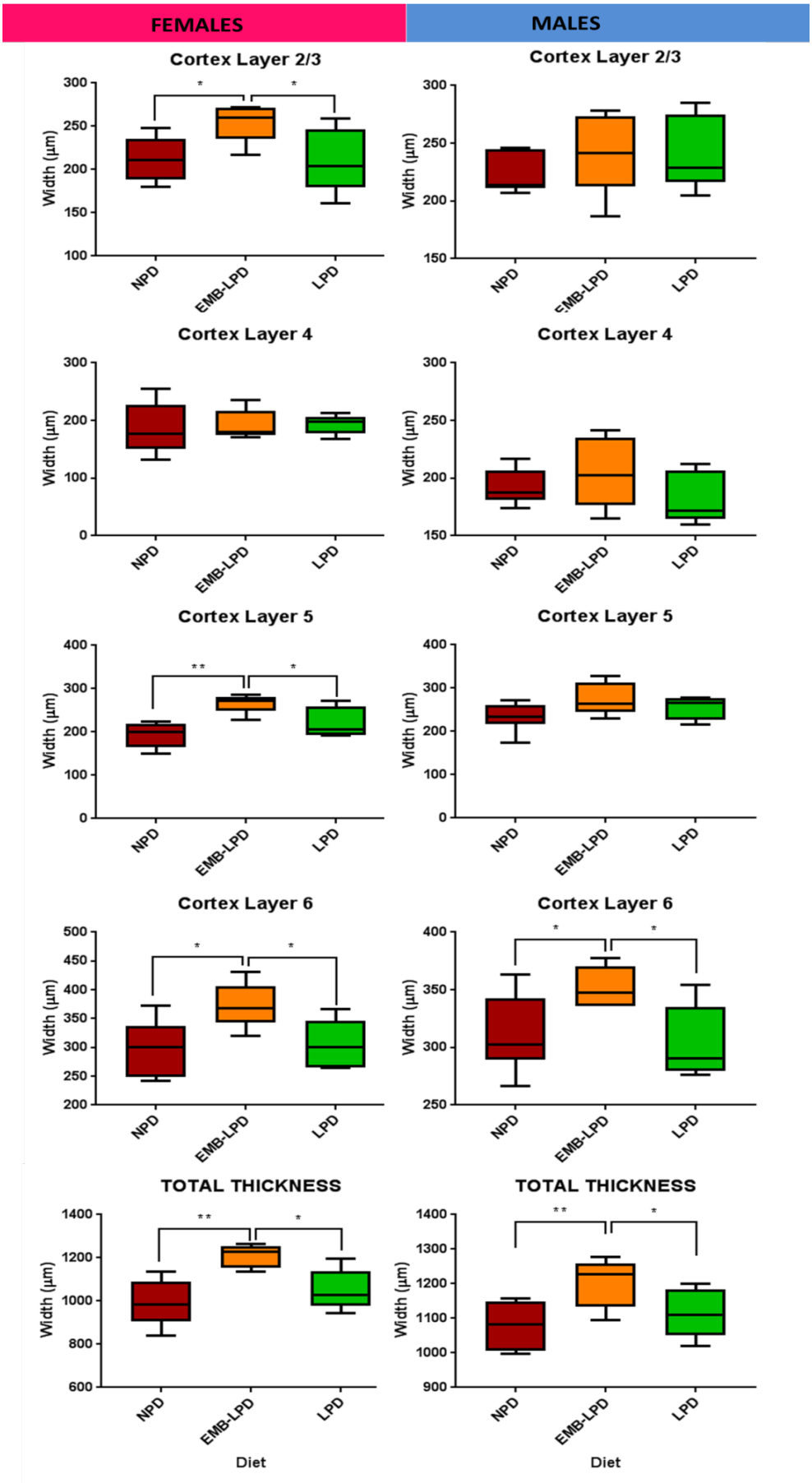

FEMALES
MALES
Cortex Layer 2/3
Cortex Layer 4
Cortex Layer 5
Cortex Layer 6
TOTAL THICKNESS
Width (µm)
NPD
EMB-LPD
LPD
Diet

Figure 4.7. Cortical layer thickness in offspring from different maternal diets

Thickness in µm of layers, 2/3, 4, 5 and 6 in the female and male offspring cortex and total thickness of all cortical layers including width of layer 1 (data not shown individually). * $p<0.05$, ** $p<0.01$. n = 5 per maternal diet for female offspring with n=6 for male NPD and n=5 for male EMB-LPD and LPD.

4.5.2 Protein restrictive diets cause a decreased number of cells in the offspring brain.

To identify if maternal diet caused a change in number of cells within the cortex a count of DAPI positive cells was taken per unit area in each cortical layer. Emb-LPD and LPD female offspring showed a significantly decreased number of DAPI positive cells compared to the control NPD group for all layers quantified within the cortex. This shows that both protein restriction diets caused a reduction in cells within the cortex for female offspring. Unlike the females, quantification of DAPI positive cells in the male offspring cortex showed a significant reduction in cell number for cortical layer 4 in Emb-LPD maternal diet offspring only, with trends of decreased cell number for the other three layers in Emb-LPD offspring although not statistically significant. Male LPD offspring display no change in cell number across all layers with cell counts comparable to the control NPD offspring. Combined counts for all the layers it showed the Emb-LPD offspring to have reduced number of cells compared to NPD and LPD offspring. These results show for both male and female Emb-LPD offspring, protein restriction in early pregnancy results in offspring with reduced cell number in cortical layers, with all layers reduced for females and a lesser effect on male offspring who have the reduction in layer 4 only as well as an overall decrease. While this reduction is also present in LPD females it is absent in the male offspring of the LPD group. This data shows a sex effect on response in offspring to the LPD diet.

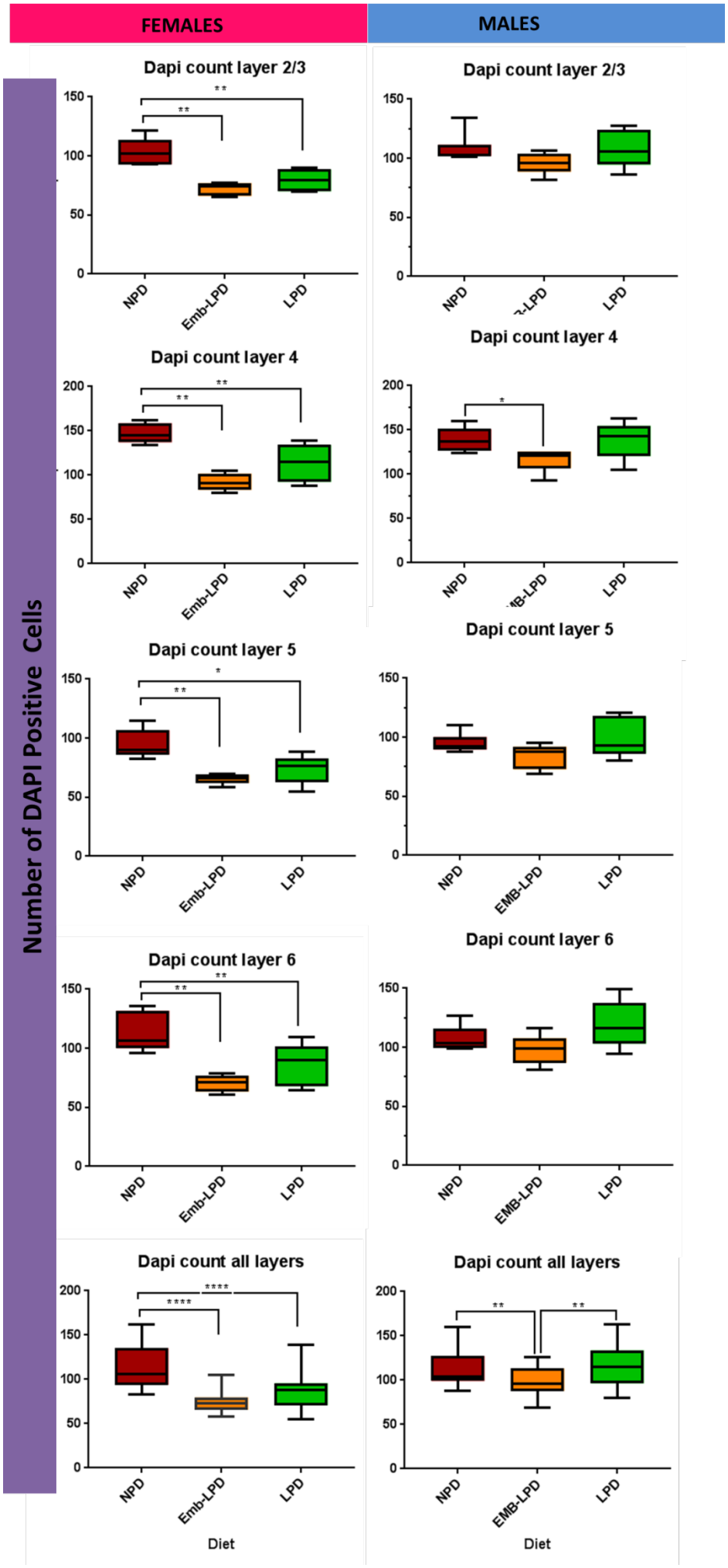

FEMALES
MALES
Number of DAPI Positive Cells
Dapi count layer 2/3
Dapi count layer 4
Dapi count layer 5
Dapi count layer 6
Dapi count all layers
NPD
Emb-LPD
EMB-LPD
LPD
Diet

Figure 4.8. Quantification of cell number in cortical layers following different maternal diets

Number of cells present in layers, 2/3, 4, 5 and 6 in the female and male offspring cortex was assessed using a count of DAPI positive cells per 41,250μm^2. Data was analysed by Multi Level Regression Analysis, * p<0.05, ** p<0.01, ***p<0.001. n = 5 per maternal diet.

4.5.3 Protein restriction causes alternations of neural populations of the adult offspring brain.

We further investigated whether the altered number of cells in the cortex was due to modifications in the neuronal population. To this end, we stained for NeuN, a neuronal specific marker. Results showed in the female offspring Emb-LPD mice displayed an increased NeuN to Dapi ratio in cortical layer 4 compared to NPD and LPD offspring, p=0.007 and p=0.01 respectively. Emb-LPD NeuN to Dapi ratio was also increased when compared to LPD in cortical layer 6, p=0.01. Looking at all the layers both Emb-LPD and LPD had a higher NeuN to Dapi ratio than NPD as well as Emb-LPD being higher than LPD. LPD was also found to be significantly different from NPD. For males this increase in NeuN to Dapi ratio for EMB-LPD offspring was absent however the trend is present for layer 6.

For the count of neurons in the females there was a reduction in NeuN positive cells for both Emb-LPD and LPD offspring in layer 6 of the cortex, with the combined total for all the layers showing a reduction in LPD offspring compared to NPD and EMB-LPD mice. For male offspring, layer 2/3 displayed also displayed a reduction in the EMB-LPD group compared to NPD mice.

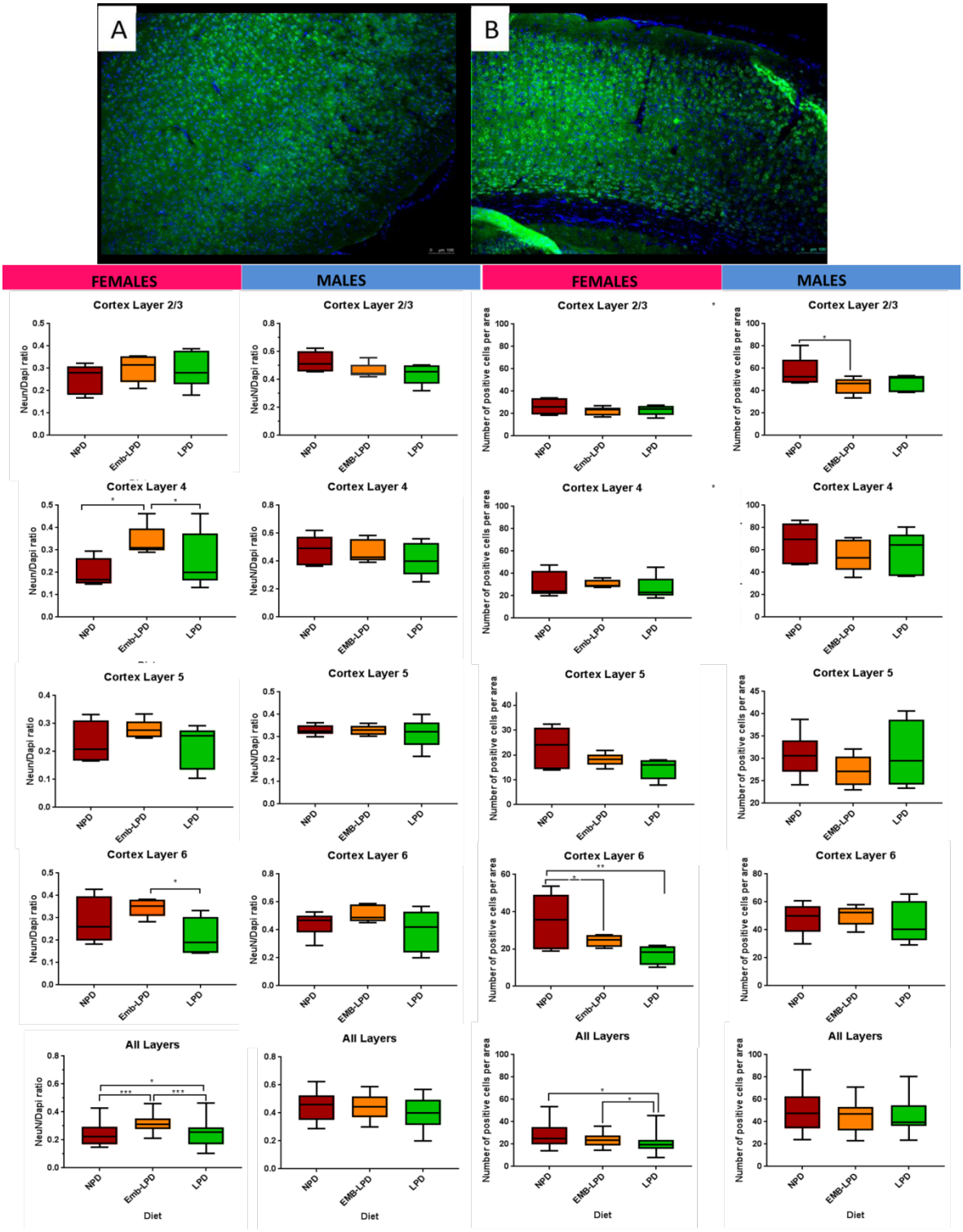


Figure 4.9. Quantification of neuron numbers within the cortex

Quantification of neurons within the offspring cortex for layers 2/3, 4, 5 and 6 as well as a global measure of all layers. The graphs on the left show a ratio of neurons which are positive for NeuN, to DAPI counterstain. A simple cell count of NeuN positive cells per 41,250µm2 was also taken with the data displayed in the two columns on the right. A) Representative immunofluorescence staining of NeuN stain shown in green and DAPI counterstain in blue in the female offspring cortex and B) male offspring cortex

again stained for NeuN shown in Green and DAPI counterstain in blue. Images taken at x10 magnification on fluorescent microscope. Data analysed by Multi Level Regression Analysis, * $p<0.05$, ** $p<0.01$, ***$p<0.001$ n = 5 per maternal diet for female offspring, male NPD n = 6, EMB and LPD n= 5.

4.5.4 Parvalbumin expression in the male cortex

In the cortex of male offspring EMB-LPD mice showed a higher Parvalbumin to DAPI ratio (P : D) within layer 4 compared to NPD and LPD offspring along with the global measure including all the layers indicating EMB-LPD mice have a significantly higher P: D when compared to the LPD group. Cortical layer 5 and 6 also showed a trend of increased P: D for EMB-LPD offspring but these were not significant. This data indicates the proportion of interneurons to all cell types is significantly increased when mice are exposed to the Emb-LPD diet but not when exposed to the LPD diet. A ratio was also calculated for interneurons to neurons using Parvalbumin positive cells to NeuN positive cells (data is not shown) but no difference was seen between the three diets. For the cell count of Parvalbumin positive cells in the cortex no significant difference was observed for any of the measure layers or global measure. Although there is increased P: D in layer 4 no significant increase in Parvalbumin cell count was seen in layer 4 for EMB-LPD offspring however a trend was present.

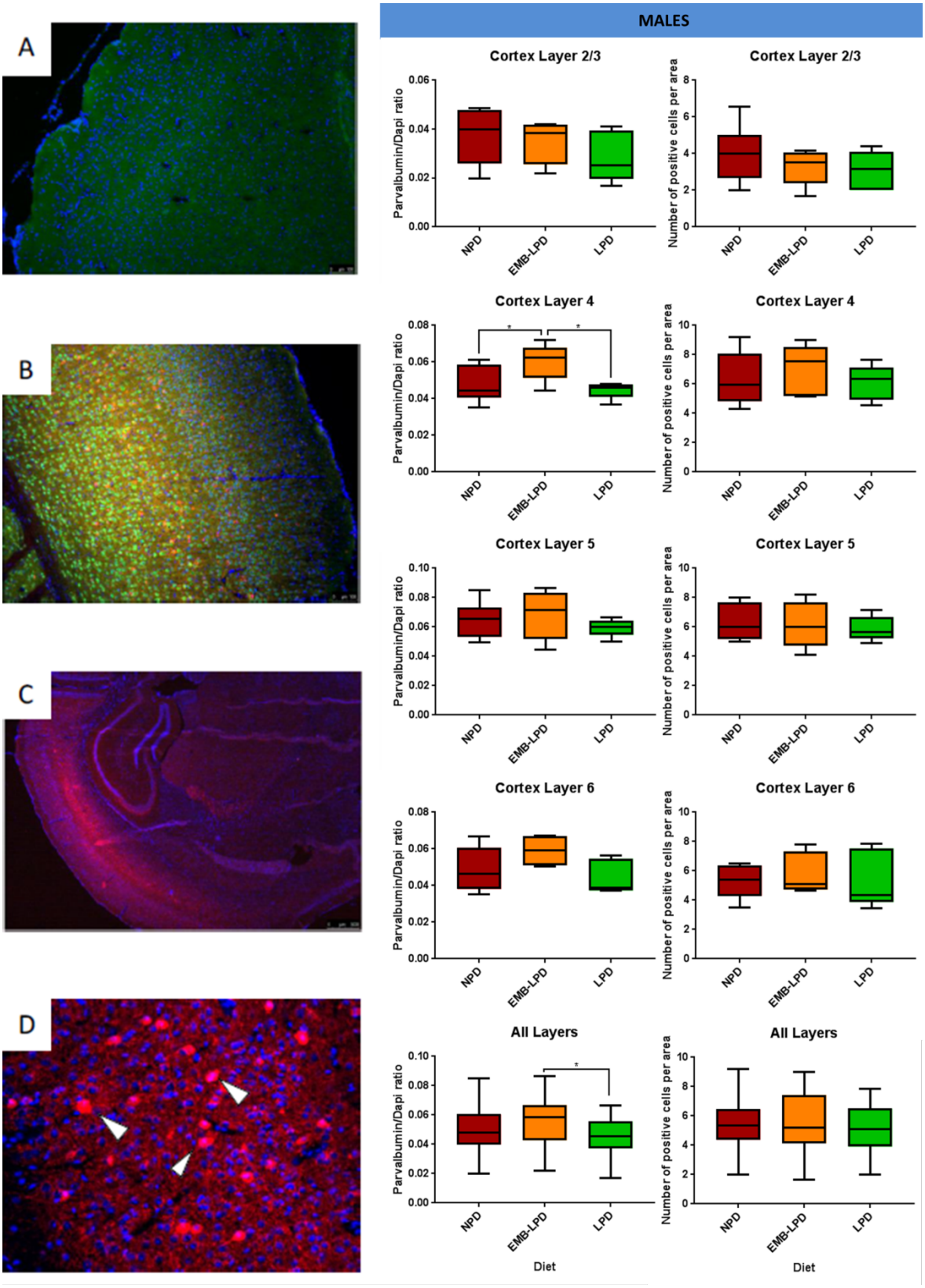


Figure 4.10. Quantification of parvalbumin expressing cells in the male offspring cortex.

Parvalbumin is expressed by interneurons and co-staining was performed with NeuN and DAPI. Data for both parvalbumin to DAPI ratio and parvalbumin+ cell count per 41,250µm^2 are shown along with representative immunofluorescence pictures. A)

Negative control for NeuN and Parvalbumin stain in cortex, only positive stain present is the DAPI counterstain. B) Representative stain of NeuN in green and Parvalbumin in red with DAPI counterstain in blue, image taken at x10 magnification. C) Fluorescence image at x2 magnification of coronal section of male hemisphere showing positive parvalbumin staining in red throughout the whole brain. D) Zoomed in image of parvalbumin staining in layer 2/3 and layer 4 of cortex with arrows indicating parvalbumin positive interneurons. Analysed by Multi Level Regression. * $p<0.05$, ** $p<0.01$, ***$p<0.001$. NPD n = 6, EMB-LPD n = 5, LPD n = 5.

4.5.5 Protein restriction during gestation populations in the dentate gyrus of the hippocampus

Radial glia-like (RGL) cells, a main type of NSC in the dentate gyrus can be capable of generating neurons, astrocytes and oligodendrocytes. We see no changes compared to the NPD for both of the maternal protein restriction diets in male and female adult offspring. In the female offspring, the number of non-RGL cells co-expressing GFAP and SOX2 was increased in the LPD offspring when compared to both the NPD and the Emb-LPD. The population of total GFAP SOX2 contained cells which includes RGL cells was also significantly increased for LPD females as well as for the count of total GFAP positive cells. When we focused on the GFAP positive SOX negative astrocytes population, there was not a significant difference between the three groups. For all of the cell populations counted, there was no significant change within the male offspring. Analysis between male and female offspring in the same diet showed a significant increase in the female LPD offspring compared to male LPD offspring for non RGL cells, total GFAP SOX2 co-staining and all GFAP cells. This indicates the LPD diet has a stronger effect on females than males for this marker. This data shows a change in the cell population which is expressing the stem cell marker SOX2 along with GFAP and the increase seen in LPD for total GFAP positive cells is due to these populations rather than mature astrocytes. The lack of changes in the RGL population suggests the intermediate populations are selectively increased in LPD females. Type 2a and type 2b progenitor cells only express the SOX2 marker and so were not counted and are not contributing to the change seen in this data.

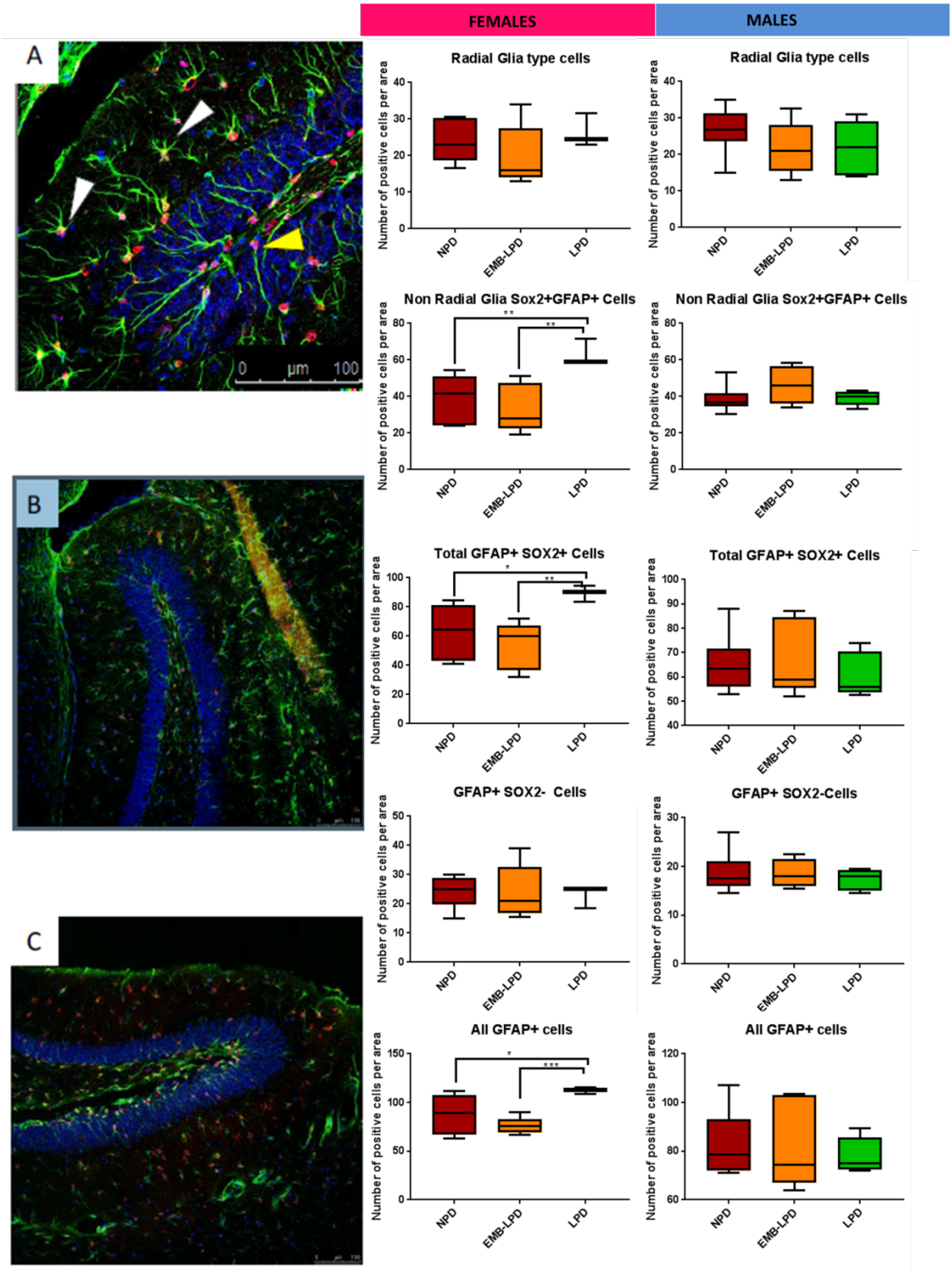

FEMALES
MALES
A
B
C
0 µm 100
Radial Glia type cells
Radial Glia type cells
Non Radial Glia Sox2+GFAP+ Cells
Non Radial Glia Sox2+GFAP+ Cells
Total GFAP+ SOX2+ Cells
Total GFAP+ SOX2+ Cells
GFAP+ SOX2- Cells
GFAP+ SOX2-Cells
All GFAP+ cells
All GFAP+ cells
Number of positive cells per area
NPD
EMB-LPD
LPD

Figure 4.11. Quantification of cell populations in the dentate gyrus

The expression of GFAP, SOX2 and DAPI was analysed for the dentate gyrus region in both the female and male offspring adult hippocampus. Counts for several populations are shown; radial glia like cells (RGL) that express GFAP and SOX2 and are not found in the dense DAPI+ layer; non-RGL cells expressing GFAP and SOX2 in the dense DAPI+ layer; cells only expressing GFAP and the DAPI co-stain. A) Close up view of the dentate gyrus with the yellow arrow indicating a RGL cell and the white arrows indicating example GFAP+ SOX2+ non RGL cells. B) Representative Female NPD dentate gyrus and C) Female LPD example showing increased non RGL cells. Graphs show number of positive cells per 202,500µm2. Male: NPD n= 6, EMB n= 5, LPD n= 5. NPD for total GFAP n=7. Female: NPD n= 5, EMB n= 5, LPD n= 3. Analysed by Multi Level Regression Analysis, * $p<0.05$, ** $p<0.01$, ***$p<0.001$. Mean value and standard deviation plotted. * indicates significance between the sexes.

4.5.6 Protein restriction alter glial populations in the CA1 region of the hippocampus

Quantification of the cells in and immediately surrounding the dense DAPI layer of the CA1 region showed no significant change in GFAP and SOX2 positive cells, mature astrocytes (GFAP positive, SOX2 negative cells) or total number of GFAP positive cells for either female or male offspring. The female offspring do however show a trend of decreased number of cells for all three of the counts with Emb-LPD and LPD offspring having a reduction in positive cells compared to the NPD group. The males do not show this trend with the protein restriction groups remaining similar to the control. Cell counting at the site further away from the dense DAPI layer showed a significant increase in GFAP and SOX2 co-stained cells in the LPD group when compared to the EMB group but not when compared to the NPD control group. This was the only significant difference seen in this region.

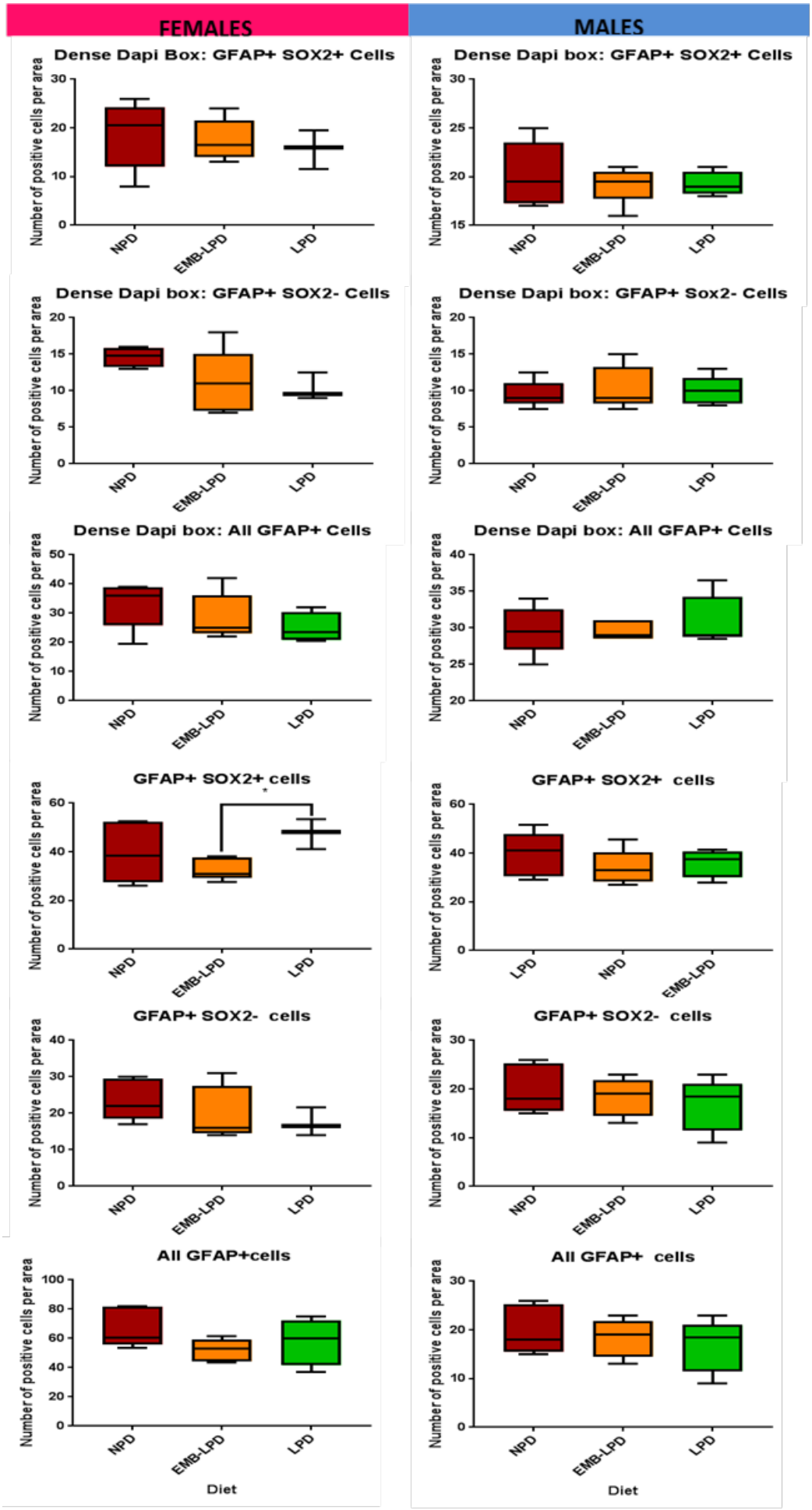

FEMALES
MALES
Dense Dapi Box: GFAP+ SOX2+ Cells
Dense Dapi box: GFAP+ SOX2+ Cells
Dense Dapi box: GFAP+ SOX2- Cells
Dense Dapi box: GFAP+ Sox2- Cells
Dense Dapi box: All GFAP+ Cells
Dense Dapi box: All GFAP+ Cells
GFAP+ SOX2+ cells
GFAP+ SOX2+ cells
GFAP+ SOX2- cells
GFAP+ SOX2- cells
All GFAP+cells
All GFAP+ cells
Number of positive cells per area
NPD
EMB-LPD
LPD
Diet

Figure 4.12. Quantification of cell populations in the CA1 region of the hippocampus

Different cell populations in the CA1 region for both the female and male offspring adult hippocampus were assessed based on expression of GFAP and SOX2 markers and DAPI co-stain. Cell counts were conducted both on the dense DAPI layer of this region as well as just inside of the layer, with the box between the layer and the dentate gyrus region. Graphs show number of cells per 90,000 µm2 for both sites of quantification. Male: NPD n= 5, EMB n= 5, LPD n= 5. Female: NPD n= 5, EMB n= 5, LPD n= 3. Analysed by Multi Level Regression Analysis, ** $p<0.01$. Mean value and standard deviation plotted.

4.5.7 Protein restriction alters glial populations in the CA3 region of the hippocampus

For the area on and immediately surrounding the dense DAPI layer female offspring in the EMB-LPD group showed a significant decrease in the number of GFAP and SOX2 positive co-stained cells as well as for the total GFAP+ count when compared to NPD. Female LPD and all male groups did not show any significant difference compared to the controls. The area further away from the dense DAPI layer showed a significantly reduced GFAP and SOX2 co-stained cells as well as the total GFAP count in the female EMB-LPD offspring again when compared to the NPD and the LPD group. This suggests the number of immature astrocytes which express both GFAP and SOX2 are reduced by the Emb-LPD group in the CA3 region opposed to the response of the female LPD offspring in the dentate gyrus which showed an increase in GFAP positive and SOX2 positive cells. For mature astrocytes characterised by expression of GFAP and absence of SOX2 expression, there was no significant change for any of the protein restricted diets in all three hippocampal regions analysed (dentate gyrus, CA3, CA1).

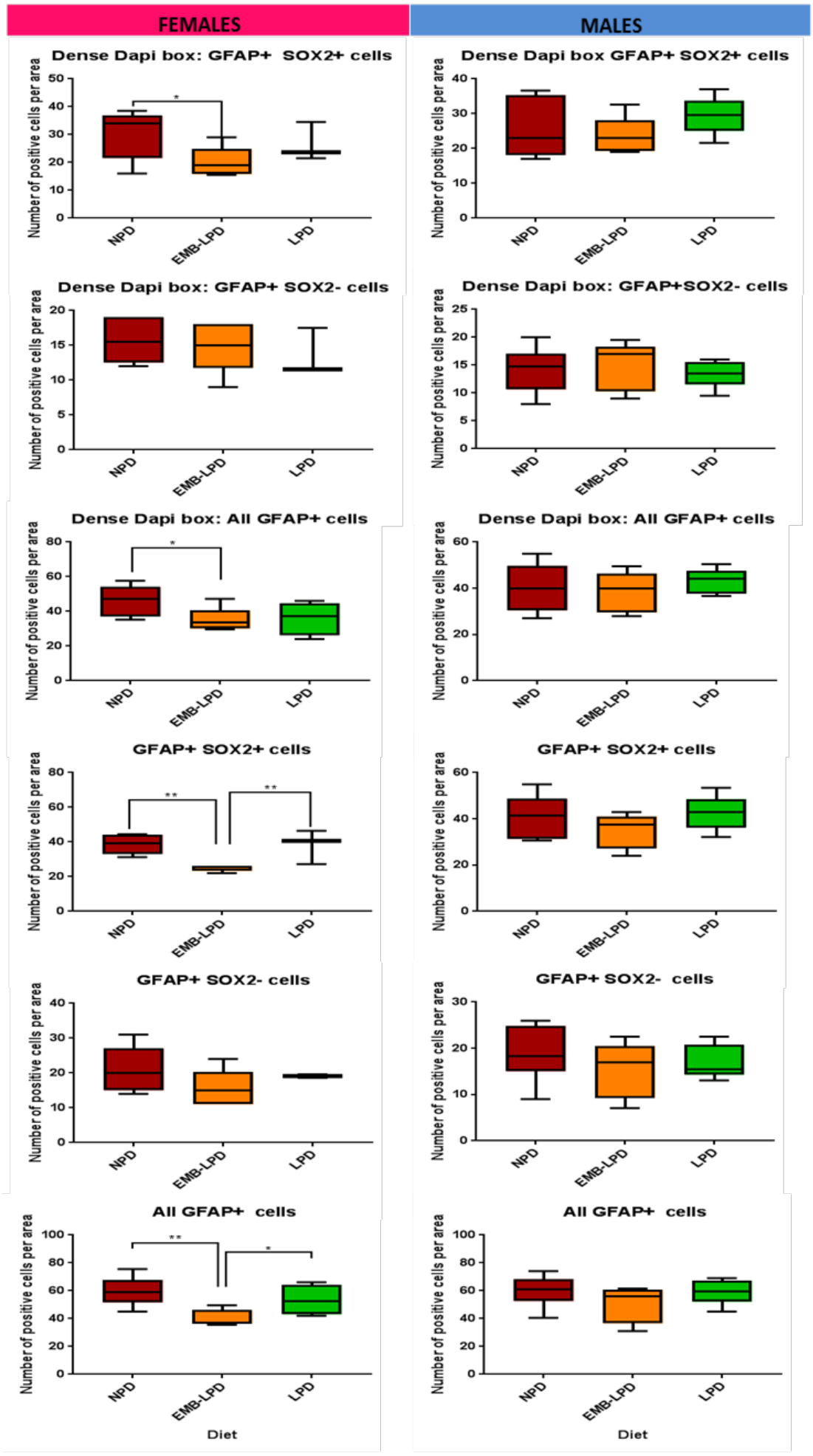

FEMALES
MALES
Dense Dapi box: GFAP+ SOX2+ cells
Dense Dapi box GFAP+ SOX2+ cells
Dense Dapi box: GFAP+ SOX2- cells
Dense Dapi box: GFAP+SOX2- cells
Dense Dapi box: All GFAP+ cells
Dense Dapi box: All GFAP+ cells
GFAP+ SOX2+ cells
GFAP+ SOX2+ cells
GFAP+ SOX2- cells
GFAP+ SOX2- cells
All GFAP+ cells
All GFAP+ cells
Number of positive cells per area
NPD
EMB-LPD
LPD
Diet

Figure 4.13. Quantification of cell populations in the CA3 region of the hippocampus

Quantification of different cell populations in the CA3 region for both the female and male offspring adult hippocampus based on expression of GFAP and SOX2 markers and DAPI co-staining. Images were quantified by two boxes, one placed on top of the dense DAPI layer of the CA3 region and one slightly further in so it sits between the dentate gyrus region and the CA3. Graphs show number of positive cells per 135,000 μm^2. Male: NPD n= 6, EMB n= 5, LPD n= 5. Female: NPD n= 5, EMB n= 5, LPD n= 3. Analysed by Multi Level Regression Analysis, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

4.5.8 Microglia are decreased by protein restricted diets in the hippocampus.

Microglia are another type of glial cell found throughout the brain. Due to the changes, we saw earlier in other glial populations these were quantified within the offspring hippocampal regions CA3, CA1 and the dentate gyrus. Both male and female offspring displayed a marked reduction in the number of IBA1 positive cells for the Emb-LPD and LPD diets within the dentate gyrus when compared to NPD offspring. Although both males and females were significant the females showed a greater decrease with female NPD compared to Emb-LPD ($p = 0.00005$) and males ($p = 0.05$). Quantification on the dense layer of the CA3 region visualised with Mayer's haematoxylin counterstain didn't show any significant change between the diets for both male and female offspring although a trend of decrease in the Emb-LPD females is still present. Analysis just within the CA3 dense layer showed a reduction in IBA1 positive cells for EMB-LPD female offspring. Only females showed a reduction in IBA1 positive cells in the two areas of analysis for the CA1 region, with the EMB-LPD showing a reduction when compared to both NPD and LPD offspring. The area inside of the hippocampus just below the dense layer of the CA1 also showed a decrease for both EMB-LPD and LPD female offspring.

The data shows the Emb-LPD leads to a decrease in the number of microglia present within the dentate gyrus and CA1 region but for the CA3. LPD females in this region were close to significance with $p = 0.09$ and shows the same trend of decrease. In males only the dentate gyrus showed this reduction in microglia for both protein restriction diets. Analysis between the sexes showed females are significantly lower than males for IBA1 stain in the Emb-LPD group. There is no change present between males and females of the NPD group for this region suggesting the Emb-LPD diet has a greater effect on the females then the male offspring.

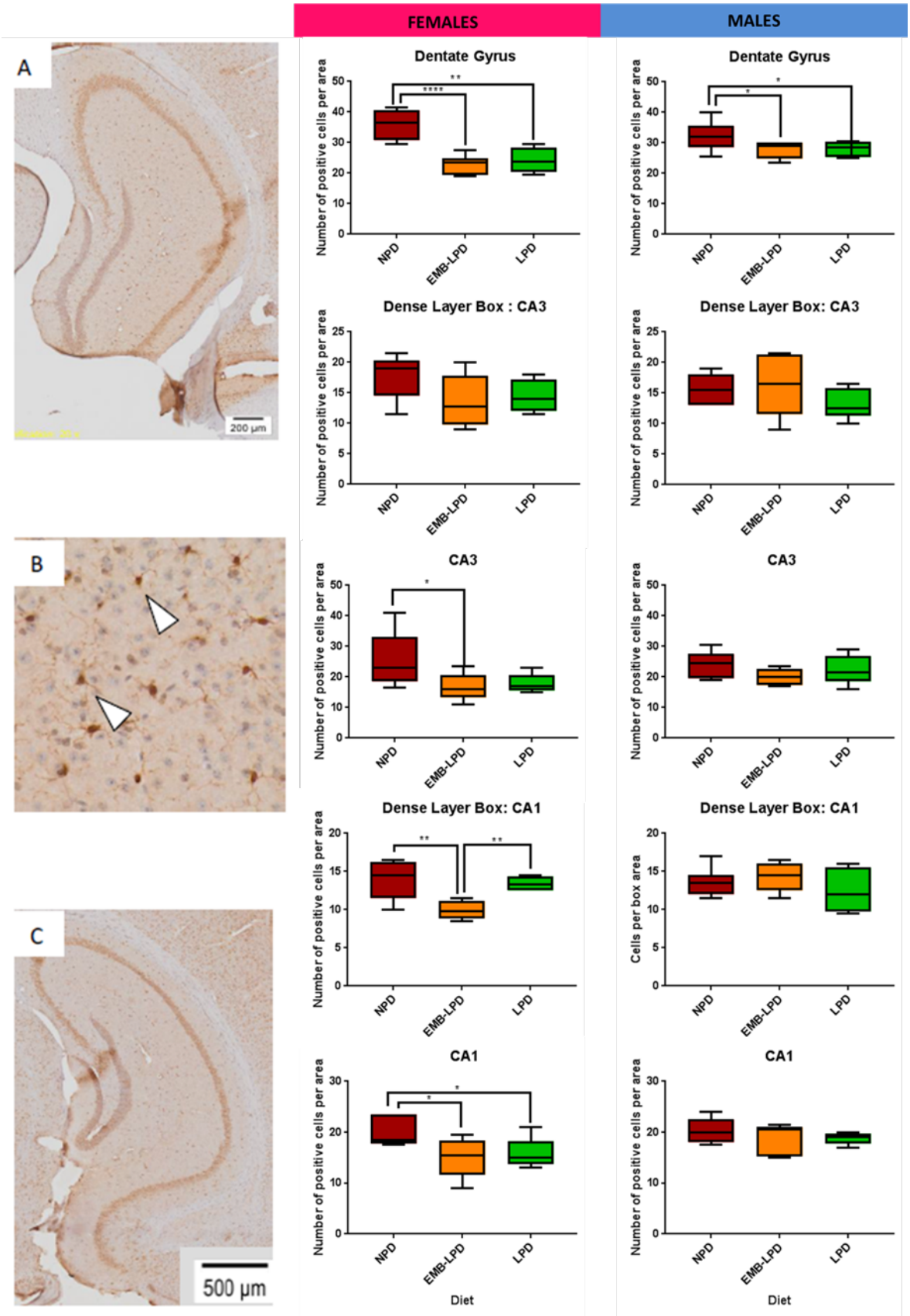

FEMALES
MALES
A
B
C
200 µm
500 µm
Dentate Gyrus
Dense Layer Box : CA3
Dense Layer Box: CA3
CA3
Dense Layer Box: CA1
CA1
Number of positive cells per area
Cells per box area
NPD
EMB-LPD
LPD
Diet

Figure 4.14. Microglia Iba1 staining in 3 regions of the hippocampus

Quantification of Iba1+ cells in the dentate gyrus, CA3 and CA1. A) NPD hippocampus stained with IBA1. B) A close-up of representative IBA1 stain with white arrows indicating microglia. C) Representative stain of EMB-LPD hippocampus. Results shown as cells per box area, size of box for Dentate Gyrus = 202,500 µm2, CA1 = 90,000 µm2 and CA3 = 120,000µm2. Female NPD n = 5, EMB-LPD n = 6, LPD n = 4. Male NPD n = 7, EMB-LPD n = 5 and LPD n = 5. Analysed by Multi Level Regression Analysis, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

4.5.9 Tyrosine hydroxylase expression in the VTA

Quantification of dopaminergic cells in the VTA was achieved through measuring percentage area stain of tyrosine hydroxylase stain, which is a crucial enzyme for the synthesis of dopamine (and other catecholamines) through the conversion of tyrosine to dopamine[145]. Analysis of this stain showed no significant difference for all three diets in adult male and female offspring.

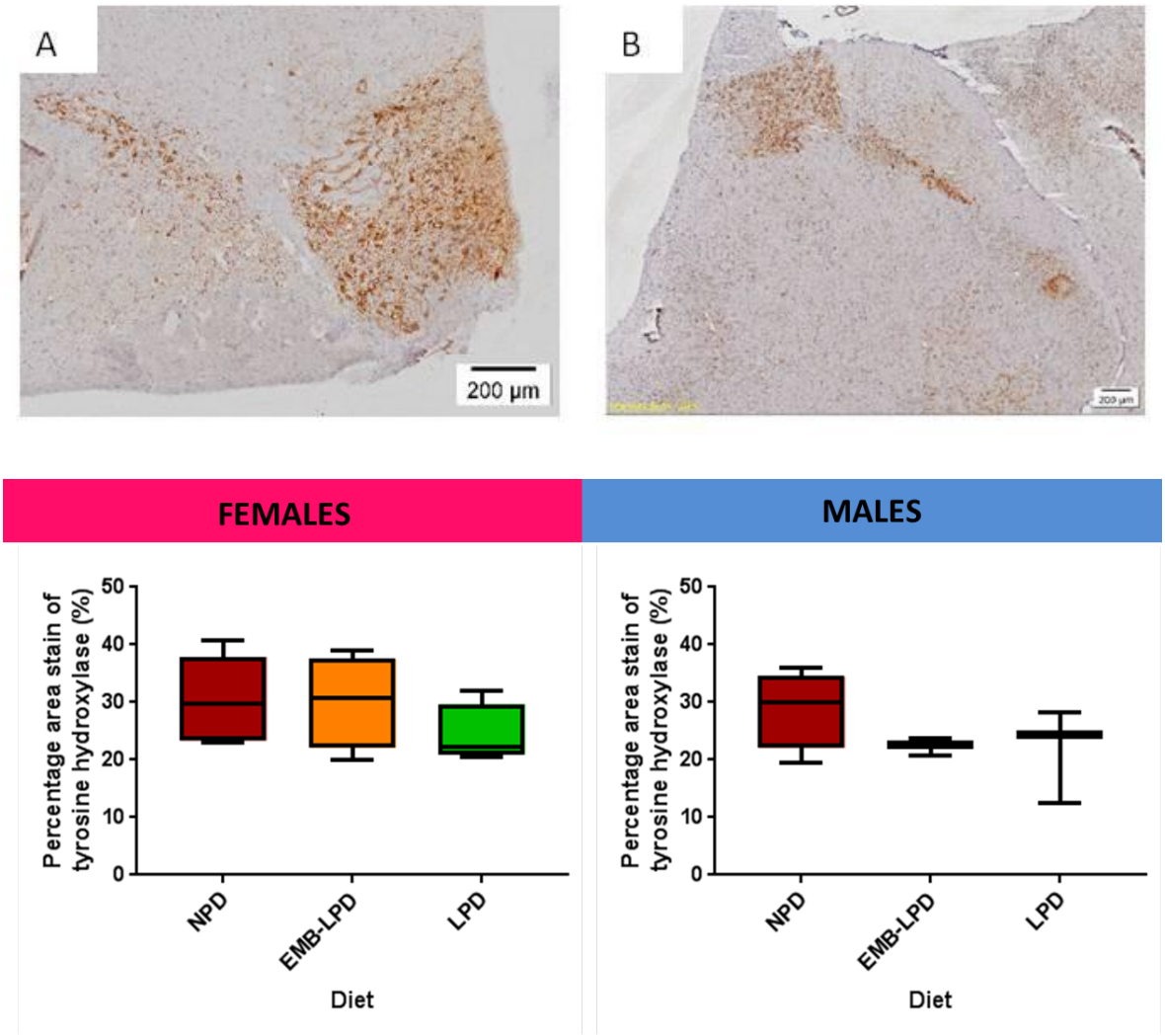


Figure 4.15. Tyrosine hydroxylase expression in the VTA following different maternal diets

A&B) Representative DAB stain of tyrosine hydroxylase positive cells in the VTA. Quantification of tyrosine hydroxylase stain using percentage area stain in the VTA for both male and female adult offspring. Male NPD n = 4, EMB n = 3, LPD n = 3. Female NPD n = 6, EMB n = 4, LPD n = 4. Analysed by Multi Level Regression Analysis. Mean value and standard deviation plotted.

4.5.10 Protein restriction decreases vasculature in the adult offspring brain.

The brain is dependent on the continuous blood supply for oxygen, nutrients and clearance of waste. Compromised vasculature can lead to problem in both morphology and function of the brain. Vasculature was investigated in the brain using collagen IV in the female brain was achieved through measuring percentage area stain. Analysis of this stain led to a significant decrease in collagen IV in the Emb-LPD and LPD in adult female offspring.

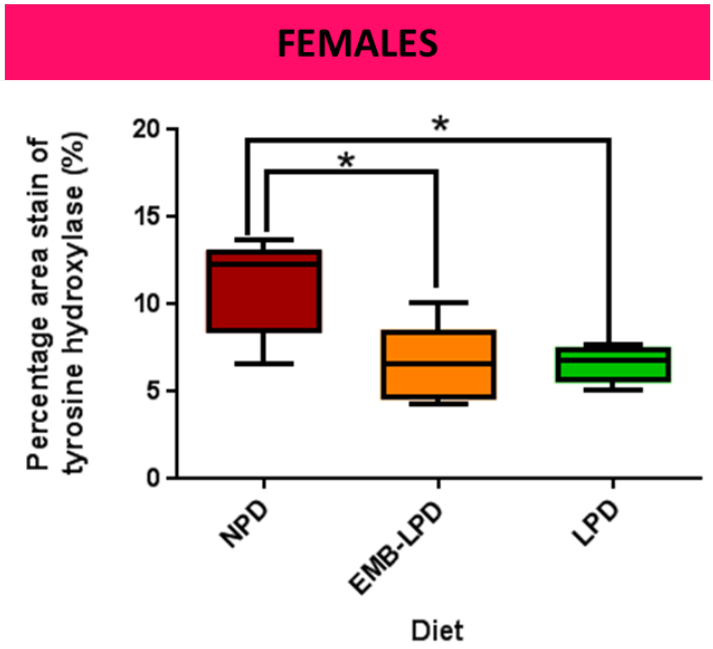


Figure 4.16. Quantification of collagen IV expression across a brain hemisphere

Collagen IV staining was quantified using percentage area stain in the female adult offspring brain. Female NPD n = 6, EMB n = 6, LPD n = 5. Analysed by Multi Level Regression Analysis. * $p<0.05$.

4.5.11 RNAseq data from the female hippocampus

RNA seq data from the female hi Gene data from female Hippocampi was generated using the illumina platform. A significant result was considered to have less than a 0.07 padj value meaning it had passed the false discovery statistics. The LPD females showed a significant result for 4 genes illustrated in table 3.1. Other genes showed significant p values but did not score highly enough to gain a significant padj value in both the Emb-LPD and LPD.

Gene Name	log2FoldChange	stat	pvalue	padj
FXYD domain-containing ion transport regulator 7	-0.339639488	-4.609227027	4.04E-06	0.065466
TDP2 Tyrosyl-DNA Phosphodiesterase 2	-0.355032944	-4.575349672	4.75E-06	0.065466
Bace2	0.400938014	4.405824047	1.05E-05	0.066768
Nuf2	0.402586263	4.375383411	1.21E-05	0.066768
Gas2	0.386877	4.234943989	2.29E-05	0.104931
Basic Transcription Factor 3 Like 4	0.171714184	3.968455143	7.23E-05	0.250149
acyl-CoA thioesterase 13	-0.330292111	-3.888751655	0.000100761	0.250149
AA474408	-0.266520995	-3.869191256	0.000109197	0.250149
methyltransferase like 16	0.231380008	3.855285222	0.000115595	0.250149
Fas Activated Serine/Threonine Kinase	-0.22213843	-3.85007733	0.000118081	0.250149
PABPC4	-0.237890923	-3.823330177	0.000131661	0.258997
GAS2L1	-0.33285559	-3.764708891	0.000166743	0.288585
Zfp87	0.274474354	3.743704408	0.000181327	0.288585
Cercam	-0.34347432	-3.733792881	0.000188618	0.288585
PPIA	-0.330542928	-3.694649752	0.00022019	0.31916
Vasp	-0.311288341	-3.649398303	0.000262855	0.343887
FOXRED1	-0.24569012	-3.642045686	0.00027048	0.343887
BIRC2	0.202878187	3.629617316	0.000283842	0.343887
FAM179A	0.305691119	3.626582783	0.000287197	0.343887
TARDBP	0.178404286	3.561943055	0.00036812	0.390736
GLS2	-0.260775457	-3.508787195	0.000450155	0.419152
Neurensin 1	-0.240215498	-3.488720015	0.000485339	0.419152

Table 4.5 RNA Seq top 22 results for LPD Hippocampi compared to the NPD.

Gene Name	log2FoldChange	stat	pvalue	padj
acyl-CoA thioesterase 13	-0.355778687	-4.301511152	1.70E-05	0.467180944
Brip1os	0.331552758	3.761398359	0.000168966	0.992611795
TDP2 Tyrosyl-DNA Phosphodiesterase 2	-0.280649996	-3.746819396	0.000179091	0.992611795
FBXL13	0.317413283	3.73693747	0.000186275	0.992611795
MAPK15	0.285828844	3.680014571	0.000233221	0.992611795
Neurensin 1	-0.238497303	-3.616129382	0.000299041	0.99998475
LCORL	0.216560816	3.478440446	0.00050434	0.99998475
Gm10516	0.316532864	3.468358483	0.000523648	0.99998475
dynein, axonemal, heavy chain 3	0.25556425	3.340621654	0.000835911	0.99998475
SV2C (Synaptic Vesicle Glycoprotein 2C)	0.298582783	3.289183859	0.001004784	0.99998475
OR2J2 (Olfactory Receptor Family 2 Subfamily J Member 2)	-0.284061944	-3.254342825	0.00113655	0.99998475
Fxr2	-0.152964744	-3.251386796	0.001148435	0.99998475
TNFSF13B	0.294586229	3.242335054	0.001185546	0.99998475
PPM1J	-0.176427618	-3.22769182	0.001247933	0.99998475
Nudcd3	-0.11421712	-3.129091338	0.001753478	0.99998475
Pcdhb3	-0.24084996	-3.085545651	0.00203179	0.99998475
tRNA methyltransferase 6	-0.22054354	-3.07760414	0.002086719	0.99998475
Armadillo Repeat Containing 10	-0.168120529	-3.061410828	0.002202966	0.99998475
Aldehyde Dehydrogenase 5 Family Member A1	-0.149867975	-3.042135023	0.002349065	0.99998475
N-Acetyltransferase 8 Like	-0.178739316	-3.032899332	0.002422164	0.99998475
cercam	-0.27731026	-3.010880402	0.002604914	0.99998475
Transcription Factor AP-2 Gamma	0.139219322	2.947915315	0.003199247	0.99998475

Table 4.6 RNA Seq top 22 results for Emb- LPD Hippocampi compared to the NPD

4.5.12 qPCR analysis of FXR2 and FMR1 in the female offsprig cortex

qPCR analysis of genes in the fragile X complex FXR2 and FMR1 in the male and female cortex, showed a significant decrease in both FMR1 and FXR2 in the Emb-LPD and LPD in adult female offspring.

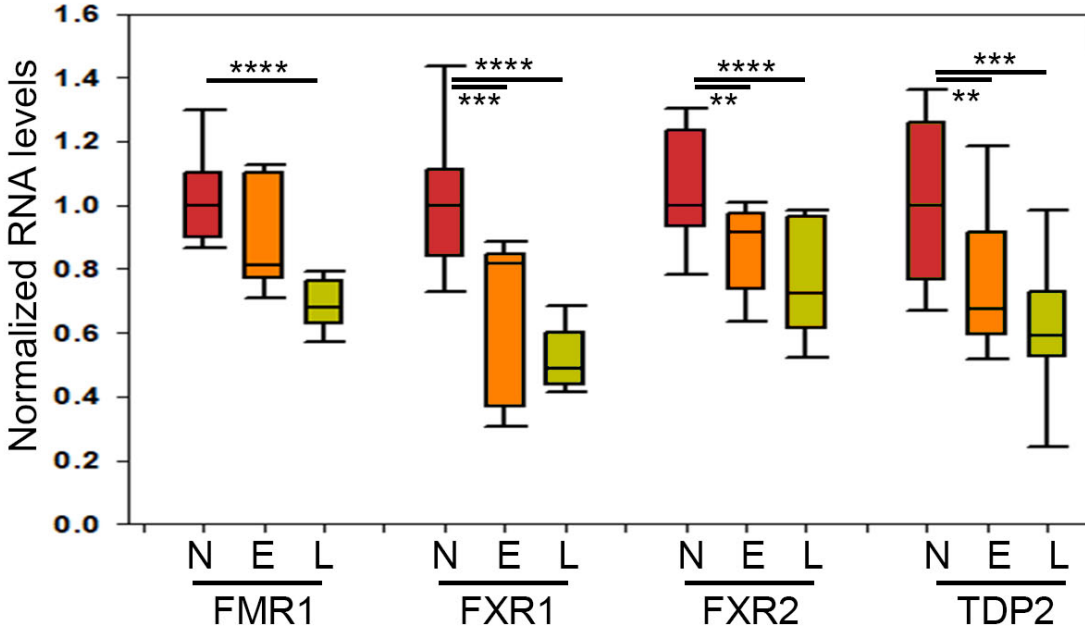


Figure 4.17. qPCR analysis of FXR2, FMR1, FXR1 and TDP2

Quantification of *Fmr1, Fxr1, Fxr2* and *Tdp2* RNA levels by qRT-PCR, normalized to housekeeping genes *Ap3d1* and *Gapdh*. Data represent 1 sample/brain of 12-19 brains from 3 or 4 different mothers per group. qPCR of FXR2 FMR1, FXR1 and TDP2 female and male adult offspring cortex showed significant decreased in the Emb-LPD and LPD. ***p<0.001, ****p<0.0001.

4.6 Discussion

The aim of this chapter was to investigate the morphology and gene expression of the adult offspring of our LPD and Emb-LPD groups. We discovered that protein restriction during pregnancy has lasting consequences for the development of the brain with the morphological differences summarised in the table 4.6. The key findings show maternal dietary quality from conception to be a critical factor in brain developmental capacity with enduring consequences on brain organization with the majority of changes occurring in the Emb-LPD.

	Emb-LPD Females	Emb-LPD Males	LPD Females	LPD Males
Cortical thickness	↑	↑	No change	No change
Cell number in the cortex	↓	↓	↓	No change
Neuron/ Dapi ratio cortex	↑	No change	No change	No change
Neuron count Cortex	↓	No change	↓	No change
Parvalbumin number in hippocampus	Not assessed	↑	Not assessed	No change
Radial glia in dentate gyrus	No change	No change	No change	No change
Early astrocytes GFAP +SOX+ cells in dentate gyrus & CA1	No change	No change	↑	No change
GFAP+ cells CA3	↓	No change	No change	No change
Iba1 in dentate gyrus	↓	↓	↓	↓
Iba1 CA3	↓	No change	No change	No change
Iba1 CA1	↓	No change	↓	No change
Tyrosine Hydroxylase In VTA	No change	No change	No change	No change
Collagen IV	↓	Not assessed	↓	Not assessed

Table 4.7. Summary of immunohistochemistry data from the adult offspring brain

4.6.1 Impact of maternal protein restriction on brain cytoarchitecture

This study showed gross changes to the cortical cytoarchitecture after exposure to the EMB-LPD. Female EMB-LPD mice showed an increase in the thickness of all cortical layers measured except for layer 4 while males only showed this increase in layer 6 and total thickness. These thicknesses are seen however there was also no increase in brain weight (data not shown). There is a higher variability in adult brain morphology data in males than females. Traditionally it is usually suggested female rodents show more variation in their data however we see the opposite[214]. Variability in

females is increased by group housing despite the absence of fighting and our mice are group housed. The source of intrinsic variability in both sexes remains an important question and one that is still unanswered. Studies have shown variance in male data is as much females if not greater in behaviour, immunohistochemical analysis and gene expression. Variability associated with the females data can be due to estrous cycle in some examples but the reason for male data variance is still unknown. The differences we see between genders in cortical thickness maybe gender specific, an increase in the n number may give more power behind the statistics and return significant data for the other layers. If this is not the case and the males only change for layer 6 and overall thickness it shows a sex specific effect with the morphology of the female offspring being influenced to a greater extent than the males by maternal protein restriction in the pre-implantation period. While the Emb-LPD shows an increase in cortical thickness both the Emb-LPD and the LPD females and the Emb-LPD males show a decrease in cells per unit area in the cortex compared to the NPD mice. This suggests there is a reduction of cells in the cortex after a protein restriction diet in utero, but this isn't necessarily the case for Emb-LPD offspring. Dapi positive cells were counted with a box of a specified size for every brain, however instead of a change in the number of cells present within the cortex could be due to the increase in thickness causing the cells to be more spread out leading to fewer cells present within the box. As the LPD group does not show this change in cortical thickness and for females there is still a clear reduction in cells, ($p=0.0001$) for both EMB-LPD and LPD, it is likely that for both diets there is a true decrease in the number of cells present. However, there is a possibility that the extra cellular matrix could be compromised and causing these cells to be further apart. The extra cellular matrix is mainly composed of proteoglycans of the lectican/ hyalectan-family, hyaluronan, and small amounts of collagen and fibronectin[215]. It has been shown in the mouse that mature nervous system express perineuronal proteoglycans and nerve cell glycoproteins are linked by certain collagenous ligands. Future work would be interesting to see if the extra cellular matrix of the low protein diet brains had been compromised. Another study showed a significant reduction in hippocampal cell density after a prenatal undernutrition challenge with mums receiving 50% food intake [216]. While this study is not exclusively a protein restriction model and so cannot be directly comparable to this current study, it does provide support to our data. Specific protein restriction of 8% during pregnancy has been shown to reduce the amount of DNA in the offspring brain, thereby supporting our findings.

Similarly, prenatal protein restriction in rats, with a 5% casein diet commenced at conception and until two weeks into gestation reported a reduction in cortical thickness and brain weight in fetal animals but a normal brain structure in postnatal animals[128]. This contradicts our findings in the Emb-LPD where we see alterations in the adult brain. The differences here could be attributed to a more severe protein restriction imposed (5% casein vs 9% casein) and a longer duration than the

EMB-LPD group. These discrepancies may also suggest the changes to cortical thickness are transient due to the absence of altered cortical structure in the postnatal mice, which may reflect a high plasticity within the brain over the prenatal and early postnatal periods, leading to a compensatory process[128]. Our results also showed that the LPD offspring do not show any differences for cortical thickness in adulthood, again highlighting the importance of duration of the exposure to the modified diet.

4.6.2 Impact of maternal low protein restriction on neurogenesis

Our findings revealed that maternal protein restriction affects the number of cells present in the brain of adult offspring. We further assessed whether this observation was due to alterations in the number of neurons. We found a reduction in the number of neurons in both EMB-LPD and LPD females for layer 6, and in EMB-LPD males for layer 2/3, this would suggest that the protein restriction experienced during pregnancy leads to a loss of neurons in the adult offspring for these two layers. Female Emb-LPD mice show no detectable change in neuron number for layer 4 but as they have a reduced number of cells, this results in an increase in a NeuN to Dapi ratio (N : D) in layer 4 when compared to both NPD and LPD mice, as well as layer 6 also showing this when compared to LPD mice. This change could be brought about by a higher differentiation of progenitors into neurons or more of the neurons surviving to maturity, meaning the layer 4 cortical neurons are spared in EMB-LPD females similar to what we see in the foetal mouse brains. This increase in the proportion of neurons is not necessarily beneficial as they may be not functional. An increase in neuronal differentiation may mean there is less selectivity during development, which will give place to neurons with poor synaptic functionality or reduced ability to integrate into a circuit. Astrocytes and microglia have both been shown to have important roles in synaptic development through controlling excitatory transmission and synaptic pruning among other functions[97 101]. The data in this project has shown a decrease for both of these glial cells within this hippocampus, if this decrease extends to the cortex the increase in differentiating neurons in development combined with a reduction in glia may mean there aren't enough glia to optimally regulate all the neurons. This could result in non-functional synapses as well as reduction in apoptosis of defective neurons, a process which microglia are actively engaged in, enhancing neuronal survival. This increase in neuron proportion is supported by earlier fetal data presented within this model where using flow cytometry EMB-LPD mice showed an increase at E14.5 and E17.5 for β-III-Tubulin, which is a neuronal specific marker, suggesting increased neural differentiation in development which seems to persist into adulthood at least for cortical layer 4 and 6 in EMB-LPD females. These extra neurons may just be making unnecessary connections not actively contributing to the inter-cortical and intra-cortical circuits meaning their presence is detrimental to cortical function.

Parvalbumin is a member of calcium binding proteins and most of the Parvalbumin reactive interneurons in the hippocampal formation are inhibitory GABAergic interneurons[171]. EMB-LPD males also showed a reduction in the number of Dapi positive cells present in layer 4. This same layer has shown an increase in the P : D ratio compared to the LPD and NPD diet suggesting the Parvalbumin positive interneurons were spared this reduction. Although not significant the other layers also show a trend of increased P : D ratio in EMB- LPD male offspring however the global measure for EMB-LPD does show significant increase compared to the LPD group. The count of Parvalbumin positive cells did not show any change in cell number for any of the cortical layers including layer 4 so the change in ratio isn't due to more cells compared to NPD but an altered proportion of interneurons to other cell types, especially for layer 4. A ratio of interneurons to neurons displayed no difference in these male offspring implying for the cortex there isn't a changed proportion between these neuronal subgroups.

Within the cortex Parvalbumin positive cells make up 40% of the GABAergic interneuron population, the increase in the proportion of interneurons within the male cortex may indicate an increase in inhibitory tone altering the neuronal signalling capacity and circuitry. This combined with the increase in relative proportion of neurons within the female layer 4 indicates the functionality of this layer in Emb-LPD mice may be compromised. The influence of the GABAergic cells is needed to ensure the correct balance of excitation and inhibition allowing for distinct perception of sensory inputs[217] , the changes in neuron proportion and GABAergic inhibition for females and males respectively may cause disruption in this circuit for the EMB-LPD mice.

The results for male and female NeuN cannot be directly compared due to the female sections selected being more anterior/rostral compared to the males which were selected further along the axis towards the caudal end to include hippocampus as well. Because of this changes between the two sexes cannot be confidently attributed to the maternal diet as they may be due to differences in the neocortex along this axis. Females have not yet been analysed for Parvalbumin positive cells in the cortex. As a change in P : D ratio in males has already been shown by this data it could be possible that females, which seem to be more sensitive to maternal protein restriction in this model, will also show differences in P : D ratio and this would be interesting future work.

For both males and females the hippocampal formation has not been examined for NeuN and Parvalbumin stain. Previous studies revealed that protein restriction in two month old rats results in a reduction in the number of granule neurons in the granule cell layer and hilus region of the dentate gyrus, as well as increasing the number of Parvalbumin positive interneurons in these regions, showing alterations in the proportion of interneurons to neurons[218]. Prenatal protein restriction has also been shown to cause a reduction of 20% in neuron numbers for the CA1 hippocampal field [219]. Evidence of this imbalance favouring GABAergic interneurons in the hippocampus after protein restriction has been postulated as a possible mechanism to explain the

learning and memory impairments seen in protein restriction models. An increase in inhibitory signalling and a reduction of neurons in the hippocampus will alter the signalling between hippocampal regions. Increased inhibitory input may also change the rate of fire of granule neurons in the dentate gyrus which project axons to connect with pyramidal neurons in the CA3 for memory formation[218]Given that our results showed that EMB-LPD mice suffer from short term memory deficits in adulthood, it would be interesting to further analyse these different neuronal populations in the context of the hippocampus.

4.6.3 Radial glia and astrocytes within the hippocampus

Staining for GFAP and SOX2 allowed quantification of type 1 NSC (radial glia) within the dentate gyrus, as well as investigation into changes in astrocytic cells by counting cells positive for GFAP throughout the hippocampus. Radial glia was chosen for analysis as they are one of the progenitor pools for neuronal and glial lineages within the brain, located within the sub granular zone of the dentate gyrus and actively engaged in neurogenesis and glial genesis throughout adult life. Adult neurogenesis can be influenced by environmental conditions, particularly maternal undernutrition [79] so this area is of particular interest to this study. For both males and females there was no significant change in the number of radial glia present for both EMB-LPD offspring and LPD offspring. This shows the original stem cell pool in the dentate gyrus is not impacted during adulthood. However we have not measured other neural progenitors within the hippocampus; type 2a and 2b neural progenitors still express SOX2 but do not express GFAP and therefore were not taken into account in our analysis. The number of non-radial glia cells which co-expressed GFAP and SOX2 was significantly increased within the dentate gyrus of LPD females. The expression of GFAP, a well categorised astrocytic marker strongly suggests these are cells which have committed to differentiating into mature astrocytes and therefore are part of the glial genesis rather than neurogenesis pathway, unlike the radial glia who are part of the neurogenesis pathway as well. These cells are most likely immature astrocytes which are still expressing the SOX2 stem cell marker but are differentiating into mature astrocytes. It was noted that these cells had similar morphology to mature astrocytes. The opposite however was seen in the Emb-LPD females for the CA3 region where a significant reduction in these cells was observed. This suggests the pre- implantation insult influences glial genesis in offspring differently to the protein restriction throughout pregnancy. When considering the cells which only express GFAP and therefore are mature astrocytes there were no differences in cell count between the diets in all three regions investigated. This pattern suggests there are a normal number of radial glia which give rise to a higher number of immature astrocytes but not all of these reach maturity. Looking at all the GFAP positive cells collectively LPD females have a higher number in the dentate gyrus while Emb –LPD females show a reduction in

the CA3 region. This suggests the Emb-LPD results in different developmental programming compared to the protein restriction throughout pregnancy, with an early insult decreasing the astrocytic populations and a longer insult increasing the total astrocytic populations and there could be a number of programming reasons for this we will discuss later.

It is well-known that radial glia plays a crucial role as scaffold for migrating immature neurons. In addition, radial glia in the dentate gyrus give rise to new granule neurons and glia populations such as astrocytes throughout adulthood. It has been suggested that the unbranched process of radial glia may convey information about the immediate environment back to the nucleus which allows each cell to develop the correct phenotype for the environmental conditions and region [220]. If this is indeed true and the radial glia are sensing the environment during the development of new glial cells and neurons, the maternal LPD challenge may be sensed by these precursors and changes seen in LPD group may be in part due to fate decisions that the radial glia make, leading to the generation of more intermediate astrocytes. It has also been suggested that radial glia support the outgrowth of neurites in immature neurons and influence the organisation of the granule cells into a compact layer[62] so any environmental impact such as low protein diet during the initial development of the hippocampus could lead to changes in cell organisation and structure.

Overall comparison of both sexes shows changes within the female offspring but not the male, with female offspring having a normal number of RGL cells but a higher number of immature astrocytes, this however does not lead to a higher number of mature astrocytes within the dentate gyrus. These populations taken as a total show LPD females have a higher number of astrocytic cells in the dentate gyrus.

To be able to efficiently process information in the brain, the correct number and types of synapses need to be formed to allow functionality in the greater neural circuits. Astrocytes have been shown to be critical in the formation and maturation of synapses as well as controlling their function [221]. The ability to receive synapses has to be acquired by neurons and is not an ability they innately possess. Physical contact with astrocytes induces alterations in the location of synaptic adhesion molecule neurexin in neuronal dendrites and allows the process of receiving synaptic input to begin[221]. Direct contact between astrocytes and neurons has been shown to increase activity of presynaptic terminals, amplitude of postsynaptic currents and number of excitatory synapses. Astrocytes are also critical for the maturation of synapses through the expression of glypicans, a family of heparin sulphate proteoglycans, anchored on the extracellular side of their membranes[222]. These glypicans have been shown to increase the frequency of glutamatergic events by increasing the expression of GluA1 subunit of the AMPA glutamatergic receptor, with astrocytes expressing glypican 4 at high levels within the hippocampus[222]. With this in mind, changes to astrocyte number may have an effect on the functionality of neuronal circuits, and may partially explain the behavioural alterations we found in in the Emb-LPD offspring.

Alterations in glial populations have been implicated in neurological conditions such as schizophrenia[220]. One of the proposed causes of schizophrenia is perturbed neurodevelopment, radial glia are involved in neurogenesis and migration during development and have been proposed to contribute to the condition due to cytoarchitectural abnormalities in schizophrenic patients[182]. Post mortem brains have shown an increase in the number of large neurons in deeper layers of the cortex and decreased numbers of small neurons in the superficial layers of the cortex suggests impaired migration of neurons[223]. Both increases and decreases in the numbers of astrocytes have both been reported in the literature regarding schizophrenia and it is still under debate as to whether these changes are caused by the pathology itself or the use of antipsychotic drugs[223]. A reduction in the number of mitochondria in astrocytes has been reported suggesting changes in astrocytic energy metabolism in schizophrenia as well as increased S100B, a Ca^{2+} binding protein expressed by astrocytes, in the cerebrospinal fluid of schizophrenic patients[182]. With the link to schizophrenia shown in the Dutch Hunger Famine[26] , and alterations to behaviour seen in famine and protein restriction models such as increased aggression[208], changes to astrocyte populations like the ones seen in this study may contribute to behavioural differences in offspring protein restriction models.

4.6.4 No change in tyrosine hydroxylase protein levels with the VTA

Recent literature on maternal nutrition has indicated that dopaminergic pathways are sensitive to nutritional changes both postnatally and prenatally with a sub-optimal environment leading to altered dopamine circuitry. This had been shown in obesogenic models where three weeks old mice were fed the diet for a period of 12 weeks, leading to the gene expression of tyrosine hydroxylase doubling[224] as well as in protein restriction models. The main evidence for changes in dopaminergic circuitry in maternal protein restriction models comes from the previously discussed mouse model where mums fed a diet of 8.5% protein throughout pregnancy and lactation. The offspring had a 6-8 and 3-4 fold increase in TH mRNA expression within the VTA and DAT, respectively, and an overall increase in the the number of TH positive cells within the VTA [145].

The VTA has been shown to form a loop with the hippocampus where dopaminergic input from the VTA is crucial for novel and long-term memory formation [145]. Given that we found memory impairments in the EMB-LPD offspring, we further assessed for potential changes in the VTA in these mice. Contrary for previous findings, our results showed no change in percentage area stained for TH for the three diets in male and female offspring. That being said gene expression doesn't necessarily translate into protein expression levels and it could be the changes seen within our model are only seen at the genetic level. The other study also showed evidence of epigenetic modification with the Cdkn1c gene promoter in protein restricted models displaying

hypomethylation[145]. This gene has roles in both growth retardation with *in utero* growth restriction being well categorised as a consequence of protein restriction as well as development and differentiation of dopaminergic neurons, investigation into the methylation status of this gene in the current project may yield interesting results.

The VTA is not the only centre which projects to the hippocampus. Along with the VTA-hippocampus loop, projections from the LC to the hippocampus also enhance memory formation and persistence after a novel stimulus[225]. The VTA and LC tyrosine hydroxylase positive dopaminergic neurons both show a higher frequency of fire in novel environments, but there are substantially more axons from the LC projections than from the VTA, suggesting the LC projections are important mediators of memory consolidation in situations of environmental novelty[225]. While the VTA-hippocampus loop novelty pathway has been supported with animal[88] and human data[226], recent studies have suggested that this loop has a small effect on consolidating novelty memory compared to the effect that the LC projections to the hippocampus do[225]. The LC can therefore be investigated in future studies on this model for changes in tyrosine hydroxylase as well as analysing other targets in the dopamine pathway.

4.6.5 Reduction in microglia number present in offspring hippocampus

The number of microglia was reduced in the dentate gyrus in EMB-LPD and LPD offspring for both male and female mice, with females also showing reductions in the CA1 and CA3 of the hippocampus. Male EMB-LPD offspring only showed a reduction in the latter. With this point raised for the other cell populations discussed, as the EMB and LPD groups show a reduction in cell count in the cortical layers suggesting reduced cell density throughout the brain a proportion of IBA1+ cells to haematoxylin stained cells needs to be taken to see if there is still a reduction in microglia in a decreased cell density background. If there is changed proportion of microglia or simply a reduction proportional to the reduction in other cell populations as EMB-LPD mice show increased cortical thickness and there is no reduction in brain size reported for the LPD group there is still less microglia available to survey the brain parenchyma compared to the NPD group. As microglia are the main immune cells in the brain, responsible for phagocytosis and surveying the brain for damage or cellular dysfunction, a reduction in this protein restriction model may lead to a reduced capacity of the brain to respond to damage or pathogenic events. Microglia are responsible for releasing inflammatory mediators such as TNFα and interleukin-1β. Obesity has been shown to induce chronic inflammation which translates to offspring in maternal high fat diet and obesogenic models through an increase in number of active microglia in offspring CNS and a higher inflammatory status[227].

Microglia have recently been proposed to have roles in synaptic plasticity and memory through their release of cytokines which along with inflammation are important in consolidation of memory and learning processes. IL-1β, TNF, IL-6 and prostaglandins are cytokines implicated in learning processes which are released by microglia[100] as well as IL- 1 which can activate endothelial cells to produce trophic factors such as IGF-1 and VEGF which also facilitate neural plasticity, neurogenesis and memory[228]. Memory formation is a period of time with high synaptic structural plasticity. Microglial processes are in close contact to neuronal cell bodies and dendrites and have neuronal activity dependent functions as well as being regulated by sensory experiences. Microglia engage in synaptic pruning during development, removing excess synapses and synapses with weak connections, with the rate of spine elimination exceeding the rate of spine formation[100]. This has been shown through the colocalisation of GFP labelled microglial processes and post synaptic density 95 (PSD95) a marker of synapses in the developing mouse hippocampus during synaptic maturation [229]. It has been proposed that they may carry out this function in a controlled manner, phagocytosing specific subsets of synapses activated in the plastic process of learning and memory, facilitating the remodelling and survival of these newly formed spines and in doing so have an integral role to formation of memory. It has been shown that an absence of microglia leads to a significant reduction in the formation of these learning or experience dependent postsynaptic dendritic spines both in young and mature mice[230]. Selective removal of microglia derived BDNF, also led to a decrease in learning dependent spine formation[230] with BDNF being well known for its importance in memory consolidation and formation. Therefore, the reduction in microglia seen in this project for both Emb-LPD and LPD offspring within the hippocampus may lead to a reduction in the availability of BDNF or disruption to the dendritic spine pruning impairing memory and learning.

Microglia perform many roles in neurodevelopment the mechanism behind the reduction in this population should be investigated.

Brain blood vessels which invade the developing brain during neurogenesis and microglia have a common origin in the in the embryonic yolk sac. Interestingly, we found a similar decrease in the vasculature of Emb-LPD and LPD mice shown by a reduction in collagen IV, which may suggest fundamental changes undergone by the progenitor cells embryonic yolk sac after protein restriction during pregnancy. The yolk sac has already been shown to respond to Emb-LPD and LPD diet within this model, showing increased endocytosis as well as a higher number of endocytic vesicles suggesting a compensatory mechanism in response to protein restriction[12]. The plasticity of the yolk sac to the maternal environment could have altered the progenitor pool or the differentiation pathways for these two cell types. Elucidating this mechanism would be interesting due to the implications a reduction in microglia and blood vessels has for neural health of protein restricted individuals.

4.6.6 Change in gene expression of the Emb-LPD and LPD

Having carried out our RNA seq in a small set of our female hippocampi we saw significant change with a padj value less than 0.07 in FXYD domain-containing ion transport regulator 7 *(FXYD7),* Tyrosyl-DNA Phosphodiesterase 2 (TPD2)*, Bace2* and *Nuf2* in only the LPD diet. The Emb-LPD showed no genes that passed the stringent false discovery reading/ padj value however we did see large fold changes and future work would be to confirm these changes by qPCR.

The FXYD protein family are homologues of the Na+,K+-ATPase, and FXYD7 is selectively expressed in brain, where its presence has been reported in both neurons and astrocytes[231], apparently with considerably more astrocytic contribution. Na+,K+-ATPase is vital in memory formation and FXYD7 decreases the apparent affinity for external K+ over a large range of membrane potentials in the absence and presence of external Na+. K+ affinity, FXYD7 does not produce an effect on the affinity for intracellular Na+[231] . Na+,K+-ATPase determines the rate of recovery of baseline external K+ during neuronal activity and is responsible for the postactivity extracellular K+ undershoot. A Na+,K+ ATPase/FXYD7 complex with a low affinity for K+ may be of physiological relevance in the brain to ensure proper excitability of neurons and/or to prevent an excessive K+ undershoot[231]. As we have a significant increase in astrocytic cells and we see this decrease in expression of FXYD7 in just the LPD female hippocampus it would suggest these mice may have reduced availability to Na K ATPase which is vital for spatial and working memory. Na K ATPase decreases with age and it would be interesting to see if this decrease in FXYD7 had an effect on LPD female behaviour later in life and if the males also had this decrease. FXYD7 decrease could be a compensatory mechanism from the LPD to maintain a normal behavioural phenotype by regulating neuronal excitability which we don't see in the Emb-LPD causing a more dramatic behavioural phenotype.

Tyrosyl-DNA Phosphodiesterase 2 (TPD2) has a key role in protecting the nervous system by preventing DNA breaks induced by aberrant topoisomerase II activity[232]. Topoisomerases are a unique group of enzymes that relieve torsional stress in DNA by transiently breaking and rejoining DNA strands. This process is important to allow access to cellular machinery necessary for DNA transactions associated with replication and transcription. Type II topoisomerases induced double strand breaks into the DNA[232]. However, severing DNA strands is risky, if topoisomerase function is perturbed during this process, the enzyme can undergo an abortive reaction whereby the topoisomerase becomes trapped on the DNA. If this occurs and the DNA break persists, something needs to resolve this to avoid accumulation of DNA damage. The solution to these breaks often comes from TDP2, an enzyme that is required to release DNA from a trapped topoisomerase. Failure

of this enzymatic activity will contribute to DNA damage that can affect cellular function[232]. TDP2 specifically cleaves the linkage between topoisomerase II (TOP2) and DNA, and failure to do this is associated with neurological disease characterized by cognitive defects, seizures and ataxia in Humans and knock out studies[233].

Recently studies have shown other roles for topoisomerases in preventing neurological disease. Topoisomerases 3β has been shown to be an RNA topoisomerase in addition to being a DNA topoisomerase and to interact with the protein affected in fragile-X syndrome (FMRP) to promote the expression of mRNAs important for neurodevelopment and synapse formation[234]. This is very interesting as we identified a decrease in the FXR2, FXR1 FMR1 from the Fragile X complex in Emb-LPD and LPD from the RNA seq data and validated it by qPCR. TDP2 is significantly decreased in the LPD RNA seq data and qPCR data confirms it is decreased in the Emb-LPD and LPD cortex. What is interesting about these genes is our behavioral and cellular phenotype in Emb-LPD and LPD. FXR2P is a selective neuronal RNA-binding protein, in the family of FMR1P, involved in fragile X syndrome. FXR2 knockout mice have memory and cognition defects, without anxiety changes[235], similar to our maternal protein restriction model. We thus speculate that the decrease in FXR2, FMR1, TDP2 RNA levels highlights a molecular mechanism that could be responsible for our behavioral and cellular phenotype. Suggesting the synaptic function of these mice maybe compromised.

Moreover, population studies in Finland have identified loss of Topoisomerases 3β in a group of individuals with a high incidence of schizophrenia similar to what is witnessed in the dutch hunger winter[233]. Additionally, mutation of Topoisomerases 1 has been implicated in autism, and direct inhibition of TOP1 was found to reduce the expression of genes associated with autism(autimsim paper). This data suggests Topoisomerases as critical but potentially dangerous enzymes. Although they are necessary for normal brain function, they must be monitored by DNA repair enzymes to prevent their detrimental activity from causing neurological disease.

BACE2 is a close homolog of BACE1, a protease known to be an important enzyme involved in the cellular pathways that some believe lead to Alzheimer's disease. The physiological function and role of BACE2 in Alzheimer's disease is unknown[236]. BACE2 is located on chromosome 21 in the Down's syndrome obligate region and may contribute to amyloid abnormalities in these individuals. In our RNA seq data we see an increase in BACE2 in just the LPD females, BACE2 is found mainly on GFAP positive astrocytes[237] and as we see an increase in GFAP positive cells in the hippocampus this rise in mRNA could be due to the increase in astrocytes. Studies however showed an increase in BACE2 expression showed no detrimental effect on memory and cognitive function and when co-overexpression of amyloid precursor protein (APP) and Bace2 is not involved in down syndrome showed that age-dependent cognitive impairment. BACE2 over expressed alone also showed no

significant cognitive decline but did lead to increased anxiety phenotype. This data suggests BACE2 over expression could occur to have some protective effects in specific behavioural and cognitive domains and a possible reason our female LPD mice show no cognitive impairments but more data on the role of BACE2 would be needed to confirm this.

Nuf2 plays an important role in kinetochore-microtubule attachment and thus is involved in regulation of the spindle assembly checkpoint in mitosis. Nuf2 is an evolutionarily conserved centromere protein, is localized to the centrosomes during G1 and S phases and then moves through the nuclear membrane to the centromere in G2 phase. We see an overexpression of Nuf2 in our LPD females but not in the Emb-LPD. Overexpression of Nuf2 has been shown to cause defective spindles, misaligned chromosomes, and activated spindle assembly checkpoint, and thus inhibited chromosome segregation and metaphase-anaphase transition in oocyte meiosis[238]. This would suggest the cell cycle of the LPD females may have been compromised and that proliferation may have been altered like we see in the foetal stages for the LPD.

There are many other potential genes to look at but as they did not pass the false discovery reading and not been validated by Qpcr yet further work would need to be done to see if there is a mechanism for the changes we see in the Emb-LPD and LPD. As we only had 24 mice in our RNA seq study and our mice are an outbred strain an increased sample size may have brought back more significant results.

4.7 Conclusion

This project has shown clear changes in both neuronal and glial populations within the adult cortex and hippocampus, evidenced by changes in cell number and proportion of different cells populations across the brain areas. As well as changes to individual populations, gross morphology of the mature brain has also been shown to change in response to maternal protein restriction. Adult brains show increases in cortical thickness in response to pre- implantation protein restriction as well as reduced cell count in all cortical layers in the primary somatosensory cortex for both pre-implantation and throughout gestation insults. The pre-implantation period has been shown to be a critical window for neurodevelopment with cortical thickness, Dapi positive cell count, neuron number and proportion, interneuron proportion, astrocyte number in the CA3 and microglia numbers throughout the hippocampus all showing significant change compared to the control group, suggesting nutrition in this period has long lasting consequences for the morphology and gene expression of the brain.

4.8 Future work

The reduction in Dapi has only been shown in the cortical layers, the hippocampus still needs to be analysed for total cell number to help clarify the changes seen in astrocytes and microglia within the different hippocampal regions, allowing analysis into whether the proportions of these cells have changed as well as the total number. Also the proteoglycans essential for Extra cellular matrix of the brain would be essential to see if they are the reason for our decrease in Dapi positive cells. Further to this the dentate gyrus is made from different layers; the sub- granular zone, granule cell layer and molecular layer, analysis in this region for glial populations did not differentiate between these layers in this project. More intricate analysis of the glial populations in each layer would provide more precise information about the distribution of these cells between diets and provide information about which layer is changing if it isn't a global change in the dentate.

Morphology and functionality of the neural populations has not been investigated in this study. Changes to dendritic morphology and spine number have been shown in previous protein restriction models, investigation into whether these changes are also present in the Emb-LPD group would contribute to the literature and provide a narrower time point to the developmental programming behind these changes. Techniques like the Golgi Method would allow detailed analysis of morphological changes and electrophysiology experiments would give some insight into the functionality of the neurons.

Carrying out RNA seq on the male Hippocampus and female and male cortex of the 3 diet groups would be essential to see if there are any sex specific differences between the diets and more interesting targets to help elucidate a potential mechanism.

Chapter 5 General Discussion

5.1 Rationale for using the periconceptional protein restriction

Mouse studies from Adam Watkins and colleagues using the model presented in this these have shown maternal isocaloric low protein diet fed exclusively during preimplantation development with control diet thereafter and postnatally (Emb-LPD) was sufficient to induce cardiometabolic and behavioral abnormalities in adult offspring[12] . The 9% casein isocaloric diet was used as it deemed a mild protein restriction sufficient for a non-pregnant mouse however, causes a mild challenge for the mouse when pregnant[13]. It also mirrors the human scenario were women from SES and developing countries receive most of their energy from grain sources and can be deficient in protein [241]. Watkins *et al* showed this animal model of protein restriction has compensatory mechanisms to maintain viable growth of the developing foetus by altering cellular characteristics of the placental lineages. For instance, NPD compared to Emb-LPD produced blastocysts with a higher number of cells in the trophectoderm (TE)[12], augmented endocytic activity in TE cell[152], and increased spreading capacity during in vitro outgrowth formation[165]. Similarly, in the primitive endoderm lineage and derivative yolk sac placenta, increased endocytic activity is stimulated by maternal protein restriction[165]. These phenotypic alterations seem to be induced at the blastocyst stage, around the time of cell lineage determination, in another study carried out in the Fleming group showed that when dams fed NPD had embryos transferred from protein-restricted embryos produced conceptuses with increased weight [109]. In the Emb-LPD murine model, they showed decreased levels of insulin in blood and branched-chain amino acids (BCAA) in uterine luminal fluid (ULF) were detected at the time of blastocyst formation and coincided with a reduced blastocyst mTORC1 signal mediated through these metabolites[19]. Further invitro experiments have revealed that exposure to insulin and amino acids (AA) during the preimplantation period can affect not only early embryo development[10] but also fetal growth[12 19]. This highlighted the first 3.5 days of pregnancy as a period were the embryo vulnerability to maternal dietary quality may represent a form of developmental plasticity to coordinate fetal growth and metabolism with prevailing maternal conditions but if conditions change, maladaptation may have consequences for disease risk in adulthood[146].

Many animal studies to date show maternal malnutrition during pregnancy and lactation may affect diverse aspects of brain development associated with impaired physical and coordinated movement, hyperactivity, altered social activity and motivation, as well as reduced mental and cognitive function, potentially in a gender-specific manner[107 129 130]. Maternal protein restriction for the duration of pregnancy has shown to have an affect the proliferation and differentiation

capacities of neural stem cells (NSCs) [127]. However, the consequences of maternal protein restriction specifically on the periconceptional period on the foetal brain development, the adult offspring brain and memory was unknown until this study.

In this chapter I am going to summarize the effects of maternal Emb-LPD and sustained LPD on mouse brain development and consequences in adult offspring.

5.2 Overview of findings

The first major novel finding of this thesis is, using *in vivo* and *in vitro* techniques, that Emb-LPD and sustained LPD reduce neural stem/progenitor cell numbers through suppressed proliferation rates in both ganglionic eminences and cortex of the fetal brain at three different time points. Moreover, we find that the diminished stem cell pool after dietary treatment exhibits distinct differentiation dynamics with Emb-LPD inducing an increase in late neuronal progenitors and young neurons while LPD induced an increase in late neuronal progenitors but not in young neurons due to an increase in apoptosis.

The second major novel finding is that Emb-LPD and LPD lead to adverse behavioural phenotypes. These results indicate a reduction in learning capacity in the male LPD offspring and both male and female Emb-LPD offspring, showing altered working memory. Moreover, Emb-LPD offspring show significant short-term memory deficits. We also saw altered brain morphology due to the diets, with different effects between genders. These data demonstrate the marked sexual dimorphism observed in the effects in offspring cognitive ability due to maternal protein restriction throughout pregnancy, and highlight the importance of assessing both sexes to obtain a complete picture.

5.3 Protein restriction during pregnancy alters the foetal brain

Our study shows that maternal LPD affects stem/progenitor cells even during a very short and early period (Emb-LPD), possibly due to a decrease in proliferation and an increase in apoptosis. Our data further indicate that compensatory processes are put in place to alleviate the effects of the stem cell deficit and the excess of neurons, possibly due to activation of apoptosis (LPD). However, this compensation, activated before implantation, is present but redundant in Emb-LPD, thus leading to an overproduction of neurons.

How might the altered patterns of brain development reported here, and with distinct outcomes

between Emb-LPD and LPD treatments, derive? Previously, Tom Fleming's group has shown the early embryo before implantation may sense maternal Emb-LPD through deficient nutrient availability within uterine luminal fluid leading to suppression of blastocyst mTORC1 signaling [14], a dietary-induced mechanism that may also function in the human [153]. Blastocyst sensing of poor maternal nutrients activates compensatory responses within extra-embryonic lineages which collectively lead to the development of a more efficient placenta and yolk sac [11 12 146 152]. However, in contrast, early undifferentiated embryonic lineages, studied using embryonic stem cell lines derived from Emb-LPD and NPD blastocysts, exhibit reduced cellular survival including reduced ERK-1/2 signaling and increased apoptosis [154], a phenotype consistent with the reduced NSC pool found in the Emb-LPD and LPD fetal brain and associated increase in detection of cellular apoptosis. A further characteristic of later embryonic lineages in fetal somatic tissues such as liver and kidney relates to differential growth rate with continued LPD challenge suppressing ribosome biogenesis whilst release from this challenge (as in Emb-LPD) stimulating ribosome biogenesis relative to NPD controls, thereby coordinating growth with nutrient availability [155].

Within the context of this study on brain development, we interpret the initial reduction in neural stem cells found in both Emb-LPD and LPD samples as a consequence of adverse dietary programming of undifferentiated cells, although the mechanism of induction is currently unknown. Subsequently, during fetal development, whilst both the Emb-LPD and LPD fetuses will be responsive to compensatory extra-embryonic systems, only the Emb-LPD fetuses will be in a 'catch-up' growth environment. Indeed, Emb-LPD offspring have increased mass during late gestation and postnatally compared with LPD and NPD offspring [242]. Given these distinct characteristics, the Emb-LPD brain phenotype of reduced neural stem cells but leading to stimulated neurogenesis across the E12.5 to E17.5 time course and increased neuron ratio in the adult, compared to NPD, may reflect these systemic changes in programming environments. In contrast, the LPD brain, following neural stem cell loss, will be within a more restrained growth environment, limiting the rate of neurogenesis.

Whilst these systemic factors may induce and contribute to the distinct embryonic programming mediated through maternal Emb-LPD and LPD, we need to further consider more specific factors that may influence fetal brain development.

One candidate linking maternal LPD with offspring brain phenotype is docosahexaenoic acid (DHA). DHA concentration has been shown to be reduced in maternal liver and plasma after maternal LPD leading to a specific impaired accumulation of DHA in the offspring fetal brain [135 162]. DHA has been shown to increase neurosphere formation [163], which is consistent with the decrease in neurosphere

formation shown here with LPD and Emb-LPD. DHA has also been shown to increase neuronal differentiation (beta-III-tubulin and MAP-2 positive cells) by decreasing Hes1 and increasing p27, thus leading to a cell cycle arrest of the NSCs [166]. Hes1 itself is important for NSC maintenance [167] [168]. This was confirmed by others showing increase in beta-III-tubulin and MAP2 via activation of the PKA and CREB pathway [169]. This might explain the effect of LPD on NSCs, but what about the Emb-LPD? In our Emb-LPD group, the LPD diet stops at E3.5, a few days before the NSC population is formed. However, the half-life of DHA in the rat brain (several weeks) [170] suggests that the effect of LPD may be long lasting and potentially retained in Emb-LPD up to the time of *in vivo* analysis.

Apart from its effect on NSCs, we show here that maternal LPD has an effect on neuronal differentiation. Indeed, LPD decreases the proportion of early progenitors while it increases the proportion of late progenitors. These results could be explained by a specific alteration of the markers Nestin and beta-III-tubulin expression. Indeed, higher expression of beta-III-tubulin in early progenitors or higher expression of Nestin in late progenitors would have led to higher proportion of late progenitors and lower proportion of neurons respectively. Such altered expression of Nestin has for example been observed in ischemic tissue damage [173], or following maternal restricted diet in the postnatal hippocampus [127]. Alternatively, maternal LPD during fetal development (as opposed to Emb-LPD) induces inhibition of differentiation of late progenitors into neurons. This disconnection between progenitor and neuron numbers has been described before in other contexts, where an increase in progenitor cells is not fully translated into the generation of mature neurons following a calorie-restricted diet [174] or in hippocampal adult neurogenesis [175]. In our model, the mechanism ensuring the elimination of excess neurons is conserved in LPD, whereas it is disturbed in Emb-LPD, suggesting that an early event in embryo development still affects neurogenesis days later (discussed above) and neuron ratio months later. E14.5 is the time in cortex development where 70% of the cells generated undergo programmed cell death [176] and our data suggest LPD and Emb-LPD could affect this process, as also identified in undifferentiated embryonic stem cell lines [154]. Figure 5.1 summarizes the effect the Emb-LPD and LPD diet on the foetal brain.

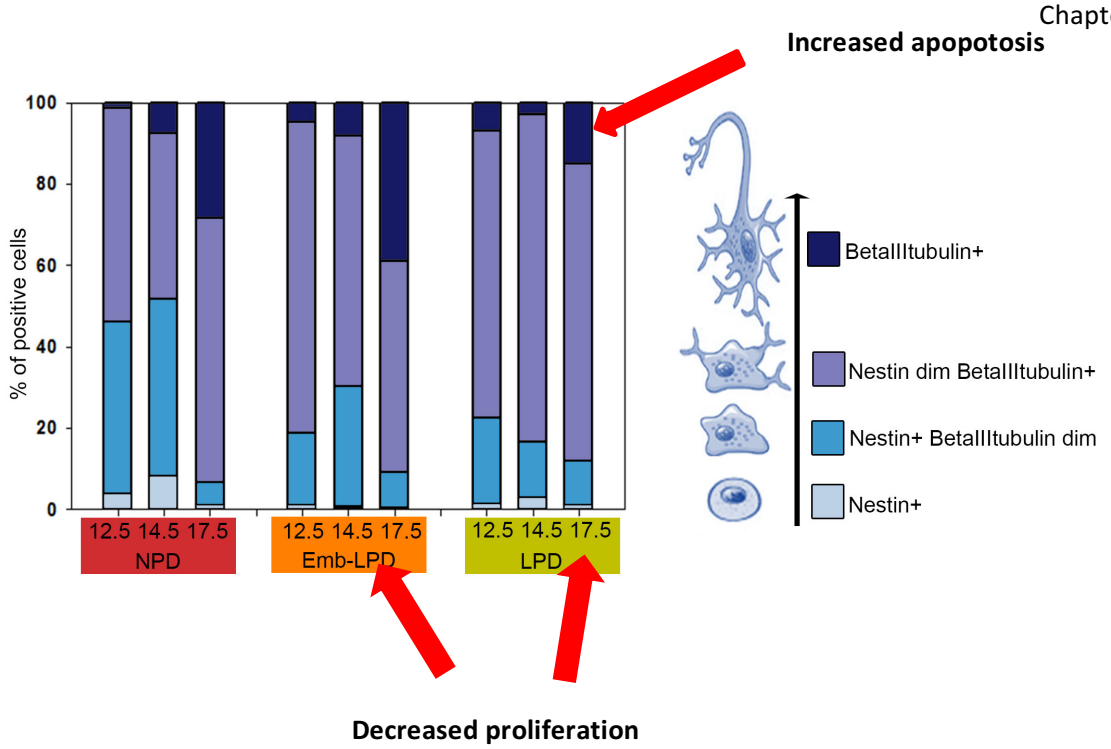


Figure 5.1. Summary of foetal brain changes by diet at E12.5, E14.5 and E17.5

5.4 Maternal protein restriction has an effect on offspring cognition and brain morphology

Using a range of behavioural techniques, that Emb-LPD and sustained LPD have differing behaviour compared to the NPD. We find that the Emb-LPD has significantly poorer short term memory and long term memory based on the NOR, T-maze tests and OFT, with the LPD males only showing poor long term memory. We see no significant change in anxiety and social interaction, unlike similar previous studies had reported. Our study therefore shows that maternal LPD affects cognitive outcomes for the offspring there are sexual dimorphic changes as summarized in the following table 1.

5.5 Maternal protein restriction potentially alters proliferation and apoptosis in the adult brain

When we consider our behavioural data with our gene expression and morphology analysis a number of conclusions can be drawn. The Emb-LPD and LPD have reduction in cell density and this could derive from our data about the fetal brains where both the Emb-LPD and LPD were seen to have reduced proliferation compared to NPD and therefore seems likely this led to the reduction in cells seen in the two protein restriction diets during adulthood levels. However, from our RNA seq data in the female hippocampus suggests a decrease in an interesting gene Acyl-CoA thioesterase

13 which has an essential role in sustained cell proliferation, our data suggests mRNA expression is decreased for this gene in both the Emb-LPD and LPD females of this model and further qPCR is needed to validate this decreased in the cortex, but if true it suggests the Emb-LPD and LPD has managed to reduce proliferation into the adult offspring.

We also see an increase in Gas2 in just LPD RNA seq data, the protein encoded by this gene is a caspase-3 substrate that plays a role in regulating microfilament and cell shape changes during apoptosis[243]. It can also modulate cell susceptibility to p53-dependent apoptosis by inhibiting calpain activity[243]. Again, if this increase in Gas2 is promoting apoptosis in the adult offspring this mirrors what we see with an increase in apoptosis at E17.5 in the LPD offspring preventing the excess neurons.

5.6 Cortical thickness and evidence of catch up growth in the adult brain

Previous work from our group using this model as well as separate studies have reported the phenomenon of catch up growth in offspring who experienced protein restriction in utero as discussed earlier [244]. In previous work the female Emb-LPD offspring have been shown to maintain an increased body weight throughout adulthood which is what we also see in our mice [12]. A relationship also exists between weight and cortical thickness so the increased size of the Emb-LPD mice may contribute to the cortical changes seen in this group. This developmental mechanism could be used to try and explain the increase in cortical thickness for the Emb-LPD offspring, in which after the initial insult is lifted just before implantation into the uterus the embryo sensing the increase in available nutrition and overcompensation in growth resulting in the changes to cortical structure, however offspring brain weight remains unaltered. Using this rationale, it could be expected the LPD group would also show an increased cortical thickness however, these offspring present with a normal cortical thickness and therefore have a mechanism in place to ensure normal cortical thickness development similar to the increased apoptosis mechanism we see in the LPD foetal stages. After birth, there is still some neurodevelopment but the major structures and circuits have been formed, it may be that a normal postnatal diet doesn't have the same effect due to the loss of plasticity in the offspring brain. To be able to determine when this change in cortical structure first becomes apparent foetal offspring as well as early postnatal mice should have their cortical structures measured to provide some more insight into when these structural changes begin in neurodevelopment.

Maternal protein restriction during gestation alters neurogenesis and gliogenesis.

Quantification of NeuN positive neurons and Parvalbumin positive interneurons in the cortex showed maternal protein restriction can affect the number of cells present as well as changing the proportion of these cells when compared to other neural populations. A reduction in the number of neurons was seen in both Emb-LPD, LPD females and Emb-LPD males. This result would imply that the protein restriction pregnancy leads to a loss of neurons in the adult offspring. However, as the Emb-LPD and LPD groups suffer from a reduction in cell number within the cortex. Female Emb-LPD mice show no detectable change in neuron number for layer 4 but this reduction of cells results in an increase in a NeuN to Dapi ratio (N : D) in layer 4 when compared to both NPD and LPD mice, as well as layer 6. This change could be brought about by a higher differentiation of progenitors into neurons or more of the neurons surviving to maturity similar to what we see in the fetal stages with an increased neuronal differentiation. This increase in the proportion of neurons isn't necessarily beneficial as the functionality of these neurons is not known. An increase in neuronal differentiation may mean there is less selectivity during development meaning neurons with weak connections or reduced ability to perform optimally are allowed to survive instead of being removed. We see a decrease in Tyrosyl-DNA Phosphodiesterase 2 (TDP2) gene expression in the female hippocampus Emb-LPD and LPD, TDP2 has a has a key role in protecting the nervous system by preventing DNA breaks induced by aberrant topoisomerase II activity. Interestingly TDP2[233] also prevents Topoisomerases 3β which has been shown to be an RNA topoisomerase in addition to being a DNA topoisomerase and to interact with the protein affected in fragile-X syndrome (FMRP)[234] to promote the expression of mRNAs important for neurodevelopment and synapse formation. This is very interesting as we identified a decrease in the FXR2 and FMR1 from the Fragile X complex in Emb-LPD and LPD with our qPCR data reduced in the females. As TDP2 is significantly decreased in the LPD RNA seq data and RNA seq data suggests it could be decreased in the Emb-LPD (but further validation would be needed to confirm this) and we also see a decrease in these 2-key fragile X genes this could be a potential mechanism suggesting synaptic function of these mice maybe compromised. What is interesting about these genes is the show a similar our behavioral and cellular phenotype to the Emb-LPD. FXR2P is a selective neuronal RNA-binding protein, in the family of FMR1P, involved in fragile X syndrome. FXR2 knockout mice have memory and cognition defects, without anxiety changes[235], similar to our maternal protein restriction model. We thus speculate that the decrease in FXR2, FMR1, TDP2 RNA levels highlights a molecular mechanism that could be responsible for our behavioral and cellular phenotype possibly by effecting the synaptic formation. FXYD7 was also decreased in the female LPD hippocampus and this could be a compensatory mechanism from the LPD to maintain a normal behavioural phenotype by regulating neuronal excitability which we don't see in the Emb-LPD causing a more

dramatic behavioural phenotype.

5.7 Maternal protein restriction alters gliogenesis.

Astrocytes and microglia have both been shown to have important roles in synaptic development through controlling excitatory transmission and synaptic pruning among other functions [97 101]. The data in this project has shown a decrease for both of these glial cells within this hippocampus, if this decrease extends to the cortex the increase in differentiating neurons in development combined with a reduction in glia may mean there aren't enough glia to optimally regulate all the neurons. This could result in non-functional synapses as well as reduction in apoptosis of defective neurons, a process which microglia are actively engaged in, enhancing neuronal survival again supported by our earlier fetal data. These extra neurons in our female Emb-LPD may just be interfering and not actively contributing to the inter-cortical and intra-cortical circuits meaning their presence is detrimental to cortical function. Staining for GFAP and SOX2 allowed quantification of type 1 NSC (radial glia) within the dentate gyrus, as well as investigation into changes in astrocytic cells by counting cells positive for GFAP throughout the hippocampus. The literature states that adult neurogenesis can be influenced by environmental conditions, particularly maternal undernutrition[79] so this area is of particular interest to this study. For both males and females there was no significant change in the number of radial glia present for both EMB-LPD and LPD offspring. This shows the original stem cell pool in the dentate gyrus in adult offspring was not impacted in the regard to the number of cells by the two maternal low protein diets unlike in the foetus.

The number of non-radial glia cells which coexpressed GFAP and SOX2 was significantly increased in the LPD females within the dentate gyrus. The expression of GFAP, a well categorised astrocytic marker strongly suggests these are cells which have committed to differentiating into mature astrocytes and therefore are part of the glial genesis rather than neurogenesis pathway. These cells are most likely immature astrocytes which are still expressing the SOX2 stem cell marker but are differentiating into mature astrocytes. The opposite however was seen in the Emb-LPD females for the CA3 region where a significant reduction in these costained cells was observed. These data suggested pre-implantation insult influences glial genesis in offspring differently to the protein restriction throughout pregnancy. When considering the cells which only express GFAP and therefore are mature astrocytes there was no differences between the diets in all three regions investigated. This pattern suggests there are a normal number of radial glia which give rise to a higher number of immature astrocytes but not all of these reach maturity however as all

diets show a normal number of mature astrocytes. However, GFAP only stains 15% of mature astrocytes so more characterisation would be needed to see if this was true.

Diet during pregnancy is crucial for the provision of methyl donors which allow epigenetic modifications to DNA and histones resulting in the correct developmental programming through the silencing of different gene targets and expression of others. Epigenetics provides a route by which the environment can alter the development of an organism and has frequently been identified as the mechanism behind developmental change seen in maternal protein restriction models[119 245]. 1.5 fold overexpression of *Gfap* and *Nestin* in offspring has been reported in a maternal 8% protein restriction model with the diet including the minimum requirement of methyl donors needed for the rats[127]. This indicates that maternal undernutrition can make changes at the genetic level as well as cellular, the latter shown in this study through the increased GFAP+ cell count in the LPD females. This study also showed treatment of neural progenitor cells in culture with 5-AZA which inhibits DNA methyltransferase, leads to the overexpression of N*estin* and *Gfap* and the down regulation of *Dcx* with hypomethylation on CpG sites of Gfap and *S100* β. This data points to the inhibition of methylation or a reduction in available methyl donors leading to increased expression of astrocyte specific genes such as *Gfap* and a reduction in neuronal specific genes skewing the differentiation of NSCs and neural progenitor cells towards the astrocyte lineage. A similar epigenetic change may be occurring in the LPD group, which would account for the increase in astrocytic cells seen in the dentate gyrus.

As the increase in immature astrocytes doesn't lead to an increase in GFAP+ mature astrocytes in the LPD group there may be a mechanism to remove excess immature astrocytes in a similar was displayed with the increased apoptosis in the E17.5 fetal brains.
Overall comparison of both sexes shows changes within the female offspring but not the male, with female offspring having a normal number of RGL cells but a higher number of immature astrocytes, this however does not lead to a higher number of mature astrocytes within the dentate gyrus but further work would need to be done to see if this is true. These populations taken as a total show LPD females have a higher number of astrocytic cells in the dentate gyrus.

Astrocytes are critical for the formation and maturation of synapses[221]. To be able to efficiently process information in the brain the correct number and types of synapses need to be formed to allow functionality in the greater neural circuits. Physical contact with astrocytes induces alterations in the location of synaptic adhesion molecule neurexin in neuronal dendrites and allows the process of receiving synaptic input to begin [221]. Direct contact between astrocytes and neurons

has been shown to increase activity of presynaptical terminals, amplitude of postsynaptic currents and number of excitatory synapses. Astrocytes are also critical for the maturation of synapses through the expression of glypicans, a family of heparin sulphate proteoglycans, anchored on the extracellular side of their membranes[222] . With this in mind changes to astrocyte number may have knock on effects to the functionality of neuronal synapses and information processing. Astrocytes have been shown in vitro studies to instruct synaptic formation and function in the rodent hippocampal neurons[222] with the hippocampus vital for memory formation. This data has shown a deficit in the number of astrocytes in EMB-LPD mice in adulthood within the CA3 region, with the same mice displaying a short term memory impairment shown by the NOR impairment to spatial working memory. There may be a link between the alteration of astrocyte number and an apparent memory deficit observed due to astrocyte involvement in formation, maturation and functionality of synapses and causing a memory defecit[246].

Alterations in glial populations have been implicated in neurological conditions such as schizophrenia. One of the proposed causes of schizophrenia is perturbed neurodevelopment, radial glia are involved in neurogenesis and migration during development and have been proposed to contribute to the condition due to cytoarchitectural abnormalities in schizophrenic patients[182]. Post mortem brains have shown an increase in the number of large neurons in deeper layers of the cortex and decreased numbers of small neurons in the superficial layers of the cortex suggests impaired migration of neurons[223]. Both increases and decreases in the numbers of astrocytes have both been reported in the literature regarding schizophrenia and it is still under debate as to whether these changes are caused by the pathology itself or the use of antipsychotic drugs[182]. With the link to schizophrenia shown in the Dutch Hunger Famine[26], and alterations to behaviour seen in famine and protein restriction models such as increased aggression[208], changes to astrocyte populations like the ones seen in this study may contribute to behavioural differences in offspring protein restriction models.

5.8 The role microglia could play in protein restriction.

IBA1 is a marker used to identify microglia within the hippocampus of adult offspring. The number of microglia was reduced in the dentate gyrus in EMB-LPD and LPD offspring for both male and female mice, with females also showing reductions in the CA1 hippocampal field and CA3 hippocampal field although only EMB-LPD offspring showed the reduction in the latter. With this point raised for the other cell populations discussed, as the Emb -LPD and LPD groups show a

reduction in cell count in the cortical layers suggesting reduced cell density throughout the. If there is changed proportion of microglia or simply a reduction proportional to the reduction in other cell populations as Emb-LPD mice show increased cortical thickness, there is still less microglia available to survey the brain parenchyma compared to the NPD group. Our decrease in microglia may mean cellular debris cannot be removed as efficiently and which is essential for normal neural function.

Microglia, have been shown to have roles in synaptic plasticity and memory through their release of cytokines which along with inflammation are important in consolidation of memory and learning processes as discussed further in chapter 3 [100], facilitate neural plasticity, neurogenesis and memory[228]. Memory formation is a highly plastic process where learning and new experiences induce experience-dependent synaptic structural plasticity. For example; motor skill learning leads to creation of new postsynaptic dendritic spines in the motor cortex. Microglial processes are in close contact to neuronal cell bodies and dendrites and have neuronal activity dependent functions as well as being regulated by sensory experiences. Microglia engage in synaptic pruning during development, removing excess synapses and synapses with weak connections, with the rate of spine elimination exceeding the rate of spine formation[100]. It has been proposed that microglia carry out this function in a controlled manner, phagocytosing specific subsets of synapses activated in the plastic process of learning and memory, facilitating the remodelling and survival of these newly formed spines and in doing so have an integral role to formation of memory. It has been shown that an absence of microglia leads to a significant reduction in the formation of these learning or experience dependent postsynaptic dendritic spines both in young and mature mice[230]. Selective removal of microglia derived BDNF, also led to a decrease in learning dependent spine formation[230], with BDNF being well known for its importance in memory consolidation and formation. A reduction in microglia seen in this project for both Emb-LPD and LPD offspring within the hippocampus may lead to a reduction in the availability of BDNF or disruption to the dendritic spine pruning impairing memory and learning. Optimal levels of BDNF are required to form stable memories within the hippocampus[247].

The formation of recognition memory leads to an increase in the amount of BDNF in the dentate gyrus, as well as a blockade of BDNF within the hippocampus impairing object recognition memory retention[247] . Deletion of the *Bdnf* gene has been shown to impair spatial learning shown by the water maze test as well as novel object recognition[247]. This is interesting as the Emb-LPD mice which showed short term memory impairment through poor performance in a novel object test earlier in this project also show this reduction in microglia within the hippocampus, particularly within the dentate gyrus. It could be that a reduction in BDNF availability due to lower numbers of microglia contributing to this apparent memory deficit.

Due to the many roles microglia performs in neurodevelopment the mechanism behind the reduction in these cells should be investigated. Blood vessels which invade the developing brain during neurogenesis also arise from progenitors in the embryonic yolk sac like microglia. A similar decrease is seen for vasculature of EMB-LPD and LPD mice shown by a reduction in collagen IV in the adult. A reduction in these two cell populations, microglia and endothelial cells could be due to the embryonic yolk sac, earlier it was described that the yolk sac in this model has increased endocytosis[152]. Elucidating this mechanism would be interesting due to the implications a reduction in microglia and blood vessels has for neural health of protein restricted individuals. It is therefore reasonable to suggest individuals who experience protein restriction *in utero* may not be able to deal with neural injury or have altered synapse morphology compared to individuals who experienced a normal maternal diet and possibly contributing to the altered behavioural phenotype.

5.9 Limitations

There are many limitations to this work, firstly a lot of data has suggested there could be a change in the synaptic morphology or function and due to the fixation of the tissue made it unable to analyse.

Our next limitation was the fact our Neun staining, results for male and female NeuN cannot be directly compared due to the female sections selected being more anterior/rostral compared to the males which were selected further along the axis towards the caudal end to include hippocampus as well. Because of this changes between the two sexes cannot be confidently attributed to the maternal diet as they may be due to differences in the neocortex along this axis. Females have not yet been analysed for Parvalbumin positive cells in the cortex. As a change in P : D ratio in males is apparent it would be interesting to see if females do the same. Collagen IV would need to be investigated in the males to see if there was decrease, other markers such as Laminin would also need to confirm the decrease we see in Collagen IV but due to technical issues we were not able to get this anti body to work within the time constraints of the PhD.

Also, as we see alterations in glia populations in the adult offspring we do not have any understanding on glia in the foetal stages and this would be interesting. However, our biggest limitation is with the RNA seq data, due to financial constraints we only sent a small sample of 8 per group of female hippocampi and therefore we do not know the gene expression changes in the male hippocampi. As only a few passed the false discovery reading in the LPD group and this is partly due to the fact we have an outbred strain of mice and the variation within this sample was too large. This could have been improved by having a larger sample size but the targets that

have come up highly on this list can still be validated by qPCR which we didn't have time within this PhD.

We also saw no difference in the social interaction test and upon further reading our test may have not been the most optimal. Many studies opt to have the 3 chambers method, were a control mouse inside of a chamber within the arena e.g. a wire mesh cylinder or box, with the subject mouse given free reign of the arena and the number of approaches to the chamber and contact with the container being recorded and regarded as social interaction or interest in the other mouse (73). This is a different technique to the one used in this study where both mice were free to move about the arena. While our test aimed to be able to distinguish specific behaviours shown by mice which may have changed, the simpler 3 chambers approach would have allowed us to just quantify approaches and contacts may have been more suitable.

5.10 Conclusion

Our study shows periconceptional maternal dietary quality to be a critical factor in brain developmental capacity with enduring consequences on brain organization, morphology and adult behavior. We show that maternal protein restricted diet, even before embryo implantation, can alter the developmental program and lead to permanent deficits in the offspring brain in a sexual dimorphic manner, reducing NSCs, altering the dynamics of neuronal differentiation, glia populations and correlates with behavioral defects as summarized in figure 5.2 . As discussed, our data strengthens the existing literature on early embryo sensing of dietary quality, with new data on the adverse consequences on fetal and adult brain and adult offspring changes in behavior and strengthens the advice that women pregnant women or women trying to conceive should be consuming optimal protein levels throughout pregnancy.

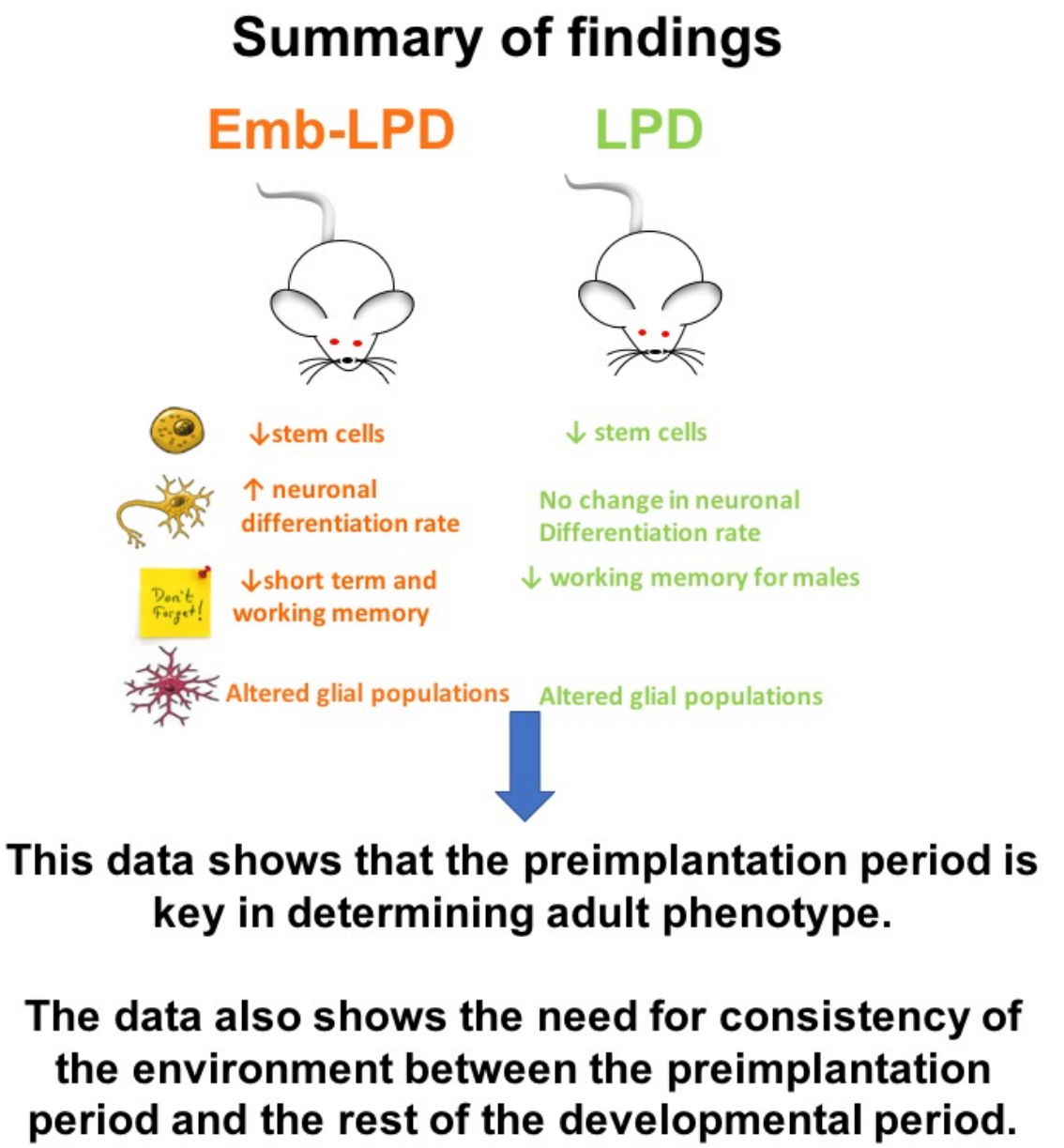


Figure 5.2 Summary of thesis findings.

This thesis in summary found that the Emb-LPD and the LPD had decreased neural stem cells in early development. The Emb-LPD shows an enhanced neuronal differentiation in the fetal stages which persists into adulthood in female Emb-LPD. Emb-LPD offspring show poor short and working memory whilst LPD males only show reduced working memory. Both Emb-LPD and LPD show altered glial populations both astrocyte and microglia. The data highlights the importance of sufficient protein consumption during the preimplantation period and that consistency of environment is critical for the embryo.

5.11 Future works and implications to offspring health

The findings of this study have implications for the health fetal neurodevelopment as well as the adult brain of offspring from mothers who receive a low protein diet during pregnancy. This work and previous work in the model highlight the importance of correct maternal nutrition during pregnancy. This project also shows that improving a diet after the mother knows she is pregnant may be too late in regards to neural development due to the many changes seen in the Emb-LPD offspring where the protein deficit is only before implantation into the uterus. This is a stage in human terms where unless the mother is actively trying to conceive, she may not know she is pregnant and therefore may not be eating adequately. While this study has given an introduction to the effect maternal protein restriction has on offspring neurodevelopment, there is much which hasn't been investigated with data from this project creating avenues for further work and if there was a potential rescue mechanism.

Although this project has shown clear changes in both neuronal and glial populations, it would be interesting to see via flow cytometry and immunohistochemistry if gliogenesis had been altered using a marker such as Glast for fetal astrocytes. GFAP is now known to only stain the primary branches of the astrocyte which only represents 15% of the glial cell volume[95] so we would need another marker to investigate if mature astrocytes had been altered in the adult brain and we would also need to investigate the cortex.

The reduction in Dapi has only been shown in the cortical layers, the hippocampus still needs to be analysed for total cell number to help clarify the changes seen in astrocytes and microglia within the different hippocampal regions, allowing analysis into whether the proportions of these cells have changed as well as the total number. Further to this the dentate gyrus is made from different layers; the sub-granular zone, granule cell layer and molecular layer, analysis in this region for glial populations did not differentiate between these layers in this project. More intricate analysis of the glial populations in each layer would provide more precise information about the distribution of these cells between diets and provide information about which layer is changing if it isn't a global change in the dentate. This study also didn't measure other neural progenitors within the hippocampus; type 2a and 2b neural progenitors still express SOX2 but do not express GFAP so were not counted in this assay. To be able to build a complete picture of neurogenesis a stain for SOX2, nestin and doublecortin (DCX) should be carried out in these offspring, type 2a neural progenitors will express nestin and SOX2 and can be differentiated from the radial glia based on their morphology as they have lost the radial projection through the granule cell layer. Type 2b cells will express SOX2, nestin and DCX and so can be distinguished from type 2a cells. A measure of

immature neurons can also be gained by counting the cells which only express DCX, this coupled with the radial glia data would give a full picture of neurogenesis and any changes seen could be attributed to specific populations.

Morphology and functionality of the neural populations has not been investigated in this study and needs to be in the future. Changes to dendritic morphology and spine number have been shown in previous protein restriction models, investigation into whether these changes are also present in the Emb-LPD group would be interesting to see if they are contributing to the adverse behaviour. Techniques like the Golgi Method would allow detailed analysis of morphological changes. Electrophysiology experiments like patch clamp would give some insight into the functionality of the neurons and micro dialysis to see if there in an alteration in neurotransmitters. Mechanisms behind the altered neurodevelopmental programming resulting from the pre-implantation insult also have not been investigated in this project and will need to be explored in the future. A potential target to look for epigenetic changes or alterations to the developmental trajectories of progenitor cells would be the previously identified yolk sac due to changes in both vasculature shown in another project and microglia shown in this one.

Another interesting area of neurodevelopment to look at is the link between vasculature and NSCs. The neural progenitors and NSCs resident in the SGZ are closely associated with the vasculature, with physical contact having been shown between the radial glia like progenitor cells and the blood vessels, these contact points were present at both the cell body and the processes of the progenitor cells[248]. In the sub ventricular zone (SVZ) of the lateral ventricle which is another neurogenic niche in the adult brain the direct contact between NSCs and endothelial cells via integins was shown to enforce NSC quiescence [249]. The vasculature in the SVZ has structures called fractones which are elongated sleeves of extracellular matrix with no cellular material which is unique to the SVZ vasculature[248] and many contact the cerebrospinal fluid filled ventricle as well as the NSCs. This has led to the suggestion that there may be direct signalling from the cerebrospinal fluid factors to the NSCs. One such factor fibroblast growth factor 2 has been shown to lead to increased SVZ cell proliferation [250] and is needed for maintenance of NSCs *in vitro*[80]. The vasculature in the hippocampus has a stereotypical pattern of vessels developing perpendicular to the granule cell layer and then turning at the granule cell layer, with some continuing to cross the granule cell layer and then travel parallel to the SGZ in a rostral to caudal fashion[248]. This patterning leads to the close proximity to neural progenitors and therefore allows establishment of physical contact between endothelial cells and NSCs. There is less evidence in the dentate gyrus that the blood vessels are directly influencing neurogenesis compared to the evidence shown for the SVZ, but it proposed that increased vasculature could provide more sites for contact

dependent signalling between the blood vessels and NSCs similar to that seen in the SVZ and that a change in vasculature in the SGZ will change the provision of systemic factors and nutrients in the blood, which may influence neurogenesis [248]. With the changes seen in vasculature in the adult brains for the protein restricted diets and supposed changes to NSCs shown in an earlier project in foetal brains investigation into the relationship between the two cell populations in the context of protein restriction would be highly interesting. Also with the increase in cortical thickness it would be interesting to see if the proteoglycan making up the ECM of the brain had been compromised.

Given data showing marked sex differences in the response to Emb-LPD and LPD challenge, investigation of placental function, which has been linked to the manifestation of sexually dimorphic maternal programming effects, would be an added benefit to asses.

Chapter 6 List of References

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