THE EFFECTS OF PRENATAL UNDERNUTRITION AND POSTNATAL ROUTINE EXERCISE ON NEUROGENESIS IN THE RAT HIPPOCAMPUS

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ABSTRACT

Early influences such as prenatal global maternal undernourishment have been well known to set up the developing fetus into what is called a predictive adaptive response *in utero* of which, manifestation of metabolic and endocrine abnormalities in these subjects have been well documented. In extension, the effects of undernutrition during early life leading to deficits and distortions of brain structure and function have also been reported in the past two decades. Following these observations, this study was set forth to investigate the effects of maternal undernourishment on prenatal neurodevelopment and postnatal neurogenesis.

By using a well established prenatal global maternal undernourishment model, market reductions in cellular densities in the CA1 and Dentate Gyrus region were observed, along with a significant reduction in cell connectivity, reflected by drastically lowered levels of spinophillin. Together, these results indicate that the adverse effects of prenatal undernourishment were exerted both prenatally during neurodevelopment and postnatally on the neurogenic process.

In order to investigate the possibility to reverse these adversities observed in the offspring of undernourished mothers, the capability of the hippocampus to regenerate neurons in the form of granule cells has been a key interest in hoping that by enhancing the process of neurogenesis, the adverse effects can be reduced or even ameliorated.

A nice contrast to the negative impacts prenatal nutritional deficits can bring about, the beneficial effects postnatal exercise has been documented to bring, has been the centre of interest. This study demonstrates that by using low-dosed, long-term routine exercise, the process of neurogenesis is enhanced in the offsprings of undernourished dams. Although a completely recovery in cellular density was not observed, the normalization of spinophillin levels indicate a possibility that beneficial affects of exercise brings about the recovery of cellular connectivity to restore normal functions of the hippocampus rather than restoring the cellular density.

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Chance favours the prepared mind.

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2.1 List of Abbreviations

AD	Offspring of Ad Libitum fed dams without postnatal exercise
ADX	Offspring of Ad Libitum fed dams with postnatal exercise
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CV	Cresyl Violet
DG	Dentate Gyrus region of the hippocampus
FGF-2	Fibroblast Growth Factor-2
IUGR	Intrauterine Growth Restriction
NGF	Neuro Growth Factor
NT-3	Neurotrophin-3

PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
RIPA	Radioimmuno-Precipitation Assay
SGZ	Sub-granular zone
SVZ	Sub-ventricular zone
TrkB	Tyrosine Receptor Kinase Beta
UN	Offspring of undernourished dams without postnatal exercise
UNX	Offspring of undernourished damns with postnatal exercise
VEGF	Vascular Endothelial Growth Factor

2.2 Neuroplasticity – Role of the Hippocampus

Situated in the medial temporal lobe of the brain, the hippocampus is perhaps one of the most important parts of the brain. Whilst it is still largely debated whether changes in hippocampal morphology or its fundamental physiological processes may directly or indirectly influence the process of learning, it is nevertheless widely accepted as the gatekeeper of memory formation. Thus the study of hippocampal neuroplasticity not only serves with great scientific importance, it may also contain significantly valuable information in terms of public health.

The structure of the hippocampus can be sub-divided into 3 major connection pathways. Cortical input into the hippocampus begins through the perforant pathway from the entorhinal cortex, to the dentate gyrus. From there, the granule cells of the dentate gyrus project to the large pyramidal cells of the CA3 region via the mossy fibre system. Finally, the CA3 pyramidal cells project to the same cell types in the CA1 subfield via the Schaffer collateral system. Figure 1-1 demonstrates a rough schematic diagram of a cross-sectional hippocampus. Pyramidal cells within the CA1 region make synaptic contacts with pyramidal cells at different locations. It has been found that damage to any one of these pathways can disrupt memory formation.

(Carter D. et al., 1999)

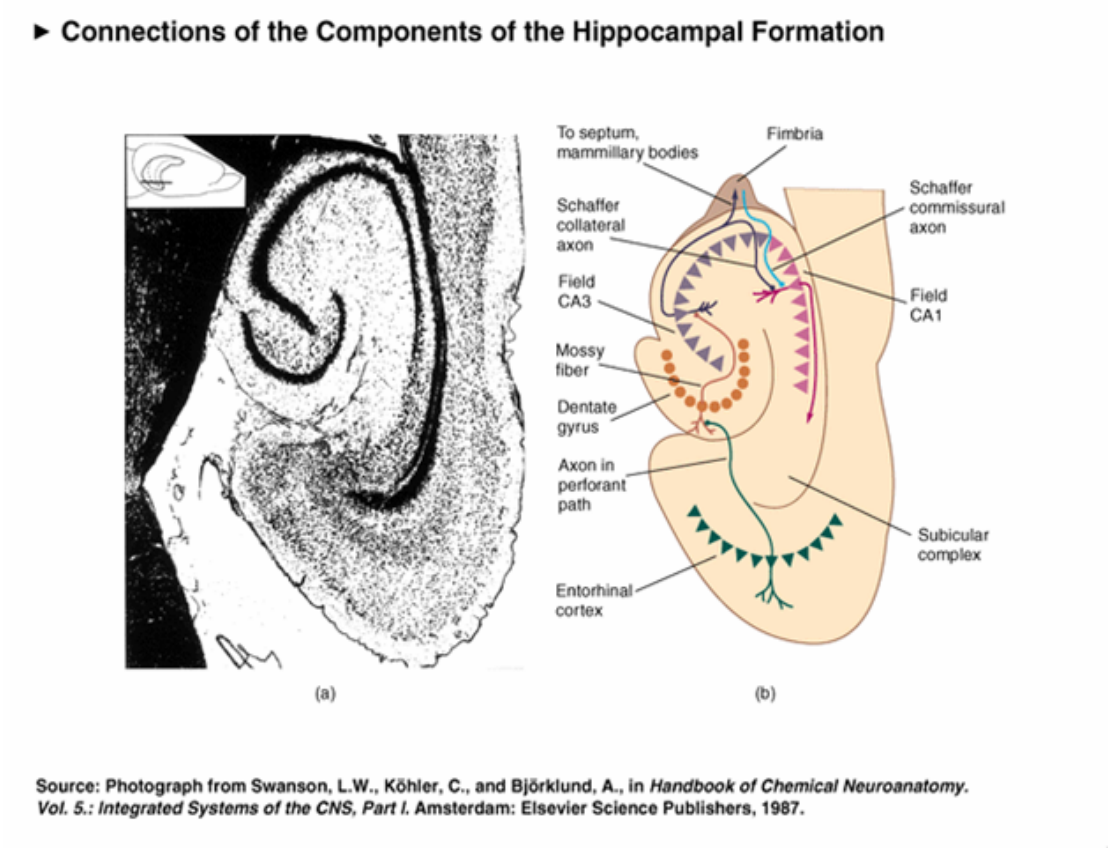

Figure 1-1 Simple diagram showing cross-section of the hippocampus

As the formation of memory has been related to the hippocampus, and the fact that LTP occurs in the hippocampus, a hypothesis was derived that adult neurogenesis participates in hippocampal functions and subsequently affects the organism's learning abilities (Leuner B. et al., 2006). This is based off the important discoveries of neurogenesis in the adult human brain (Eriksson P.S. et al., 1998) – a process which was previously only observed in animal models (Altman J et al., 1965).

Adult neurogenesis is the production of new neurons in the adult brain. As simple as it may sound, the process itself contains a vast array of events beginning with the division of a precursor cell until the existence of a new functioning neuron. The

manner of cell division (symmetric vs. asymmetric), the migratory path of the newly divided cells, and alterations in stimulating factors resulting in different type of cell development are just a few events which occur as part of the neurogenic process (Christie B.R. et al., 2006).

Although neurogenesis occurs largely during prenatal development, the adult brain does retain a small capability to produce new neurons. Most neurons are terminally differentiated and are not replaced when they die, so it is unusual that small regions of the brain retain progenitors cells that can proliferate and be integrated into the appropriate neural circuits to become fully-function neurons. The importance of neurogenesis is evident after brain injury as the only substrate for repair and recovery (Christie B.R. et al., 2006).

Adult neurogenesis is restricted to the sub-ventricular zone (SVZ) that lines the lateral ventricle, the Sub-granular zone (SGZ) of the dentate gyrus in the hippocampus and possibly the forebrain. In the SGZ of the hippocampus intermediate progenitors arise that mature locally into granule neurons of the dentate gyrus. These send axonal projections to area CA3 and dendritic arbours into the molecular layer. These adult-born hippocampal and olfactory cells are estimated to take 4 weeks to mature (Christie B.R. et al., 2006).

Because of the role the hippocampus plays in the learning process and it also being the primary site of postnatal neurogenesis, it makes sense to look into a number of

different factors which are known to alter the process of neurogenesis. Whilst it remains unethical to perform experiments on humans, vast amounts of studies have been conducted on animal models looking into the affects of antenatal maternal and early-environmental influences and also postnatal modulations on neurogenesis.

Whilst the exact molecular basis of memory formation is still unclear, Tashiro et al. (2006) described the integration of new neurons in the adult dentate gyrus through NMDA-receptor-mediated functions. As a large amount of newly born neurons die before they mature, and the survival of these new neurons are thought to be regulated in an experience-dependent manner, it has been postulated that the process of selection in neuronal survival may be the key to unlocking the mechanisms of specific memory formation.

The data obtained from their knock-out mice model indicated that the survival of new neurons are regulated by their own NMDA-type glutamate receptor during a critical phase shortly after neuron birth. These findings indicate that neuron survival may be directly regulated in an information-specific, input-dependent manner, and thus the authors postulate that circuits formed by new neurons or the selective death of others may represent information acquired within a short critical time frame after neuron birth. (Tashiro A. et al., 2006)

The trade-off between the benefits of new neurons and the problems they create for integration into the existing circuitry is evident from the fact that the degree of post-

natal neurogenesis decreases with increasing brain complexity. In lower reptilian brains, neurogenesis is capable of providing enough new neurons to completely regenerate the entire brain, whilst in higher mammalian species this process is restricted in small regions of the brain where it mainly acts as a source of cell replacement.

While the progenitor cells are proliferative neurons that retain the capacity to differentiate into a wide variety of neurons and glia, in a mouse model they have been shown not to be true stem cells. These cells are more limited in their proliferative capacity, cannot itself renew, and may or may not be multi-potent. Neurogenesis in the hippocampus is of particular interest as it receives information from each of the senses, and has been implicated to act as a gate way to learning, memory, motivation, motor behaviour and projects widely throughout the brain.

2.3 Neurotrophins and Growth Factors

In both the embryonic and the adult brain, a large scale process of cell death often occurs shortly after newly generated neurons begins to make functional connections and integrations into the existing circuitry of the brain . There appears to be a selection principle that governs the usefulness of these connections. The neuronal network is not built up neuron by neuron; instead, a vast number of neurons are generated, of which only a small functional subset survives. Currently it is estimated that for every neuron that survives and become incorporated into the circuitry, up to five times that amount is actually generated. The removal of the extra cells occurs via programmed cell death, or apoptosis, as if the brain liberates itself from excess parts in an activity dependent way (Boonman Z. et al., 1999) .

The first targets of apoptosis are often synapses and branches of neuritis, but if activity in a given cell falls below a particular threshold the cell is eliminated. Development is thus inseparably interwoven with cell death. Paradoxically, apoptosis is a principle of maturation. Activity-dependent fate is determined by secreted factors that promote either survival or cell death. To avoid being eliminated, a new born neuron must either have successfully integrated into an existing circuitry or has received sufficient survival signal to sustain itself. This "Neurotrophic Hypothesis" was developed by Rita Levi-Montalcini who demonstrated that that neuronal survival is dependent on trophic support from the target zones (Levi-Montalcini R. et al., 1942).

In a classic experiment, she showed that administration of antibodies against Neural Growth Factor (NGF) when the developing neurons innervated their targets would lead to death of the neurons, whereas exogenous NGF would rescue neurons that otherwise would have died. Conversely, when NGF or its receptor Tyrosine Kinase Alpha (TrkA) is lacking, cell survival similarly decreases.

These initial findings have been extended to the large family of neurotrophic factors, including a range of neurotrophins (NGF, BDNF, NT3, NT4), neurotrophic cytokines (CNTF, LIF-1, IL-6, etc), and the families of Glia-derived neurotrophic factor (GDNF). Individual factors act differently on different populations of neurons. Neurons read and integrate over a spectrum of neurotrophic signals. Among other

parameters, this pattern depends on the distribution of the different receptors that mediate neurotrophic factor action. Receptor expression not only differs between different cells but also over time in the same cell (Thoenen H., 1995).

Neurotrophic support on a newly integrated neuron can be provided anterogradely from the pre-synaptic neuron or retrogradely from the postsynaptic neuron in an autocrine manner from the neuron itself, or sometimes from the surrounding glia, in particular astrocytes and microglia. In the course of development, neurons become dependent on neurotrophic factors to survive. The acquired responsiveness to BDNF, for example, is mirrored in the delayed expression of the BDNF receptor Trk-B. BDNF is also transported in an anterograde fashion; it is not only involved in stimulating dendritic growth but also affects the survival of the post-mitotic neuron.

An activity-dependent autocrine signalling by neurotrophins has been shown for hippocampal pyramidal cells in vitro. In some populations of sensory neurons the preference of different growth factors can change over time, and sometimes extracellular signals are required to mediate this switch (Kaplan D.R. et al., 1997).

It is likely that similar activity-dependent switches (albeit perhaps very subtle) are more the rule than the exception and are found in other neuronal cell types as well. The means by which sequential expression of receptors is regulated is not yet known. Some brain regions are patterned independent of complete input signals, which imply that there must be an intrinsic program that makes them independent of external neurotrophic cues.

The final outcome of neurogenesis is the combination of proliferation, maturation, migration, differentiation and apoptosis. This is a complex system controlled by both intrinsic cellular and extrinsic cues. Regulation involves a wide variety of endogenous molecules including hormones, neurotransmitters, growth factors and transcription factors, which in turn are modulated by aging, nutrition, physical exercise and environmental enrichment.

2.3.1 Brain-Derived Neurotropic Factor (BDNF)

Of all the neurotrophins known to us, Brain derived neurotrophic factor (BDNF) is the most widely distributed member of the neurotrophin family which regulates the growth, differentiation, function and plasticity of nerve cells. Like other neurotrophins, BDNF is a small, soluble endogenous that becomes active when dimerised (Dieni S. et al., 2005).

They are synthesised as approximately 250 amino acid residue precursors that are secreted as non-covalently linked dimmers of two identical subunits of 120 amino acid residues. Neurotrophins share structural homology in the segments that include anti-parallel beta strands that form the hydrophobic core and are supported by 3 intramolecular cysteine disulfides. Major variation is found in three beta hairpin loops, the reverse turn, and the carboxyl and amino termini. Mature forms of neurotrophins have 90~100% homology between species (Aid T. et al., 2007).

Within the SVZ in vitro, BDNF promotes the survival and differentiation of progenitor cells and in vivo increases the number of the newly generated neurons in

the RMS and olfactory bulb through enhanced migration. It also stimulates an increase in progenitor cell number in parenchymal structures lining the third ventricle including the hypothalamus, the forebrain and cortex. It is unclear as to the mechanisms to increase in progenitor cell number, as it may be attributed to a suppression of differentiation or increased survival. However, enhanced maturation has been shown in the adult forebrain. BDNF then promotes differentiation and survival of newly generated neurons (Aid T. et al., 2007).

Growing evidence suggests that BDNF is also involved in activity-dependent synaptic plasticity. In cell cultures it promotes neural growth, synapse formation and synaptic plasticity. It has the capacity to modify hippocampal synaptic function by modulating the efficacy of neurotransmitter release. This is achieved by selectively increasing the level of synapsin 1 and synaptophysin (but has no effect on syntaxin). Synapsin 1 is involved in vesicle pool formation and neurotransmitter release, synaptophysin in biogenesis of synaptic vesicles and budding, and syntaxin in vesicle docking and fusion. Along with the regulation of synaptic efficacy, BDNF also regulates dendritic branching and remodelling, synaptogenesis in axon terminals and the functional maturation of excitatory and inhibitory synapses (Liu B., 2003).

Through its effects on synaptic efficacy and plasticity, BDNF mediates the cognitive enhancement seen with exercise while exercise in turn increases levels of hippocampal BDNF. BDNF deletion or inhibition has been shown to produce a deficit in long term potentiation, the transcription dependent electrophysiological correlate of learning and memory (Mizuno M. et al., 2003).

2.3.2 Vascular-Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor VEGF derives its name from its involvement in vasculogenesis where it enables the differentiation of angioblasts and their multiplication to form endothelial beds. It is well known that in order for new cells to form, an abundant blood supply must be present to fuel the developmental process. Thus it makes sense to look VEGF levels involved in neurogenesis. VEGF expression is known to occur in astrocytes adjacent to cerebral vessels following hypoxia and subsequently leads to angiogenesis, and also found in hippocampal neurons following ischemia (During M.J. et al., 2006). Expression of VEGF mRNA is modulated by oxygen tension and induced by hypoxia. In response to a drop in oxygen tension VEGF and VEGFR expression is rapidly but reversibly up regulated (>10 fold). VEGF expression is also regulated by a variety of cytokines such as EGF, TGF-B, keratinocyte growth factor as well as cell differentiation.

VEGF also has a variety of neurotrophic effects. It has direct effects on neurons and glial cells to stimulate growth, survival and axonal outgrowth. It also has mitogenic effects on cultured astrocytes and Schwann cells where VEGF prolongs survival and stimulates proliferation. This has been shown in various in vitro models such as in the cervical and dorsal root ganglia where it stimulates axonal outgrowth and increased the survival of both neuron and satellite cells (Matsuzaki H. et al., 2001).

VEGF has four isoforms due to the alternative axon splicing. $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. $VEGF_{165}$ is the predominant species and is a heparin-binding homo-dimeric glycoprotein of 45kDa. Neural progenitor cells express both VEGFR-1 and VEGFR-2 receptors so the neurotrophic effects may be via either one. In the

developing brain receptors are especially expressed in the ventricular zone. Dorsal root ganglia, but not cervical root ganglia, express VEGF-2 only so its effects here may be via different signaling pathways. Like BDNF and FGF-2 receptors, VEGF receptors signal via the tyrosine kinase pathway and uses the same subsequent PI3-K/Akt and MEK/ERK pathways which regulate different functions. The MEK pathway induces the differentiation of neurons, but not glia, and PI3-K pathway promotes progenitor survival. The angiogenic effects are mediated only through the VEFGR-2 and PIK-3/Akt signaling pathway (Matsuzaki H. et al., 2001).

A concentration gradient of VEGF guides the migration along the RMS of neuronal progenitors. As angiogenesis and neurogenesis occur simultaneously in the adult dentate gyrus, the recruitment of neural progenitors for neurogenesis may be through a common mechanism that is used to recruit endothelial cells for angiogenesis. In addition to neurogenic effects, neuroprotective effects have been shown in HN33 cells which are derived from somatic cell fusion of mouse hippocampal neurons and blastoma cells. VEGF reduced death induced by serum withdrawal and protected against cell death induced by oxygen and glucose deprivation, cerebral ischemia, and glutamate toxicity. These effects are also mediated via VEGFR-2 and the PI3-K pathway (Matsuzaki H. et al., 2001).

Like the possible role of BDNF in exercise-induced improvements in memory and cognitive functions, VEGF has been postulated to be the neurotrophin responsible for mediating the benefits of environmental enrichment. Cao et al. investigated the regulation of neurogenesis through VEGF levels in enriched versus control environments and spatial learning through the Morris water maze (MWM) model.

Initially, by treating rats with VEGF, these models demonstrated ~2 times more BrdU-labeled cells in the sub-granular zone of the hippocampus when compared to the control group indicating a direct increase of neurogenesis. (Cao L. et al., 2004)

This was then followed by the use of VEGF knockdown models in which lead to 70% reduction in VEGF levels, followed by the observation of the loss of increase in neurogenesis through the up-regulation of VEGF in subjects living in enriched environment. In the spatial learning analysis approach by use of MWM and also a passive avoidance task, VEGF treated mice demonstrated better performances in both exercises whilst no differences in locomotive abilities were observed in all subjects. This demonstrates the possible role VEGF plays in mediating environmental enrichment to neurogenesis, learning and memory.(Cao L. et al., 2004; During M.J. et al., 2006)

2.3.3 Fibroblast Growth Factor-2 (FGF-2)

Fibroblast growth factor-2 (FGF-2, or sometimes known as bFGF) also plays an important role as a simulator of neurogenesis. It is regarded as the most potent of all growth factors. bFGF belongs to a family of 9 different fibroblast growth factors that performs different roles in signalling. Throughout the body, bFGF plays many roles including stimulating angiogenesis, smooth muscle growth, wound healing and tissue repair. It is likely to play a role in both haematopoiesis and in the differentiation and function of the nervous system, the eye and the skeleton (Grothe C. et al., 1996).

Two isoforms of bFGF are known to exists, one with high molecular weight which is found in the nucleus and another around 18kDa is found in the cytoplasm. Because of

their spatial separation these isoforms have different biological roles – the former being involved in cell growth and migration, and the latter, in cell growth only. Because it lacks a signal sequence for active secretion, bFGF is normally found in the cytoplasm or nucleus, much less often in the matrix or on the cell surface. Therefore secretion is either from damaged cells or by alternative exocytotics mechanisms that bypass the Golgi pathway (Seddon A. et al., 1991).

bFGF and its receptors (FGFR1-4) are widely distributed throughout the CNS. Like VEGF and BDNF, they signal via the tyrosine kinase pathway. These receptors have a cytoplasmic domain that has intrinsic protein kinase activity that phosphorylates the proteins on kinase residues. bFGF null mice have reduced granule cell number in the hippocampus, which is saved by enhancing expression. This is due both to enhanced neurogenesis and reduction of neurodegeneration, suggesting the proliferative and protective roles of bFGF during development (Seddon A. et al., 1991).

The role of FGF-2 in neurogenesis in the normal adult brain has been suggested to be negligible, playing a bigger role in neuroprotection, due to its lack of secretion from healthy cells. While FGF-2 expands the progenitor population in the SVZ, the population of hippocampal progenitors is unaffected. Evidence suggests this increase in the SVZ may not be attributed to an increase in proliferation but rather to a decrease in migration as differentiation of progenitors is delayed. Contradictory evidence from others report an increase in proliferation, differentiation and migration. (Wagner J.P. et al., 1999) However, after neuronal death from traumatic brain injury, cerebral ischemia or seizure, basal levels are sufficient to facilitate proliferation of progenitor cells

2.3.4 Neurotrophin-3 (NT-3)

Neurotrophin-3 (NT-3) may serve as an excellent candidate as a diffusible factor that regulates dentate neurogenesis. The expression of NT3 gene in the adult brain is highly confined in the dentate gyrus (Maisonpierre P.C. et al., 1990). Elevated neuronal activity, either by LTP-inducing stimuli or ischemia, enhances the expression of NT-3 mRNA in the dentate.

Cell culture experiments shoed that NT-3 antagonizes the proliferative effects of basic fibroblast growth factor (bFGF) on progenitor cells, and enhances the differentiation of newborn neurons. In addition, dietary restriction enhanced the expression of NT-3 in dentate gyrus, paralleling the increase in the number of newborn neurons. However, direct evidence for the role of endogenous NT-3 in the proliferation, survival or differentiation of hippocampal progenitor cells in vivo is lacking (Shimazu K. et al., 2006).

Recently a study was published where scientists looked into the involvement of NT-3 in the proliferation or differentiation of progenitor cells in the dentate, and discovered that NT-3 specifically regulate the differentiation, but not the proliferation of neuronal precursor cells in the dentate. Their stereological analyses also showed that there were no statistical differences in the number of granule neurons per dentate gyurs between wild type and NT-3 null mutant mice (Shimazu K. et al., 2006).

2.3.5 Tyrosine Kinase Beta (TrkB)

Tyrosine Kinase Beta (TrkB) is a member of the same cell surface tyrosine receptor family as the receptors of FGF-2 and VEGF. These receptors are made of glycosylated proteins of roughly 825 amino acid residues in size and signalling action occurs

through their cytoplasmic domains by phosphorylating proteins on kinase residues of which they regulate. Binding of BDNF induces TrkB dimerization and auto phosphorylation, which subsequently attracts intracellular signalling proteins that induce either of two signalling pathways – MEK pathway which induces differentiation of neurons and the PI3-K pathway that promotes progenitor survival.

Although not a neurotrophin itself, the TrkB receptor can be activated by BDNF, NT3 and NT4 (Huang E.J. et al., 2003), whilst earlier studies focusing on actions of TrkB demonstrated the importance of BDNF-activated TrkB action for cortical development in vivo, and that alterations in TrkB signalling often lead to downstream consequences (Jones K.R. et al., 1994).

Recent studies using dominant-negative Trk receptors or genetic knockdown of TrkB receptors using shRNA caused a decrease in embryonic precursor cell proliferation both *in vivo* and *in vitro*. It was also observed that the inhibition of TrkB/C also caused a delay in the generation of neurons, but not astrocytes, and ultimately perturbed the postnatal localization of cortical neurons *in vivo*. (Bartkowska K. et al., 2007)

The importance of TrkB in relation to the process of neurogenesis has investigated in recent years. In particular, the focus have been placed in TrkB's role in the signalling pathway for BDNF, which is known to greatly influence the process of neurogenesis by promoting cellular proliferation. Several studies have also shown TrkB to be a vital element of the hippocampus in order for the formation of memories and the process of learning to take place (Mizuno M. et al., 2003).

2.4 Environmental Factors that influence neurogenesis

2.4.1 Nutrition

Over the past few decades, a vast amount of studies have shown adverse maternal influences can lead to the onset of postnatal problems (Gluckman P.D. et al., 2004). There's an old saying "we are what we eat", which has gradually ascended in the scale of factors that influences our lives thanks to the invention of fast foods and more refined products and the alarming increase of obesity rates around the world. In terms of research in neurogenesis, this has become of particular importance as people are becoming more aware of the adversities brought about by an unhealthy diet. Thus, different types of dietary fats have also been the target of many in-depth studies investigating its effects on neurogenesis.

During development, the hippocampus undergoes sequential generation of its neural subpopulations. The CA subfields CA1, CA2/3 and the subiculum are generated prenatally while the granule cells of the dentate gyrus are generated mostly after birth. Fittingly, global maternal undernourishment has been observed in previous pilot studies to significantly reduce the number of cells in the CA1 while not affecting other subfields and reduce the volume of both the CA1 and subiculum but not other subfields. Figure 1-2 shows the comparison photos taken from our pilot study, demonstrating an obvious morphological difference in the density of pyramidal cells occupying the CA1 region of the hippocampus due to maternal nutritional undernutrition.

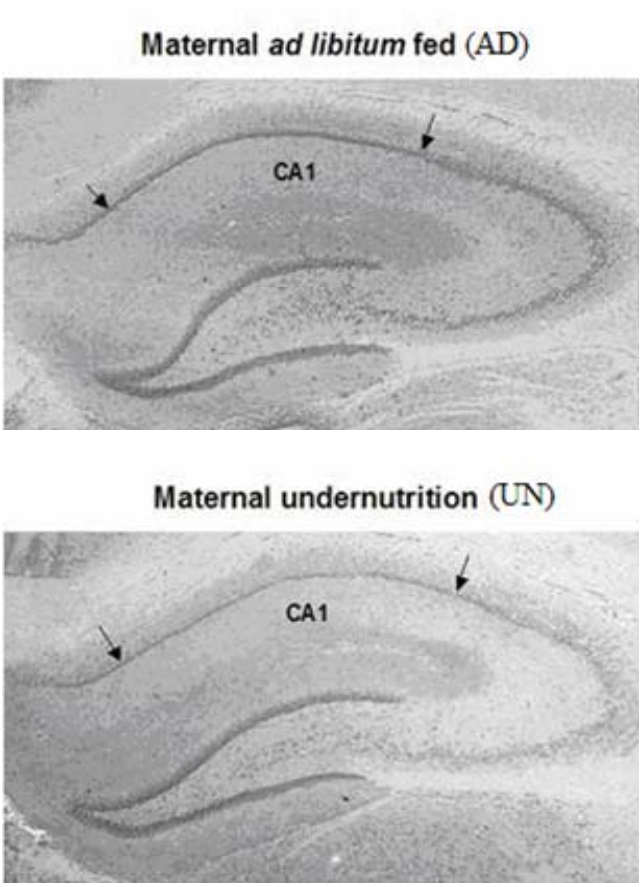

Figure 1-2 Histopathological outcome observed in the adult hippocampus
Morphological assessments indicates that the area occupied by the CA1 pyramidal layer is smaller in offspring of undernourished dams compared to offspring of *ad libitum* fed dams.

It is well known that lack of key nutrients during critical periods of development leads to alterations in neurogenesis and very often malformation, regardless of postnatal interventions. Docosahexaenoic acid (DHA) [22:6(n-3)] is essential in normal brain development and is found to be enriched in brain membrane phospholipids during neonatal development. For a fetus, the only way of obtaining DHA would be from the maternal diet, which is either transferred across the placenta directly, or synthesized from α-linolenic acid (ALA) [18:3(n-3)] (Das U.N., 2003)

A number of studies have shown insufficient DHA levels in the fetal brain leads to adversities such as decreased performance in learning tasks, membrane receptor /

protein activity abnormalities and alterations in neurotransmitter metabolisms such as dopamine (Pauline Coti Bertrand et al., 2006). This can mainly be attributed to the fact that during the S phase of the cell cycle, a doubling of membrane phospholipids is required for the process of mitosis to occur in generating daughter cells; phospholipids deficiencies would then subsequently lead to decreased proliferation rate.(Bertrand P.C. et al., 2006)

By alteration of maternal diet in rat models, Bertrand et al. demonstrated significant reductions in sizes of the cortical plate, primordial hippocampus and dentate gyrus in models of n-3 fatty acid deficiencies in the embryonic brain. In consistency with other studies of the same nature, n-3 fatty acid deficiency models (see figure 1-3) showed approximately 25% decrease in cortical plate thickness, and a 50% decrease in the dentate gyrus; whilst the thickness of the ventricular zone, the progenitor region of cerebral cortex development was significantly increased by as much as 110% (see figure 1-4) The authors postulated that the changes in morphology is likely to be due to the inhibition or delay of neurogenesis in the n-3 fatty acid deficiency models during early stages of development. (Bertrand P.C. et al., 2006)

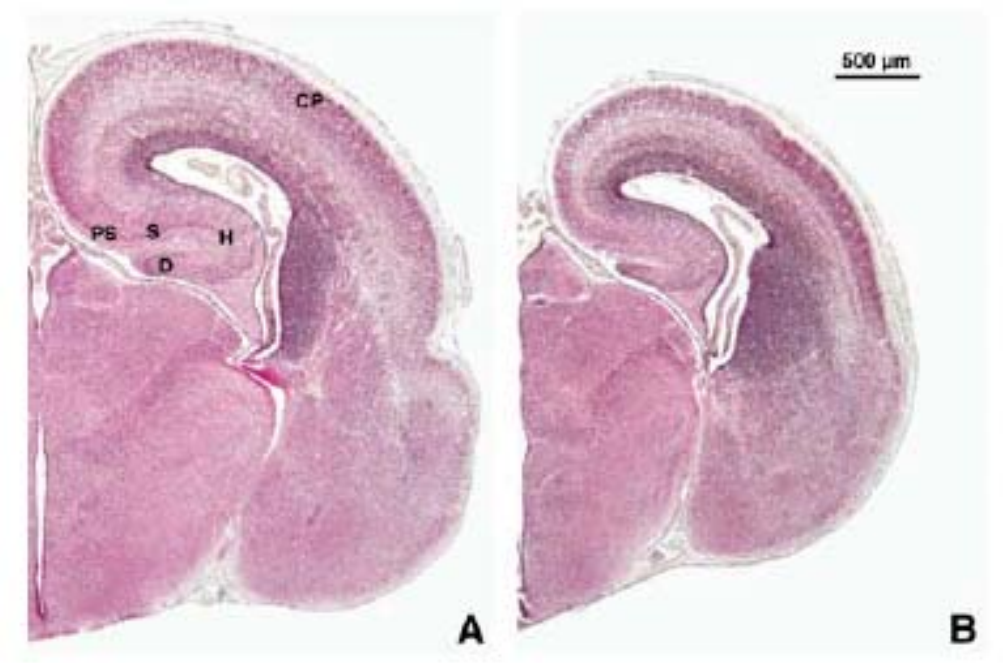

Figure 1-3 Comparison of cerebral hemisphere slides of A) normal diet and B) n-3 deficient diet in rat models. CP = cortical plate, H = hippocampus, D = Dentate Gyrus, S = subiculum and PS = pre-subiculum (Bertrand P.C. et al., 2006)

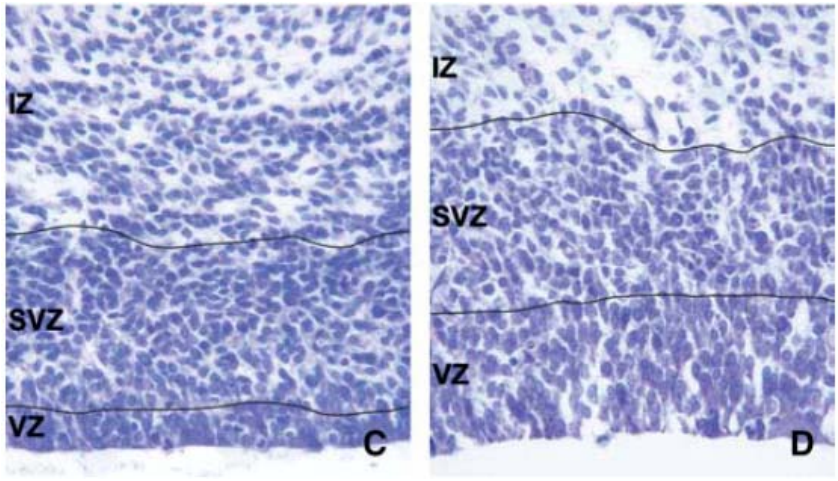

Figure 1-4 Photomicrographs comparing C) control and D) n-3 deficient diet showing Intermediate zone (IZ), Sub-ventricular zone (SVZ) and ventricular zone (VZ).(Bertrand P.C. et al., 2006)

Postnatally, it has been demonstrated that a baby needs about 10mg/day of DHA in order for normal brain development. Whilst approximately half of our daily dietary DHA intake is used up by various metabolic mechanisms, a daily intake of at least 20mg / day would be required. Though newborns have the ability to store and

synthesize DHA from breast milk where a surplus of DHA is obtained, this surplus is vital during periods when the baby is ill or cannot feed normally to ensure development is not impaired during critical periods of maturation. It has also been shown that sufficient DHA levels protects the brain from toxic actions of TNF-α and postulated to improve memory formation by up-regulating glucose uptake, and improved neurogenesis by GDNF up-regulation. (Das U.N., 2003)

Though it remains important to acquire and maintain the essential dietary needs in order for a normal developmental process to occur, it is equally as important that unhealthy diets are reduced or completely avoided. Molteni et al. described in 2002 the adversities of a high-fat and refined sugar (HFS) diet on neurogenesis. While observing higher levels of brain-derived-neurotrophic-factors (BDNF) levels exist in the hippocampal region of animals that demonstrate faster learning responses of spatial memory tasks, two months of a HFS diet was sufficient to cause the reduction of BDNF levels and reduced learning performances were observed (Molteni R. et al., 2002).

A side-effect of a HFS diet is the inevitable rise in insulin levels, thus hyperinsulinemia is also likely to have an effect in their study design. Whilst there have been suggestions that insulin may facilitate learning and memory (Wickelgren I., 1998), further studies are required to verify this issue.

Other than essential fats in diet, insufficient zinc levels during development have also been shown to impair learning and memory formation in both human infants and animal models. Though the exact mechanisms by which zinc is involved in memory formation is still unknown, it is required for neuronal differentiation, maturation and

synaptogenesis.

From data published by Chowanadisai et al., maternal zinc deficiencies have been shown to reduce NMDA receptor expression in neonatal rat brain and hippocampus, with the effects persisting into early adulthood. As NMDA receptors play a heavy role in synaptic functions, the current estimate where 82% of pregnant women world wide have zinc intake levels below the recommended amount is a rather alarming finding (Chowanadisai W. et al., 2005). Further studies in this area could bring about better public education in terms of a better recommended diet for pregnant women.

2.4.2 Exercise and Environmental Enrichment

The postnatal environments also play an important role in shaping our development and maturation of body functions. Environmental enrichments generally consists of a combination of enhanced social relations and interactions with non-social stimuli that leads to behavioral and neuronal modifications. In animal studies, exposures to enriched environments have been associated with improved spatial and non-spatial memory acquisition and retention. (Leggio M.G. et al., 2005; Lewis M.H., 2004)

Voluntary exercise is a major form of enrichment, as some studies in the past has reported running wheel exercise can lead to increase neurogenesis in the dentate gyrus of the hippocampus, and found to be correlated with the increases in spatial learning through measurements by the Morris water maze. Selective enhancements in dentate gyrus LTP was also observed (van Praag H. et al., 1999). These studies are particularly important as there has been a major interest in developing therapeutic strategies to restore impaired neurogenesis caused developmental abnormalities – a

process which was thought to be irreversible until the discovery of neuroprogenitor cells in the dentate gyrus of the human hippocampus (Eriksson P.S. et al., 1998).

Olson et. al. recently published an extensive study on the effects of environmental enrichment and voluntary exercise which demonstrated significant increases in factors that directly or indirectly affects the process of neurogenesis in mice models (figure 3A). Voluntary exercise in the form of wheel running has been found to lead to an increase in spine density, neurotrophin levels such as BDNF, GDNF, NGF and NT-3, and by either stimulating the learning and memory processes, neurogenesis is encouraged in these mice models (Olson A.K. et al., 2006).

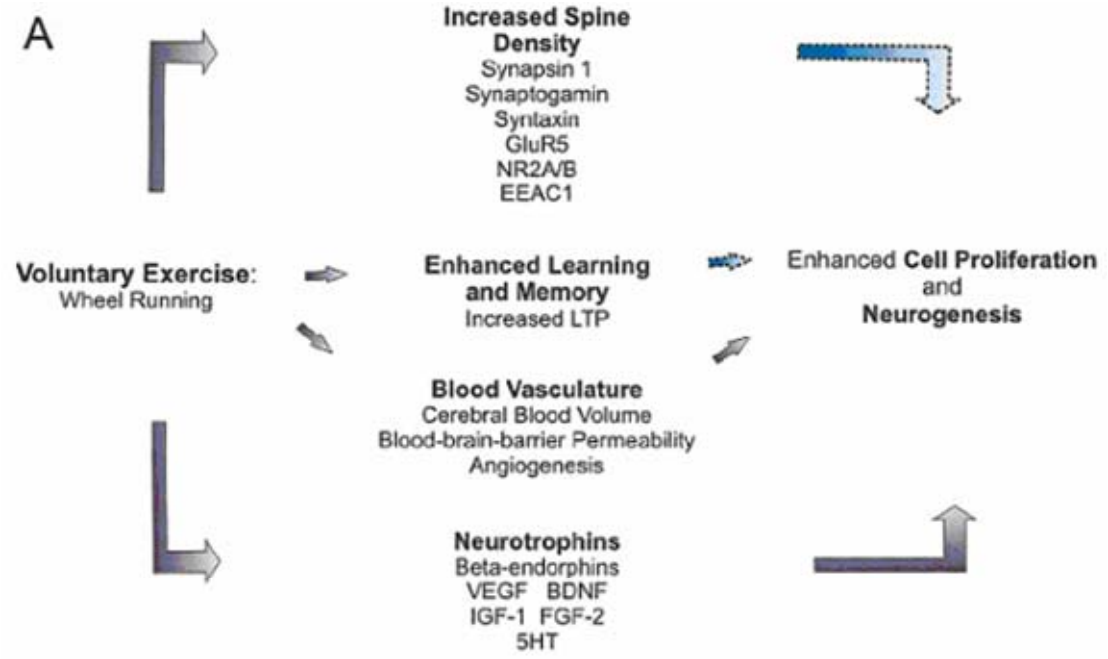

Figure 1-5 summary diagram showing effects and possible factors influencing neurogenesis from exercise (Olson A.K. et al., 2006)

Detailed researches into the molecular basis of cognitive and neuronal improvements induced by exercise has further supported this claim. Using rat models Ding et al. demonstrated the selective increase in insulin-like-growth factor I (IGF-1) expression

without alterations in the expression of IGF-2. By use of antibodies against the IGF-1 receptor, it was suggested that by blocking the receptor, while not significantly attenuate the effects of exercise on learning acquisition, exercise-induced increase in BDNF levels were reversed, and the exercise-induced augmentation in recalling abilities was abolished. This suggests a possible mechanism by where IGF-1 plays a role with BDNF in mediating exercise-induced enhancements in neuroplasticity. (Ding Q. et al., 2006)

In further investigation of how exercise influences the process of neurogenesis in the dentate gyrus, Kitamura et al. published a set of interesting results earlier this year in where they found no statistical significant increases in cell volume of the dentate gyrus in mice models exposed to running wheel exercise. The increase in total number of neurons has been thought to be the critical part of improved hippocampal functions, where this study demonstrated that neuronal cell-death of pre-existing neurons is also up-regulated as a result of exercise in the dentate gyrus. This suggests that it could be the turn-over rate of neurons, and not the total volume, to be important for brain function and memory maintenance (Kitamura T. et al., 2006a)

Conversely, there has also been some studies suggesting a deficient environment leads to an opposite effect especially via the changes of neurotrophin levels and in turn may play a part in the manifestation of developmental diseases such as Rett's syndrome, autism and other post-natal neurological disorders such as Alzheimer's and schizophrenia in some individuals (Gomez-Pinilla F. et al., 2005). Other studies also suggests early isolation rearing has diminishing effects on cell proliferation, survival and differentiation particularly in the dentate gyrus region of the hippocampus, likely

due to the reduction in BDNF expressions and the reduction of migration of new neurons to the granule cell layer by altering the morphology of radial glia cells (Rizz S. et al., 2007).

2.5 Background of Stereological Analysis

Up till recently, a large amount of histological work relied on old methodologies which either involve long grinding hours of strenuous work, or biased forms of estimation models that do not lead to convincing results. Having said that, it should also be pointed out that there is no single approach, no particular computer software capable of meeting all the demands or exploring all the possible parameters involved in stereological work.

The challenge of obtaining quantitative information about a 3D structure from 2D tissue sections has always been a major issue in the field of stereological analyses, and it is also where most of the bias is created. The "corpuscle" problem, as it is known in this field, saw many studies falling for the trap of counting the same object within a given volume more than once and/or failure to recognize the arbitrary nature of the object's shape and size.

Finally in 1925, Swedish mathematician S.D. Wicksell pointed out the core behind the corpuscle problem and explained why was not possible to obtain true unbiased estimates of object numbers in arbitrary sized and shaped objects by using "representative" slides taken from the object (Wicksell S.D., 1925). Since then, numerous attempts have been made to overcome the corpuscle problem, but none really achieved the original aim of solving the problem, if not adding more bias and

more assumptions are not suitable for biological, arbitrarily shaped objects.

In 1984, D.C. Sterio published the dissector principle (Sterio D.C., 1984), which is now widely accepted as the first unbiased method for estimating the true number of objects in a given volume of tissue. He incorporated two conventions introduced by Professor Hans Juergen Gundersen in the late 1970's– an unbiased counting frame, coupled with unbiased counting rules that ensured each and every object was counted 'for the first time, once only' (Gundersen H.J.G., 1977). This gave rise to a new stereological methodology that can now be applied onto different organs and models regardless of its size, shape and orientation in any defined region of tissue. The key to a successfully and truthful stereological investigation lies in avoiding or eliminating bias.

Since its is not feasible to count every single object down to its smallest founding particle, sampling and estimation methods are often used – unfortunately this is also where most of the bias is created. Systematic errors such as inadequate or biased selection of 'representative' tissue sections of an object, stereological biases such as counting specific regions of the tissue only, while ignoring others, may result in, and add onto the total amount of error (variation) reflected in the data.

However, aside from preventing those, there are also forms of error that cannot be avoided. These are the non-systematic errors which arise inevitably due to biological variations and errors in sampling that occurs by chance. However, as these are unbiased errors, they can be accounted for by simple calculations of Biological Variation (BV) and Coefficient of Error (CE). Combined together, the total observed

variance, given that all forms of systematic bias is removed, can be represented as:

$$\text{Total observed variance} = CV^2 = BV^2 + CE^2 \text{ (Mouton, 2002)}$$

These values are often used in describing the precision and accuracy of the data gathered, and have since been widely accepted and used by studies looking into delicate regions of the brain such as the hippocampus, sometimes with modifications to the calculation made to fit the object being studied. (Keuker J.I.H. et al., 2001).

In general, the five main steps to avoid bias are (Mouton, 2002):

1. Randomly select individuals from the population
2. Identify the border of a defined reference space
3. Perform systematic-random sampling of objects in the reference space
4. Count the total number of objects in a known volume of reference space
5. Scale the local estimate to the complete reference space.

2.5.1 The Dissector Principle

The dissector is a 3D geometric probe for counting numbers of objects (cells) with a probability that the cells being counted are unaffected by the size, shape or orientation of the object. The seminal by D.C. Sterio in 1984 described exactly how this principle can resolve the corpuscle problem – rather than counting the total number of objects directly, the dissector principle provides a theoretically unbiased estimator of the expected numerical density in a defined reference space that can be determined by investigators independently to better suit the study being conducted (Sterio D.C., 1984). Different to previous designs, Sterio used a 3D probe rather than counting

profile numbers with a 2D sampling frame. This allows for the counting of true objects within a given section rather than counting profiles which can overlap and cause the corpuscle problem.

Following this work, Gundersen and Jensen provided further detail to this approach by designing a method to calculate the number of samples and animals needed in order to reduce the amount of variation in the data gathered (Gundersen H.J.G. et al., 1987), and thy also demonstrated if sample estimates are repeated at systematic-random locations throughout a reference space, the variation around the mean estimate drastically decreases, leading to a result with higher precision.

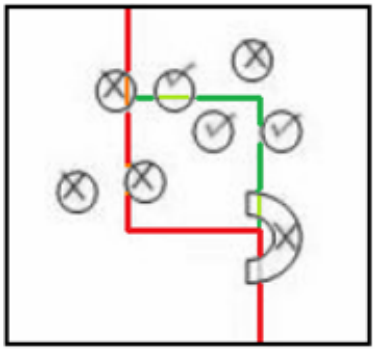

Figure 1-6 Diagram of a typical counting frame. Only cells within, or in contact with the green lines were counted, whilst all targets outside the square, or those in contact with the red lines were discarded. This is to ensure that all desired targets are counted "for the first time, once only".

Figure 1-4 shows a typical counting frame used by the dissector. Only cells within, or in contact with the designated "counting" lines (marked in green) are recorded, while all others are ignored. This ensures objects are counted only at the initial intersection between the scanning plane and the object. At subsequent intersections, the object is not recounted. This ensures that all objects have the same probability of being counted, regardless of the target volume's size, shape or orientation – thus preventing bias.

2.6 Scope of Research

2.6.1 Background

Maternal under-nutrition during pregnancy has been found to be a major factor leading to adversities in the development of the brain both functionally and morphologically. The widely used model of a general undernutrition in rat mothers during gestation exposes its offsprings to prenatal deficiencies in essential fatty acids and proteins. Recent studies have demonstrated such deficiencies during gestation can lead to significant delays in neurogenesis and malformations in particular regions of the brain thought to be responsible for the formation of new memories (Bertrand P.C. et al., 2006; Das U.N., 2003). Prenatal protein deficiencies have also been suggested to lead to a reduction in neuron number and total cell volume in specific parts of the trisynaptic pathway in the hippocampus (Lister J.P. et al., 2005).

In contrast, past studies have demonstrated that voluntary exercise can lead to increase neurogenesis in the dentate gyrus of the hippocampus. (van Praag H. et al., 1999). Extensive studies on the effects of environmental enrichment and voluntary exercise have also been conducted and showed significant increases in factors that directly or indirectly affects the process of neurogenesis in mice models (Andrea K. Olson et al., 2006).

Previous unpublished data by our group showed an increase in brain weight in offspring from undernourished mothers after they had access to voluntary free-exercise for 12 weeks – suggesting that postnatal exercise may stimulate brain growth

even after impaired intrauterine brain development has been experienced.

While it has been suggested by some that increased neurogenesis will lead to an increase in total neural cell volume, this has not always been observed in different studies. A recent study offers a possible explanation for this by demonstrating increased cell turn-over rates in animal models with exercise, therefore even thought neurogenesis was increased, the total neural cell volume did not differ significantly when compared to non-exercised animals (Kitamura T. et al., 2006a).

A number of key neurotrophins which are known to have direct or indirect influences on the memory processing of the hippocampus have also been identified to be up-regulated after exercise. BDNF, a member of the neurotrophin family known to play am important role in the growth, survival and maintenance of neurons during development was found to have an exercise-induced increase in the hippocampal region (Neeper S.A. et al., 1997; Vaynman S. et al., 2003), and the importance of its pathway involving signalling through TrkB was also found to be an indisposable part of mediating exercise-induced downstream neuroplasticity (Vaynman S. et al., 2004). Other neurotrophins, such as basic fibroblast growth factor, bFGF were also found to be increased after exercise (Gomez-Pinilla F. et al., 1997).

Other factors such as vascular endothelial growth factor, VEGF, although does not act directly in mediating the neurogenic process, is linked to indirect benefits which aids or subsequently promotes neurogenesis – in this case, by the process of angiogenesis,

which creates neurogenic hotspots (Palmer T.D. et al., 2000). Recent studies have even found VEGF a necessary factor for exercise-induced adult hippocampal neurogenesis (Fabel Kl. et al., 2003)

The combination of the adversities posed by maternal nutritional deficiencies during gestation and the postnatal treatment approach of a mild long-term routine exercise thus became an interesting topic to study.

2.6.2 Aim

The main study objectives addressed in this thesis are:

1. To investigate the effects of prenatal maternal undernourishment on hippocampal development, in the form of changes in neurotrophin expression and/or alterations in neuron number and morphological features of the hippocampus.

2. To investigate the effects of postnatal long-term exercise on the hippocampus of maternally undernourished and ad libitum fed offsprings in the forms of changes in neurotrophin expression and/or alterations in neuron numbers and morphological features of the hippocampus

2.6.3 Hypothesis

1. As prenatal undernourishment is well-documented in having adverse effects

on the developmental process, it is expected that through the reduction or inhibition of key nutrients, building blocks and neurotrophic factors, the intrauterine development of key regions in the hippocampus will be impaired, leading to a reduction in cell density or connectivity which can negatively affect the ability to learn and memorize.

2. Based on previous studies using rat models, by up-regulation of key neurotrophic factors and improved angiogenesis (Olson A.K. et al., 2006), it is likely that an increase of cellular density will be observed in the dentate gyrus - the location where neurogenesis of new granule cells occur following exposure to a mild postnatal routine exercise.

3. With the interaction of both factors, it is likely that the enhancing effects of mild postnatal routine exercise will lead to the alleviation of delayed or inhibited neurodevelopment caused by prenatal maternal undernourishment during gestation and compensate for deficiencies by enhanced synaptic connectivity, increased neurogenesis in the dentate gyrus and the normalization of key neurotrophic factors. Furthermore, it is possible that the re-activation of neurotrophic factors in prenatally undernourished subjects by postnatal long-term exercise will be in a much greater amplitude to not only normalize the adversities from the prenatal undernourishment and not only override the adverse effects of prenatal undernourishment but also raise the magnitude of neurogenesis in these subjects to that even higher than normal.

2.6.4 Proposed methodology

In order to study the objectives set forth for this study, an animal study involving the AD / UN animal model well-established in the Liggins Institute will be used to create the conditioned groups of interest (AD & UN), followed by postnatal manipulations through an exercise regime (ADX & UNX) as our treatment groups. However, as there are no current record of just how much exercise is required routinely in order to have an effect on neurogenesis, and at the same time limiting the total amount of exercise available to the animals to prevent huge variations in the amount of exercise carried out between different animal subjects in trials with no-limit exercises, this study also acted as a pilot study in this regard, as a daily cap is imposed on our animal subjects to control the amount of exercise they will carry out each day.

In conjunction with a small number of protein analyses and key neurotrophic mRNA expression analyses, this research places its main focus on the morphological changes in the neuronal composition of the hippocampal region resulting from exposure to gestational maternal undernutrition and mild post-natal routine exercise. The stereological approach used will be what is currently accepted as the most unbiased methodology in this field – the optical fractionator method (Stereo D.C., 1984).

CHAPTER 3: MATERIALS AND METHODS

3.1 Animals and study design

Virgin female Wistar rats were time-mated (age 100±5 days) using a rat oestrous cycle monitor which provided information regarding the stages of oestrous amongst the animals prior to the introduction of the male rat. After confirmation of pregnancy by the presence of spermatozoa when a vaginal plug was expelled, rats were housed individually in standard rat cages containing wood shavings as bedding. All rats had free access to water at all times throughout the experiments. Room temperature and light darkness cycles were maintained constant at 25ºC and 12 hour light - 12 hour dark cycle respectively. Pregnant rats were then assigned to one of two nutritional groups in accordance to the maternal undernutrition rodent model (Woodall S.M. et al., 1996): one which received a standard diet containing 19.9% protein, 5% fat and 3.504kcal / g digestible energy available *ad libitum* throughout. The second group of pregnant rats were given a diet which is 30% of the standard chow fed to the *ad libitum* group in order to achieve the condition of global maternal undernourishment.

Food intake and maternal weights were recorded daily until parturition. After birth, pups were weighed and litter size adjusted to 8 pups per mother with pups from UN dams cross-fostered within 48 hours onto dams that received *ad libitum* feeding sthroughout pregnancy. Likewise, offsprings of the AD dams were cross-fostered by the UN dams to balance out any effects of maternal bonding. The rationale for cross-fostering and litter size adjustment is to standardise postnatal diet until weaning. All of the ethics related to this work were approved by the Animal Ethics Committee at the University of Auckland.

3.1.1 Daily routine for animal subjects

For the purpose of this study, only male offsprings were used to avoid the effects of estrogen in female rats, a factor known to affect neurogenesis (Galea L.A.M. et al., 2006). A total of 40 pups were selected at random, 20 of which came from mothers that were undernourished during pregnancy (UN), whilst the other 20 came from mothers that were fed an *ad libitum* diet throughout gestation (AD). These two main groups AD and UN were further divided in half into exercise and non-exercise groups. Thus resulting in a total of four treatment groups for this study – AD, UN, ADX, UNX (see list of abbreviations, chap 1).

Animals in this study were also subjected to a pilot study attempting to look into the effects of maternal undernutrition and postnatal exercise on the process of learning, thus the design of the animal's daily routine also included behavioural measurements. However the results of this pilot study will not be presented nor discussed in this thesis.

The animals from the same litter were housed in pairs based on their treatment group (AD, UN, ADX and UNX) in standard cages and conditions as mentioned previously. All subjects were fed with standard chow *ad libitum* only during the hours of 2 ~ 4pm everyday, whilst clean drinking water was ensured to be available at all times. In order to minimize stress to the animals and reduce disturbance on their sleep patterns, bodyweight measurements were done between 8:30am ~ 9am every morning, once only. Food intake and total body weights of the subjects were measured once every 3 days in a systematic schedule to reduce stress.

At 35 days of age the rats were exposed to 3 days of what is termed as the "auto-shaping" process – basically exposing the rats to the exercise and behavioural experiment chambers to allow them to familiarize themselves with the environment. This was then followed by 20 days of "pre-training" aimed at conditioning the rats to the behavioural experiment apparatus.

Exercise began for the designated exercise groups (ADX, UNX) half way into the pre-training period (age 45 days) and continued throughout the experimental period. The duration and amount of exercise were monitored via computers attached to the exercise chambers. As previous data from our group demonstrated the increased preference for exercise over feeding in UN rats, and also their tendency to exercise a lot more in comparison to the AD group, an exercise cap of equivalent to 56 meters per day were set on the running wheels. When an X, or exercised, rat completes this distance, regardless of the amount of time taken to achieve this, a brake is applied. The brake forces the subject to discontinue exercise in the form of running for the remainder of the exercise period but remain in the chambers until the full 60 minute has lapsed.

Due to the number of exercise chambers available and to minimize the time lag between the first and the last animal whilst setting them up in the experimental chambers, subjects were systematically grouped by the time in which they are exposed to the behavioural testing and the exercise chambers. The rats were always placed in the behavioural study chambers first for 1 hour, followed by immediate transfer to the exercise chambers for the second hour. Behavioural measurements were taken prior to exercise to avoid short-term transient effects exercise might have.

Figure 2-1. Photograph of the exercise chamber with running wheel attachment used for the exercise study. All rats regardless of group were placed in these chambers. A computer controlled master brake allows for the application of permanent brakes for the subjects in the non-exercise groups, or in the case of the exercise groups, the brakes are only engaged once the subject has reached its daily exercise limit of 56m.

The daily routine of the rats began after weight measurements are taken,

- **Run 1** consisted of cages: AD1, AD2, UN1, ADX1, UNX1 and typically began with behavioural experiments at 9am, then transferred to the exercise chambers at 10am. Only rats of ADX1 and UNX1 had access to chambers with unlocked wheels until they have reached the daily exercise limit.
- **Run 2** consisted of cages: UN2, UN3, UNX2, ADX2, ADX3 and typically began with behavioural experiments at 10am, then transferred to the exercise chambers at 11am. Only rats of UNX2, ADX2 and ADX3 had access to chambers with unlocked wheels until they have reached the daily exercise limit.

- **Run 3** consisted of cages: UN4, AD3, UNX3, UNX4, AD4 and typically began with behavioural experiments at 11 am, then transferred to the exercise chambers at noon. Only rats of UNX3 and UNX4 had access to the chambers with unlocked wheels until they have reach the daily exercise limit.
- **Run 4** consisted of cages: AD5, UN5, ADX4, ADX5, UNX5 and typically began with behavioural experiments at noon, then transferred to the exercise chambers at 1pm. Only rats of ADX4, ADX5 and UNX5 had access to chambers with unlocked wheels until they have reached the daily exercise limit.

The guillotine doors remain firmly shut during the transfer of the rats to prevent non-recorded exercise to occur in any subjects. Only when the "begin" signals were given through our controlling computers were the guillotine doors lifted for the rats. It was ensured that all other environmental conditions were kept identical for rats of all treatment groups – i.e. Both exercise and non-exercise groups had free access to the running wheels inside completely identical exercise chambers during the study period; with the only exception being the wheels in chambers with rats from the non-exercise group were locked tight, whilst the exercise groups were allowed to run until they reach the daily limit of 56 meters.

Detachable containers with fresh drinking water were refilled between each run group and placed in the chamber only when a rat was in the chamber. **Figure 2**-1 shows the experiment chamber with a running wheel attachment used in this study. As mentioned before the behavioural pilot study will not be included in this thesis thus a picture of the device will not be shown. The running procedure continues throughout the animal study period until the subjects were processed for tissue collection.

3.1.2 Culling and hippocampal extraction

Culling of the animal groups was a particularly important process as it requires both speed and precision. This took place at age 135 ~ 139 day for the rats. All 40 of the subjects involved in this study were culled according to their daily exercise schedule at 10 animals a day - Run 1 culled on Day 1, Run 2 on Day 2 and so on. The culling procedure involves firstly anesthetization by placing the rats inside a small gas chamber filled with isoflurane.

As soon as a state of unconsciousness was confirmed, the subject was removed from the chamber, immediately measured for total body weight and nose-anus length; thereafter killed straight away by decapitation. The main body was collected and processed for metabolic and plasma measurements which were also of specific interest by other members of our group.

The whole head of the rat was then processed by Dr Thorsten Gorba in the following way: Of the ten rats in each treatment group, three were selected at random and had their hippocampus surgically dissected out, followed by quick freeze in liquid nitrogen and subsequently stored in -80ºC until required for molecular work. The other seven rats per group had their brains removed as a whole and placed immediately in a 4% PFA solution at 4ºC for 24 hours and thereafter transferred into a 10% sucrose solution (See Appendix) on the following day, subsequently stored also at 4ºC until required for cryostat and histological preparations.

3.2 Tissue Histology

In order to accurately assess the possible changes in the hippocampal morphology due to manipulations set in this study, it was essential to utilize the optimal methodology to obtain truthful representations of the changes in total hippocampal cell counts and volume. Due to the limitations of the current technologies available, such as inability to count each and every individual cell within a 3D structure, frozen sections of the hippocampal structure obtained from our animal models were used and subsequently analysed on cresyl violet and thionin stained sections. These sections were then counted and measured by using optical dissector and optical fractionator methods, applying the Cavalieri estimation principle. This method was adopted to avoid serious inaccuracies of using "representative sections" of the brain for estimates of brain volume (Glaser J.R. et al., 2000).

3.2.1 Cryostat

In preparation for frozen hippocampal slices, previously mentioned whole brain samples – 7 of 10 from each treatment groups were gathered and first separated into the left and right hemisphere. Each hemisphere were subsequently embedded using the OCT embedding medium, labelled and then stored in -80°C until required for cutting.

Cutting of frozen sections were performed on a Leica CM 3050S cryostat (Leica Microsystems Nussloch Gmbh, Heidelberger, Germany), with the thickness of sections set at 40μm; the chamber temperature was set at -20ºC whilst the object temperature which holds the OCT mounted hippocampi were set at -12ºC. The temperature settings were vital as they heavily influence the quality of the sections.

Each section cut was immediately placed in 48-well plates containing approximately 1ml of storage solution (see Appendix), collection of the sections for each hemisphere began just between appearance of the subiculum and the first appearance of the hippocampus. Plates were then labelled accordingly and stored in -20°C until required for stereological analysis. Each hemisphere netted in approximately 150 ~ 190 sections (3 ~ 4 plates) within the collection range, of which roughly 100 sections contains the hippocampus and its affiliated sub-regions – namely the CA1, CA2/3 and the Dentate Gyrus.

3.2.2 Cresyl Violet Staining

Staining was performed on 40μm rat hippocampal slices from a non-related brain to validate and test the effectiveness of the stains. Initially a staining solution containing 0.01% Cresyl Violet and 0.01% Safranin O in 1x PBS was used (Bonthius D.J. et al., 2004). However this stain was unable to produce the desired effect of marking clear neuron cellular margins and nucleus staining and was at the same time, too faint for analysis by the equipments available.

The staining solution was then changed to one that was made with 0.1% cresyl violet and 0.01% thionin mix in PBS. This stain was able to produce the desired histological effect as cresyl violet's nissil staining properties (Zhang R. et al., 2005) generated clear cellular margins whilst the thionin component marked out each cell nucleus clearly for counting purposes.

The staining procedure involves firstly a 10 min defrosting period (adjusted in accordance to ambient temperature) after the plates were removed from storage. After

defrosting, each section was transferred using a cooled glass rod from the storage well plates and washed in 1x PBS for a further 20 min for the purpose of removing residual storage solution and remnants of the OCT mounting medium. This wash step is absolutely vital and affects the quality of the stained slices as the storage solution and the remnants of the mounting medium decreases the stain quality.

Sections were then immersed in the afore-mentioned CV/Thionin staining solution for at least 20 minutes before washing off excess stain with PBS again immediately prior to mounting. The sections were then mounted in a free-floating fashion onto a non-coated slide which was then cover-slipped using apathy's mounting media (see appendix).

3.2.3 Cell Counting by Optical Dissector

Stereological analyses were performed on the slides using an optical microscope coupled with the StereoInvestigator software (MicroBrightField, Williston, VT, USA) immediately within 24 hours of staining. The hippocampal region investigated in this study was defined as its whole structure including the dentate gyrus and the CA 1 ~ 3 sub-regions. CA2 and CA3 region were grouped together as one due to the nature of CA2 region being rather small. The border of CA1 and CA2/3 region was defined where the pyramidal cells of CA2/3 gave way to the more densely packed neurons of the CA1 layer.

To further investigate any fundamental differences in morphology between the samples it was important that the exact cell counts of these selected sections can be recorded and compared. This requires the use of the Optical Dissector function in the

aforementioned StereoInvestigator program. The settings for this work were specifically adjusted in order to achieve optimal outcomes and efficiency.

The first step involves sectioning the reference space containing the objects of interest, and then a simple systematic sampling method is used to select the sections that are to be subjected to object counting. This is then followed by the generation of randomly located sampling frames that is to cover the selected area of interest in the cut-out sections that contains the objects of interest. In our case, pyramidal and granule cells in the hippocampus. Thirdly, the thickness of the sections being sampled must also be taken into account, and 'guard zones' that prevents object counting being influenced by the passing of the knives edge during sample gathering are also setup to prevent bias from occurring.

The dissector height was set at 10μm with a 10% guard zone, which buffers out the potential differences / instabilities in the section's contour created by the knife's edge passing the tissue. The sampling mesh grid set to cover a 200x200μm area. From here the counting frame size was set to be 2500μm^2, with an expected count of 10 ~ 20 cells per frame. Counting of the cells was done using the 40x objective lens. Cells were counted in a specific manner as described by the StereoInvestigator manual (see Figure 2.2).

The StereoInvestigator software also provides a range of measures for errors and variation and error within the study, which are later used to validate the results.

3.3 Molecular analysis

Quite often, changes observed in stereological studies are the result of an underlying physiological or molecular process. Thus it is equally as important to investigate the fundamental components that could have lead to these changes. For the purpose of this study, molecular experiments were conducted using two well known techniques – western blotting for semi-quantitative Spinophilin protein analyses and RT-PCR for semi-quantitative expression analyses on VEGF, FGF-2, BDNF, NT-3 and Trkβ.

3.3.1 Western Blotting for Spinophilin

Spinophilin was chosen for the protein investigation in this study due to its nature of being a marker for synaptic density (Sarrouilhe D., 2006) and also its roll in the regulation on the formation and function of dendritic spines (Feng J. et al., 2000). A higher level of Spinophilin indicates higher number of synaptic terminals present.

3.3.1.1 Protein Extraction

As described earlier in the procedures during culling, protein from the left hippocampi of 12 randomly selected rats were extracted. Immediately following the removal of these hippocampi from the -80°C they were placed on dry ice to prevent temperature related protein degradation.

The extraction requires the use of RIPA lysis buffer treated with protease inhibitor and sodium orthovanadate (see Appendix) and an extraction probe. Individual hippocampi were homogenized in the RIPA lysis buffer and between each sample the probe was rinsed in 70% ethanol and MilliQ water to prevent contamination.

The resulting supernatant was collected and transferred to eppendorf tubes then centrifuged for 15 minutes at 10,000 rpm with the temperature set at 4ºC. The clear lysates were collected and centrifuged again using the same settings. Subsequently the lysates were stored at -80ºC until required.

3.3.1.2 Protein Quantification

Protein Quantification was determined using spectrometry. The assay can be done on a single 96-well ELISA plate. By using a modification of the Bradford method, the BIO-RAD DC quantification kit (BIO-RAD Laboratories, Inc., Hercules, CA, USA), 25 µl of the working reagent was added to each of the wells required.

Protein standards were made up using BSA prepared in RIPA lysis buffer at concentrations ranging from 0.2 – 1.4mg/ml and added to the ELISA plate in triplicates at 5 µl per well. The clear lysate samples (diluted 1:10 in RIPA lysis buffer) were also pipetted in triplicates of 5 µl. Finally 200 µl of protein assay buffer was added to each well used, and the plate was left to stand in room temperature for 15 minutes.

The absorbance of the standards and samples were then read at 595nm on an ELISA plate spectrometer. By creating a standard curve using the average absorbance value read from each of the standards, the concentration of the samples can then be calculated. Standard curves were only accepted to be valid if its R value was equal or greater than 0.990.

The sample protein concentrations obtained from this procedure can then be used to calculate out the exact amount of clear lysate required for 20 µg of protein, subsequently prepared in a 15 µl mix with Laemmli's buffer (see appendix) and MilliQ. Multiple aliquots of the samples were stored in -20ºC (for immediate usage) and -80ºC (long term storage).

3.3.1.3 Antibody Validation

The concentration of anti-spinophilin (Upstate Biotechnology) used was first set at 1:250 (Marx S., 2001), this was found to produce very heavy protein bands which could not be quantified easily. Thus a lower concentration of 1:500 and 1:1000 were tested along with the secondary antibody goat anti-rabbit IgG at concentrations of 1:10000 and 1:20000.

Of these combinations it was determined that a primary concentration of 1:500 coupled with a secondary antibody concentration of 1:10000 produced the most desired and measureable protein bands on the western blot. The amount of protein loaded into each well was reduced from the normal 20µg to 10µg also to reduce the intensity of the bands formed. The resulting protein band-size of the spinophilin western blot is roughly 120kDa.

3.3.1.4 SDS Polyacrylamide Gel Electrophoresis

First, 0.75mm thick 8% Acrylamide running gels (see appendix) were cast and allowed to set for 45 minutes with a layer of water-saturated butnaol layered on the surface of the gel to exclude air bubble formations. Once the resolving gel had

polymerised, the water-saturated butnaol layer was removed and replaced by the 5% stacking gel (see appendix) was added with a 10-well well-forming comb in place. Once polymerised, the comb was removed and the gels were placed in the gel-electrophoresis apparatus with the inner chambers filled with running buffer.

Prior to loading, the samples were treated with appropriate amounts of laemmli buffer and MilliQ to ensure all samples loaded contains equal amounts of protein. 10µl aliquots which contain 10µg of protein from each sample were pre-heated to 95ºC for 5 minutes then loaded into the wells. The SeeBlue® Plus2 Pre-Stained Standard protein ladder (L) was loaded onto each gel at a volume of 7µl in order to visualise protein molecular weight.

The samples were loaded so that all the major comparisons between the treatment groups can be visualised. This setup allows direct comparisons within gel for:

AD vs. UN – the effects of prenatal undernourishment on spinophilin expression

AD vs. ADX – the effects of postnatal exercise on spinophilin expression

UN vs. UNX – the effects postnatal exercise on spinophillin expression

ADX vs. UNX – the combined effect of prenatal nutrition and postnatal exercise on spinophilin expression

Table 2-1 Table showing Western Blot loading arrangements

Lanes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Gel 1	L	AD1	AD2	AD3	C	UN1	UN2	UN3	C	-
Gel 2	L	AD1	AD2	AD3	C	ADX1	ADX2	ADX3	C	-
Gel 3	L	UN1	UN2	UN3	C	UNX1	UNX2	UNX3	C	-
Gel 4	L	ADX1	ADX2	ADX3	C	UNX1	UNX2	UNX3	C	-

Initially, only one control lane (C, lane 5) was used in each gel. This was later increased to 2 lanes (lane 5 and 9). All of the protein samples used as the control came from one single animal. This control rat belonged to a group of AD offsprings that were not exposed to any experimental procedures and were simply housed in their home cage for the entire duration of the study. This control method was used so that it can be possible to measure inter-gel variations.

Electrophoresis was then performed at a voltage of 80V for 15 minutes. Once the dye front has left the stacking gel the voltage was raised to 120V and electrophoresis was continued for another 45 minutes. The proteins separated by SDS-PAGE were then transferred from the gels onto nitrocellulose membrane (BIO-RAD) in a Criterion Transfer apparatus (BIO-RAD) for 1 hour at 100V.

Upon checking the integrity of the transfer, the membranes were then placed in 10% blocking solution for 3 hours on a rocker in a 4ºC storage room. This was followed by replacing the blocking solution with the primary antibody and incubated overnight. The primary antibodies were removed on the following morning and the membranes were then washed with PBS-Tween for 5 minutes, 5 times. After these washes the membranes were then incubated in the secondary antibody for 1 hour, immediately followed by 5 more 5 minute washes with PBS-Tween.

After the final wash the membranes were incubated again but this time with the enhanced chemiluminescence detection substrate (SuperSignal® West Dura Extended Duration Substrate Kit). Thereafter the membranes were sealed in a plastic pocket and air bubbles were avoided to optimise the effect of development of the film. The

proteins tagged with horse raddish peroxidase could then be visualised via exposure to autoradiographic film (Agfa-Gevaert, Belgium) and subsequently developed / fixed using a Kodak Xomatic X1000 processor.

After the development of the films the nitrocellulose membranes were then placed in Indian ink (or standard blue ink) for 2 hours then washed with MilliQ water to check for equal loading. Data of a gel was discarded if unequal loading was observed in its corresponding membrane.

3.3.1.5 Protein Densitometry

Densitometric analysis of the immunoblots visualized on autoradiographic film was performed on a GS800 densitometer (BIO-RAD) using Quantity One quantification software (BIO-RAD). The band analysis function was used to measure the intensity of the bands from scanned images of the films. Bandwidth detection was set at 3.619mm, there after the "Trace Quantity" function was used to obtain the data. The program expresses the intensity of each band as a relative ratio to an inbuilt reference. Statistical analyses were conducted by expressing the sample readings as a ratio of the loading control mentioned before. As the same control was applied in every single gel, these values were also corrected to one of the gels in order to take into account the inter-blot variation. The resulting mean protein content from the AD group was set to 100% and all other values were expressed relatively.

3.3.2 Neurotrophin Analysis

A number of important neurotrophins that have been well documented in the current literature are known to play a major role in the process of neurogenesis. For this study the target Neurotrophins investigated were BDNF-II, bFGF, VEGF, NT-3 and Trkβ, whilst G6PDH was used as the reference for the other Neurotrophins in order to observe any differences in expression between the groups of interest.

3.3.2.1 RNA Extraction

The extraction of RNA was carried out using the Qiagen RNA-midi kit to ensure fast extraction while ensuring the integrity of the RNA. This involves firstly grinding the pre-dissected hippocampal tissue using a small mortar and pestle with liquid nitrogen. The ground hippocampi were then suspended in 2ml of β-mercaptoethanol treated GITC-containing lysis buffer (Buffer RLT, Qiagen) as suggested by the manufacturer.

The lysates were then centrifuged for 10 minutes at 5000g, the flow through was discarded. This was followed by adding 2ml of 70% ethanol to the homogenized lysate and mixed by shaking vigorously to resuspend the precipitates. The lysate is then transferred to a 4ml RNeasy column (Qiagen) and spun again at 5000G for 10 minutes and then left to stand for 5 minutes for the remaining ethanol to evaporate.

4ml of Kit supplied RW1 Buffer (Qiagen) was then added back to the RNeasy column and centrifuged again at 5000G for 5 minutes; once again the flow through was discarded. Then 2.5ml of RPE buffer (Qiagen) was added to the RNeasy column followed by another centrifuging process of 5000G for 2 minutes. This step was repeated again with another 2.5ml of RPE buffer but this time it was centrifuged for 5

minutes to dry the RNeasy silica-gel membrane. To elute the RNA, the RNeasy column was transferred to a new collection tube and added with 150µl of RNase-free water, then centrifuged at 5000g for 3 minutes. This step was repeated to achieve a final volume of 300µl RNA extracts.

The concentration of each RNA sample was measured using a NanoDrop spectrophotometer equipped with NanoDrop v3.0.1 software (ND-1000 spectrophotometer, BioLab Ltd, NZ) by first blanking with 1µl DEPC-treated water and then 1µl of the extracted RNA from each sample. In accordance to the manufacturer, the ratio of absorbance measured at 260nm and 280nm was used to screen samples for purity and contamination. For RNA, a reading of 1.8 ~ 2.0 is optimal. A ratio lower than 1.8 often indicates protein, phenol or other contaminations that absorb strongly at 280nm. Similarly, a secondary measure of nucleic acid purity, the 260/230 ratio can also be used. The optimal reading of this ratio for RNA is around 2.0 ~ 2.2.

Due to unknown reasons the concentration yield of the extraction method was not satisfactory, either by having unacceptable values of 260/230, 260/280 readings or simply the yielding RNA concentration was too low. Thus a further step, the NaOAC-EtOH condensation step was used (Sambrook J. et al., 1989).

This method involves adding 30µl (one-tenth of sample volume) of 3M sodium acetate to the RNA extract, then 750µl (2.5x of sample volume) of ice cold pure ethanol, and stored in -20ºC for at least 12 hours. On the second day the mixture was centrifuged at 4ºC under 14000 rpm for 30 minutes; after removing the supernatant,

the residue was resuspended with 200µl of 70% ethanol. This solution is then centrifuged again at 4ºC under 5000G for 10 minutes.

The supernatants were then carefully removed without touching the RNA pellet, and allowed to air dry for up to 30 minutes in order for the remaining ethanol to evaporate. Finally the RNA pellet was resuspended in 50µl of RNase-free water. The validity and quality of the RNA yield was then re-checked using the NanoDrop machine to ensure it was viable.

3.3.2.2 First-strand cDNA synthesis

In order to produce the first-strand cDNA required for the subsequent polymerase chain reaction, the concentration readings taken from the extraction process were used to calculate the amount of lysate that would contain exactly 1.5µg of RNA. The initial step of the reaction requires a mixture with total RNA of 1.5µg, 1µl of Oligo dT (Invitrogen), 1µl of 10mM dNTP (Invitrogen) then topped up to 11µl with DEPC-treated water. The solution was then heated at 65ºC for 5 minutes, then immediately placed back on ice.

The second step of cDNA synthesis involves adding 4µl of 5x First strand buffer (Invitrogen), 1µl of 0.1M DTT (Invitrogen), 1µl of RNase Out (Invitrogen) and 1µl SuperScript III™ Reverse-Transcriptase (Invitrogen). After thorough mixing by pipetting the full solution was then incubated at 50ºC for 45 minutes, followed by 15 minutes incubation in 65ºC to inactivate the reaction. The first strand cDNA synthesis is complete after this step.

3.3.2.3 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The RT-PCR was then conducted using the first-strand cDNAs freshly created. Each PCR tube was made to contain 50µl of total products after completion of the process. The reaction requires 5µl of 10x PCR buffer (Invitrogen), 1µl of 10mM dNTP (Invitrogen), 1µl of the sense strand of the primer, 1µl of the anti-sense strand, 0.4µl of Taq DNA polymerase (Eppendorf), 2µl of the fresh first-strand cDNA and topped up to 50µl with DEPC-treated water. All primers used in this study were manufactured using Invitrogen's custom primer order system. The primer sequences and PCR settings used for this study were as follows:

BDNF-II (Maisonpierre P.C. et al., 1991) – ***239bp***

BDNF sense strand GGA CAA GGC AAC TTG GCC

BDNF anti-sense strand CAG AGG AGG CTC CAA AGG

1. *Denaturation at 95ºC for 1 minute*
2. *Annealing at 55ºC for 1 minute* **31 Cycles**
3. *Extension at 65ºC for 1 minute*

bFGF (El-Husseini A.E.D. et al., 1992) – ***722bp***

bFGF sense strand AAC GGC GGC TTC TTC CTG

bFGF anti-sense strand CTA CAA GCT CTACCA CAG GGG A

1. *Denaturation at 95ºC for 30 seconds*
2. *Annealing at 58ºC for 1 minute* **33 cycles**
3. *Extension at 65ºC for 1 minute*

NT-3 (Maisonpierre P.C. et al., 1991) – ***476bp***

NT3 sense strand AAG GAG TTT GCC AGA AGA CT

NT3 anti-sense strand TTT TGA TCT CTC CCA ACA CT

1. *Denaturation at 95ºC for 1 minute*
2. *Annealing at 55ºC for 1 minute* **33 Cycles**
3. *Extension at 65ºC for 1 minute*

TrkB(Middlemas D.S. et al., 1991) – ***509bp***

TrkBsense strand GGG AGC AAC ACT CCG TCT TCT

TrkBanti-sense strand ATG TGC AGC ATC TGC GAC TGC

1. *Denaturation at 95ºC for 1 minute*
2. *Annealing at 55ºC for 1 minute* **31 Cycles**
3. *Extension at 65ºC for 1 minute*

VEGF (Pichiule P. et al., 1999) – ***713bp & 640bp***

VEGF sense strand CCA TGA ACT TTC TGC TCT CTT G

VEGF anti-sense strand GGT GAG AGG TCT AGT TCC CGA A

1. *Denaturation at 95ºC for 30 seconds*
2. *Annealing at 60ºC for 1 minute* **32 Cycles**
3. *Extension at 65ºC for 2 minute*

Cyclophilin (Lee H.H. et al., 2006) – ***291bp***

Cyclophilin sense strand ACC CCA CCG TGT TCT TCG AC

Cyclophilin anti-sense strand CAT TTG CCA TGG ACA AGA TG

1. *Denaturation at 95ºC for 45 seconds*
2. *Annealing at 56ºC for 45 seconds* **30 Cycles**
3. *Extension at 65ºC for 45 seconds*

All PCR's began with an initial denaturation period for 2 minutes at 95ºC and ended with a final extension period for 6 minutes at 65ºC. There after, 16µl of the PCR products were removed from each PCR tube and the rest were stored in 4ºC until required again. PCR products were visualized using a 1.8% agarose gel added with 0.1 ethidium bromide (Sigma Chemical Co., St. Louis, MO, USA) and viewed under UV light using the EpiChem II Dark Room system (Ultra Violet Product, UK). Each well was loaded with a 20µl mix containing 16µl PCR product and 4µl of 6x DNA loading dye (see appendix). A 123bp ladder was loaded onto every gel in order to visualize the band size of the PCR products. The gels were run on 100V for 45 minutes.

Analysis of the band intensity was measured using area density measurements of LabWorks v4.0.0.8 (BioImaging System, UVP Inc. Upland, CA) for windows. This function produces the band intensity of the PCR gel, and the true values of each band's intensity was taken as the total intensity minus background and recorded accordingly. Resulting values were then expressed as a percentage against the average of the AD group for each individual neurotrophin tested and subsequently analyzed.

3.4 Statistical Analysis

All statistical analyses of the results were conducted using the analysis of variance (ANOVA) method with the appropriate *post-hoc* tests when required. Individual data groups were also checked for its coefficient of variance to verify validity. A special method was used to analyze the western blot data concerning the effects of exercise and undernutrition on the expression of Spinophilin in the hippocampus as it cannot be justified by using normal ANOVA. All statistical analyses were carried out using StatView® for Windows, Ver 6.0 (SAS Institute Inc. Cary, NC, USA) statistical software. Statistical significance was assumed at $p < 0.05$ level and data is presented as mean ± SEM unless otherwise stated.

A special analysis was used for the Spinophilin data under the recommendation of Jennifer Miles who obtained the knowledge from Professor Chris Trigs of the Statistics Department in the University of Auckland. The method is called synergistic contrast and is used specifically when the interaction between the two main conditions accounts for more than 1/3 of the variation in the data (Daniel C., 1976).

CHAPTER 4: RESULTS

4.1 Animal Studies

From the beginning of the animal study, birth-weights, body weights at regular intervals, food intake and exercise records were noted for monitoring purposes. The datasets were collected by a number of staff involved in the animal studies, including myself, whilst the final collating of the data was done by Amy Norman who oversaw the animal studies.

All statistical analyses were conducted made using appropriate test measures that compares mean ± SEM between groups. As explained in the methodology section, only male offsprings were used in this study, and for the initial animal preparation work prior to weaning, N = 20 in both offsprings of pregnant female rats that were fed either a Ad libitum (AD) or a Under Nourished (UN) diet during pregnancy. This was then subdivided to four different groups, AD, UN, ADX and UNX after weaning and the beginning of the exercise study.

At birth, the UN pups (4.48g ± 0.25) were observed to have significantly lower birth-weights compared to the AD (6.21g ± 0.14) controls (Figure 3-1, $p<0.001$), this AD (4.98cm ± 0.07) / UN (4.45cm ± 0.09) difference was also observed in the totally body length comparisons measuring from nose to anus (Figure 3-2, $p<0.001$). However the body length measurements for nose-to-tail was not found to be different between the two groups No significant differences were observed however in the nose-tail lengths (Figure 3-3) nor in the litter did size produce by the mothers of the AD and UN pups (Figure 3-4).

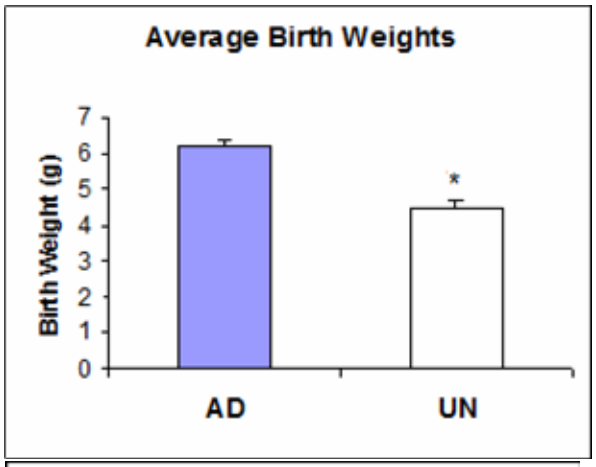

Figure 3-1. Graph showing average weight of AD and UN pups at birth, age = day 0, n = 20. Graph indicates mean weight in grams ± SEM as error value. * indicates p<0.001 calculated by One-Way ANOVA.

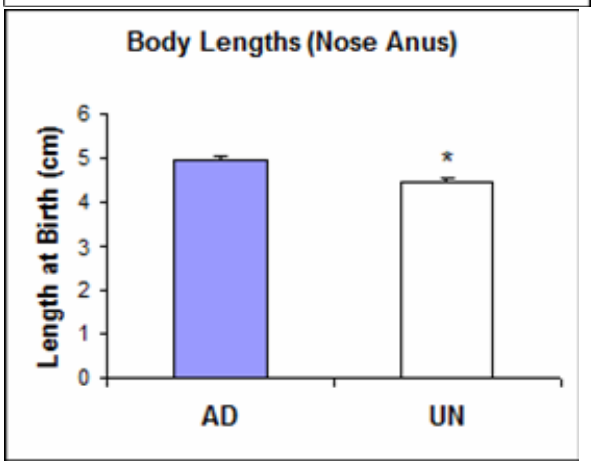

Figure 3-2. Graph showing average nose-anus length comparison of AD and UN pups. Age = Day 0, n = 20. Graph indicates mean length from nose to anus in centimetres ± SEM as error value. * indicates p<0.001 calculated by One-Way ANOVA.

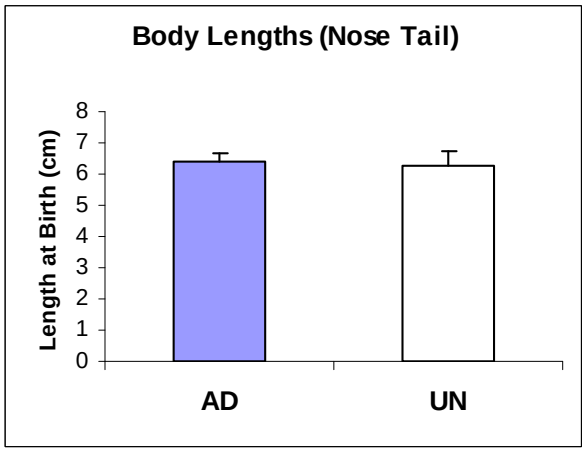

Figure 3-3. Graph showing average nose-tail length comparison of AD and UN pups. Age = Day 0, n = 20. Graph indicates mean length from nose to tail in centimetres ± SEM as error value.

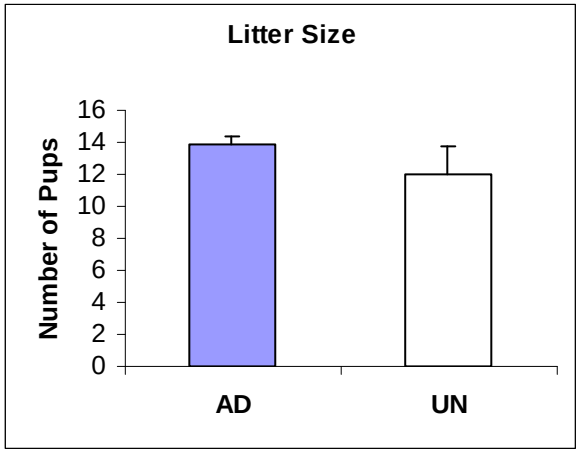

Figure 3-4. Graph showing average litter size comparison between 8 successfully pregnant AD and 9 successfully pregnant UN mothers. Graph denotes mean litter size ± SEM**.**

Table 3-1 : Physiological Parameters at Birth				
	Birth Weight	**Length (Nose-Anus)**	**Length (Nose-Tail)**	**Litter Size**
Mean AD	6.21g ± 0.14	4.98cm ± 0.07	6.43cm ± 0.24	14 ± 0.48
Mean UN	4.48g ± 0.25	4.45cm ± 0.09	6.28cm ± 0.43	12 ± 1.8
One-way ANOVA	**<0.0001**	**<0.0001**	n.s	n.s

means±SEM, n=20 / group

* p values are calculated using One-Way ANOVA. Significance denoted with bold lettering.

Table 3-1 is a summary comparison table for the physiological parameters measured at the time of birth.

Starting from the day of weaning (day 22), the bodyweights of all 40 subjects were recorded once every three days to generate the growth curve below. For a detailed breakdown of the weight records please refer to the Appendix. With exception to day 97, the body weights of the UN and UNX subjects on all other days measured were observed to be significantly lower than the AD and ADX groups ($p<0.05$, calculated with Fisher's PLSD test) due to the programming effect of prenatal maternal nutrition

The effects of exercise did not affect the total body weights of the subjects, albeit differences due to exercise were observed to be significant on days 34 and 85 ($p<0.05$, calculated with Fisher's PLSD test), they were most likely chance events over the 139 day period. The interaction effects of prenatal nutrition and postnatal exercise was not observed to be significantly influential on the body weight of the subjects on any given measured time-point. Figure 3.5 demonstrates the overall growth curve of the subjects over the entire experimental period from weaning till culling.

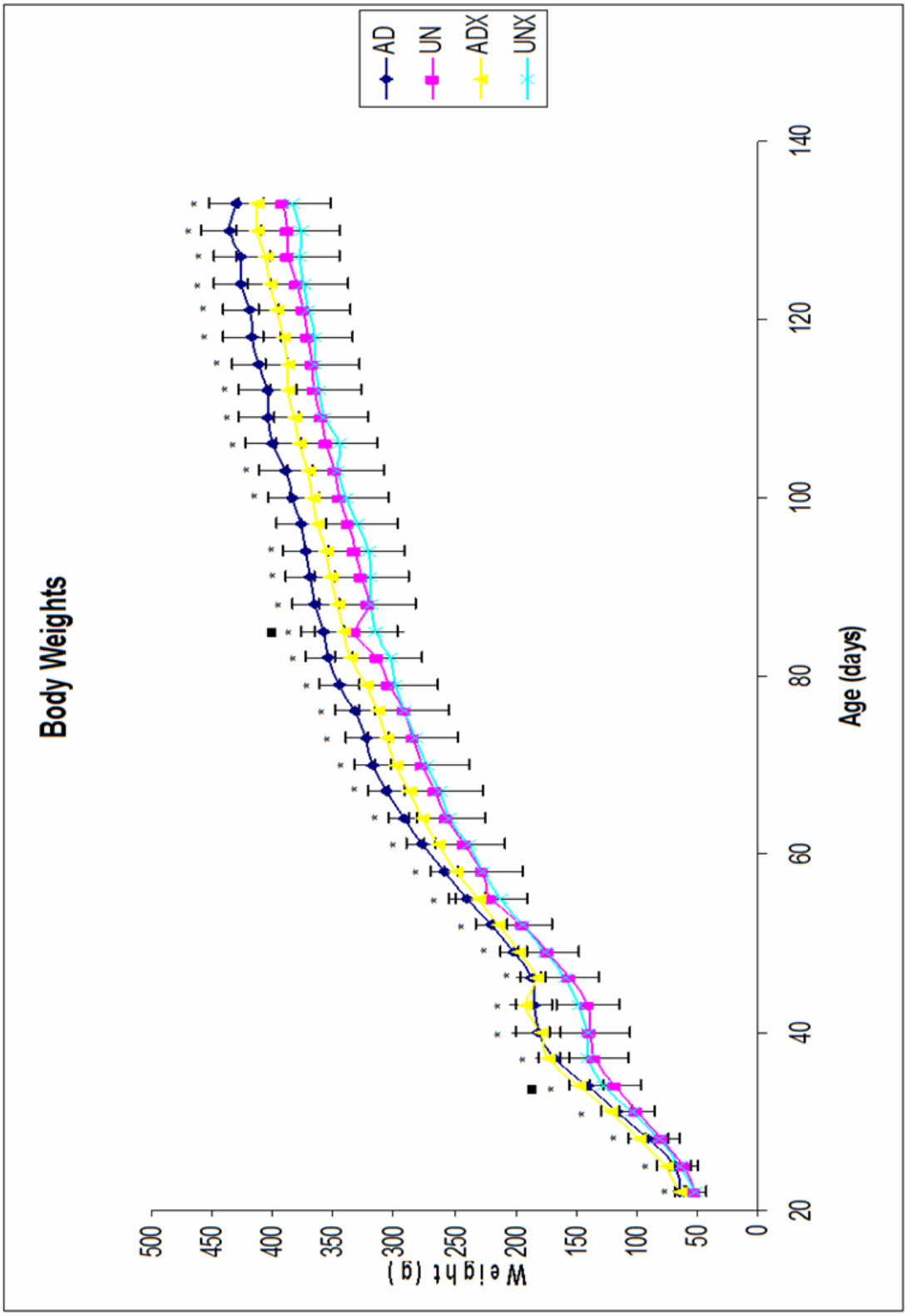

Figure 3-5 Graph showing the mean growth curves by body weight for all four groups monitored in this study (AD, UN, ADX and UNX). * indicates significant differences by prenatal nutrition ($p<0.05$, calculated by Fishers PLSD test). ■ indicates significant differences by exercise treatment ($p<0.05$, calculated by Fishers PLSD test). No interaction effects were observed on any given timepoint.

Total food consumption was also measured at regular intervals for all subjects and calculated as mean kCal ± SEM. This value included both the standard chow mix which is fed during the feeding sessions and the noyes pellets from the chambers of the pilot learning study which our subjects also took part in. Initially the UN and UNX category demonstrated lesser food intake between the day 37 ~ 40 post partum ($p<0.05$) and days 58 ~ 61 post partum ($p<0.05$), and then at the days 100 ~ 102 post partum period both exercise groups had lesser food intake compared to their non-exercised counterparts ($p<0.05$). Whilst the trend of the exercised animals eating less continued onto a later time-point, this difference was not found to be significant.

The major developmental time-points were established to be roughly 20 days apart; these time-points are good indicators of the growth stages of a rat. First time-point of days 37 ~ 40 post partum is the first major time-point after weaning, and also corresponds to the first major growth period of these rats. From figure 3-6 it is clearly evident the total amount of food consumption is significantly different between the offsprings of AD and UN mothers ($p<0.05$), regardless of the routine exercise or non-exercise.

Table 3-2: Food Intake (kCal) Averaged over 3 days at 5 major developmental time points.

Condition	Treatment	kCal (d37-d40)	kCal (d58-d61)	kCal (d79-d82)	kCal (d100-d102)	kCal (d121-d123)
AD	**Non-Exercised**	126.46± 10.03	144.36 ± 2.57	147.27 ± 4.75	144.75 ± 5.16	144.22 ± 4.92
	Exercised	124.92 ± 12.18	132.66 ± 3.41	139.41 ± 7.39	128.41 ± 3.10	130.91 ± 2.10
UN	**Non-Exercised**	87.45 ± 25.01	128.92 ± 7.40	139.09 ± 6.19	139.75 ± 6.07	143.05 ± 8.88
	Exercised	87.02 ± 19.14	124.24 ± 6.08	129.29 ± 4.52	132.92 ± 5.08	137.68 ± 3.97
Two-Way ANOVA		**P-values**				
Prenatal Nutrition		**0.044**	**0.037**	0.136	0.961	0.620
Postnatal Exercise		0.956	0.138	0.149	**0.033**	0.112
Interaction		0.975	0.513	0.870	0.354	0.485

***mean food consumption (kCal) ± SEM**, n = 10 per group. $p<0.05$ calculated with standard Two-Way ANOVA.

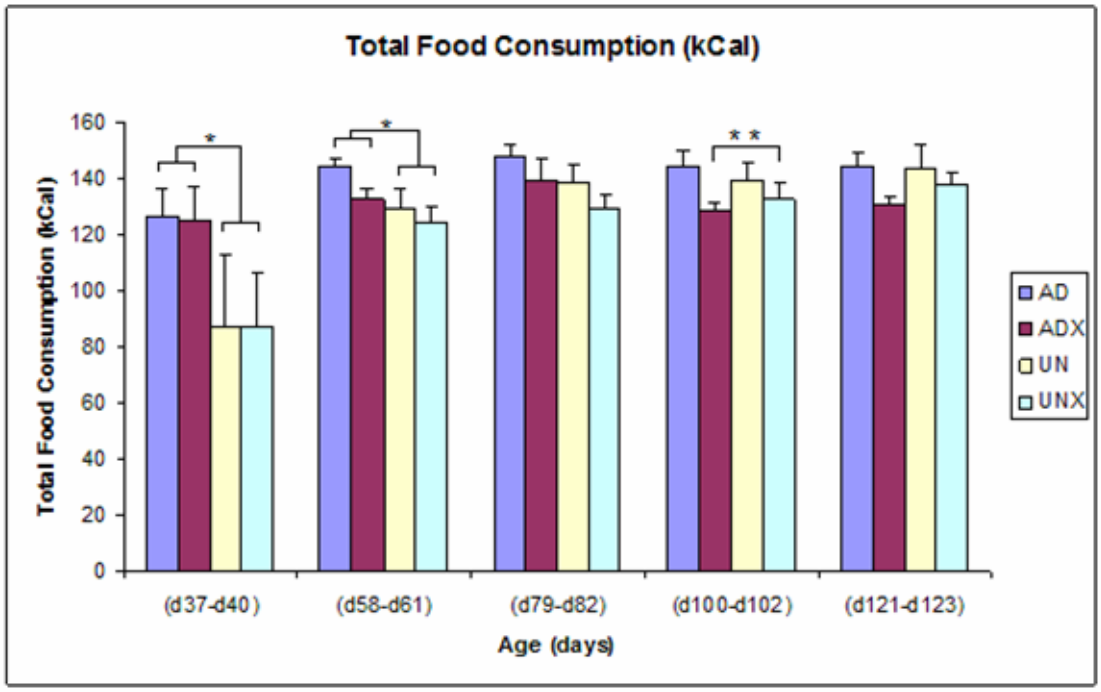

Figure 3-6 Graph showing mean food intake in kCal for 5 major time points of the study. Error bars denotes SEM. N = 10, major time points are established using the age of the rats. * indicates statistical significance of AD/ADX > UN/UNX, $p<0.05$. ** indicates AD/UN > ADX/UNX, $p<0.05$. both calculated using Two-Way ANOVA.

However as time progresses towards the 5th time point, where the growths of these rats have plateaued, the differences are no longer visible from total food consumptions. However, the analysis cannot be complete without also taking into the account the differences in sizes and weight between these subjects, as a larger critter naturally requires a higher amount of energy to maintain its normal metabolism.

Further looking into the food intake data by calculating the kCal intake per gram of body weight, the UN and UNX groups demonstrated significantly higher figures than the AD and ADX groups on the final measuring time point of days 121 ~ 123 post partum ($p<0.05$). There is also a general decreasing trend of kCal per gram body weight as the animal studies went on.

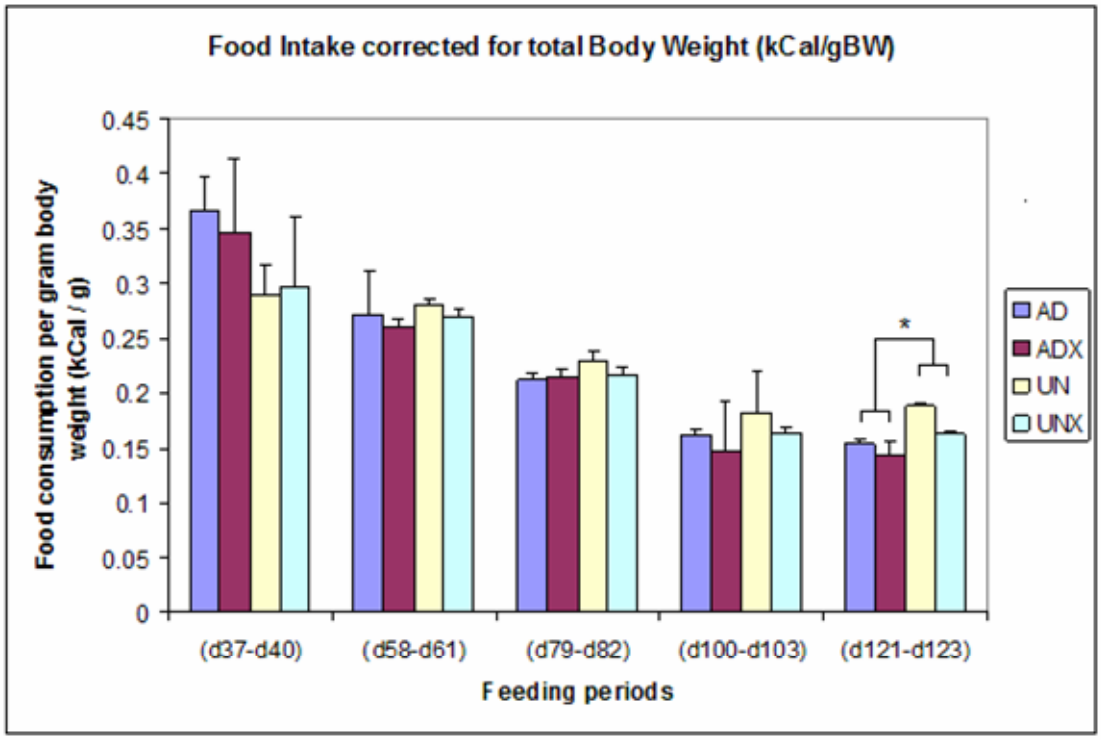

Figure 3-7 Graph showing mean food intake in kCal per gram of body weight for 5 major timepoints of the study. N = 10, major time points are established using the age of the rats.

Table 3-3: Food Intake (kCal/gBW) Averaged over 3 days at 5 major developmental time points.						
Condition	**Treatment**	**kCal / gBW (d37-d40)**	**kCal / gBW (d58-d61)**	**kCal / gBW (d79-d82)**	**kCal / gBW (d100-d102)**	**kCal / gBW (d121-d123)**
AD	**Non-Exercised**	0.366± 0.032	0.273 ± 0.04	0.212 ± 0.007	0.188 ± 0.006	0.178 ± 0.005
	Exercised	0.347 ± 0.026	0.260 ± 0.006	0.215 ± 0.010	0.165 ± 0.038	0.169 ± 0.003
UN	**Non-Exercised**	0.291 ± 0.067	0.281 ± 0.007	0.228 ± 0.008	0.152 ± 0.047	0.196 ± 0.013
	Exercised	0.298 ± 0.062	0.269 ± 0.007	0.217 ± 0.007	0.174 ± 0.005	0.191 ± 0.003
Two-Way ANOVA		**P-values**				
Prenatal Nutrition		0.2293	0.1715	0.2661	0.6419	**0.0132**
Postnatal Exercise		0.9071	0.0664	0.5999	0.9833	0.3103
Interaction		0.7970	0.9246	0.3941	0.4606	0.7469

***mean food consumption corrected for total body weight (kCal/gBW) ± SEM**, n = 10 per group. $p<0.05$ calculated with Bonferroni post tests.

Combining the two datasets, it its evident that while there were no obvious differences between the amount of food intake, the UN and UNX groups went from an initially lower food intake ratio to a gradually reversed trend and eventually reaching the point where they ate more food per gram bodyweight compared to the AD and ADX groups. These data demonstrated that exercise had no effect on the on the food intake of the

subjects of this study, whilst in comparison, as body weight plateaued at a mature age, caloric intake as a percentage of total body weight increased in the offsprings of UN mothers in comparison to their AD counterparts.

4.2 Exercise Data

As explained in the methodology section, each rat of the exercise groups ADX and UNX were exposed to a dosed, routine exercise everyday. Whilst being limited to 300 quarter-wheel turns – roughly 56m of running per day on the exercise running wheel before it is clamped down.

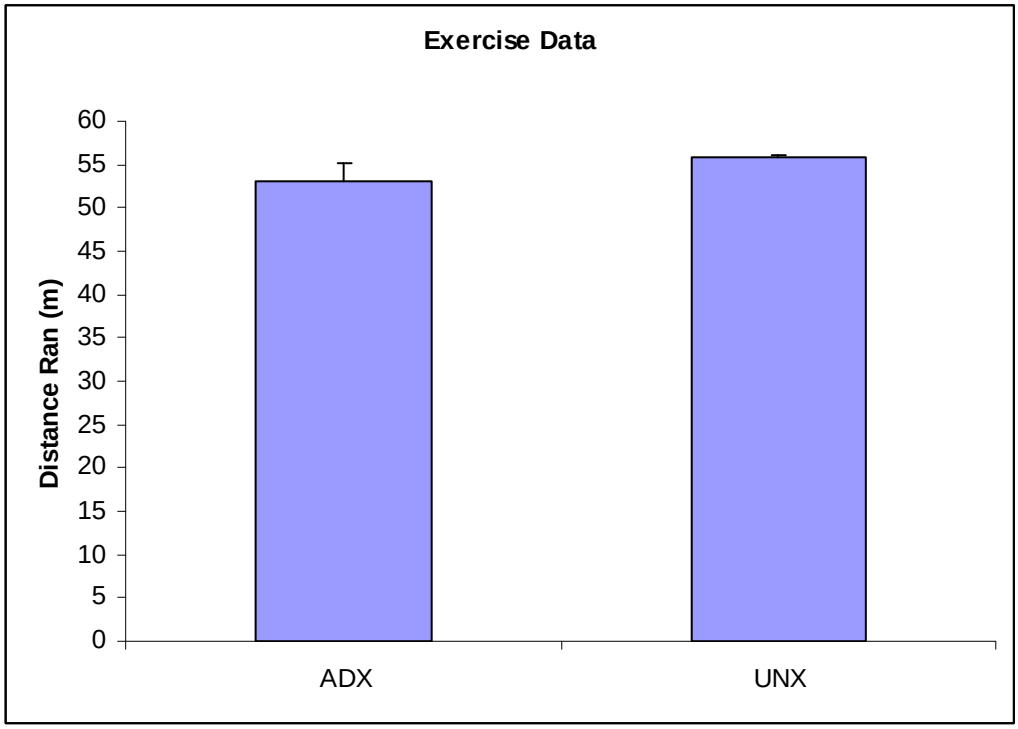

Figure 3-8 Graph showing mean distance ran by AD and UN exercise participants. Error bars denotes SEM. N = 10.

Table 3-4 : Average exercise data throughout the study	
	Distance Exercised on Running Wheel
AD	53.10m ± 2.15
UN	55.91 ± 0.20
One-tailed T-test	0.1127

A one-way ANOVA was not performed on this analysis as there was a daily limit of 56m of exercise allowed on the subjects. In order to accommodate for this feature of the study design, a one-tailed T-test was used to better analyze the result. From the records of the daily exercise routine, we found no differences in the mean total distance travelled by the AD (53.10m ± 2.15) or UN (55.91m ± 0.20) exercise participants during their allocated exercising time frames. This demonstrates that *both groups were exposed to the same amount of low dose routine exercise throughout the study.*

4.3 Plasma Measurements

At the end-point of the study, another batch of important physiological parameters were recorded, these included the total body weight, body length (nose-anus and nose-tail), liver weight, brain weight and plasma measurements for a number of metabolic markers. The collated data was again provided by Amy Norman.

Total Body Weight (BW) in the UN groups were observed to be significantly lower ($p<0.001$) than their AD counterparts at the end-point of the study, as did both measurements of body length; nose-anus ($p<0.001$) and nose-tail ($p<0.001$). Whilst none of the other organ measurements demonstrated any statistical differences – thereby demonstrating normal proportional growth in all groups monitored.

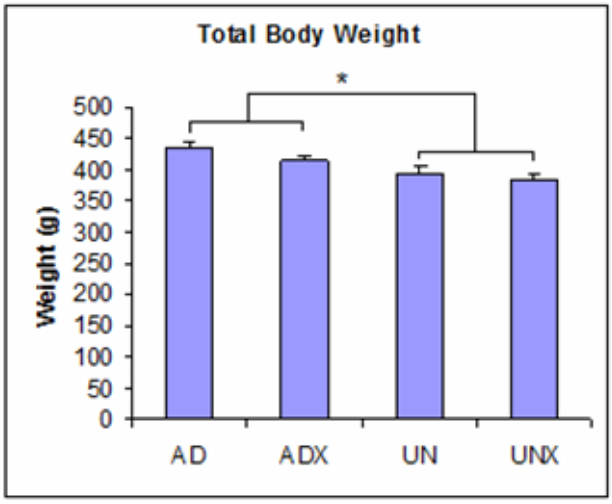

Figure 3-9 Graph showing total body weight of subjects at the end-point of the study. Error bars denotes SEM. N = 10. AD/ADX > UN/UNX, $p<0.001$ calculated by Two-Way ANOVA.

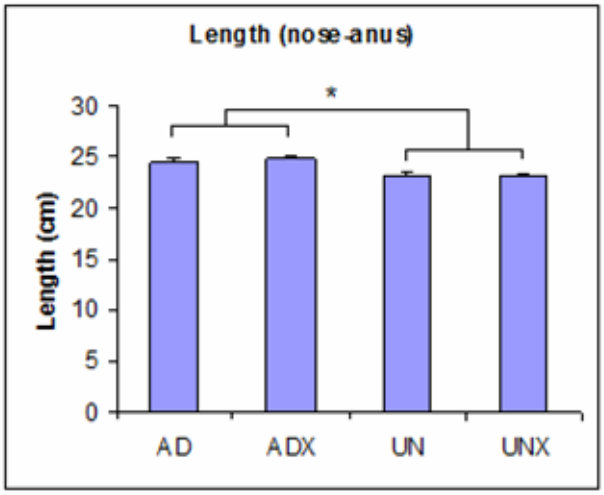

Figure 3-10 Graph showing body length (nose-anus) at the end-point of the study. Error bars denotes SEM. N = 10. AD/ADX > UN/UNX, $p<0.001$ calculated by Two-Way ANOVA.

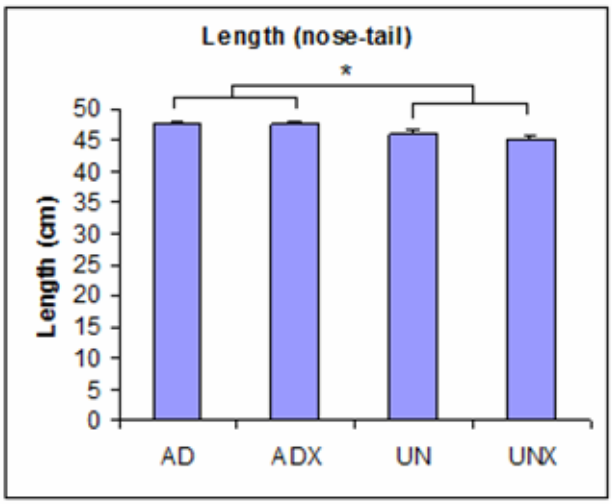

Figure 3-11 Graph showing body length (nose-tail) at the end-point of the study. Error bars denotes SEM. N = 10. AD/ADX > UN/UNX, $p<0.001$ calculated by Two-Way ANOVA.

Table 3-5 Table of Physiological Measurements at end-point of the study.							
Condition	**Treatment**	**BW** (g)	**Length (nose anus)** (cm)	**Length (nose tail)** (cm)	**Liver** (%BW)	**Brain** (%BW)	**BMI** (kg/m^2)
AD	**NE**	437 ± 7.88	24.5 ± 0.34	47.7 ± 0.37	2.79 ± 0.07	0.479 ± 0.01	7.212 ± 0.19
	EX	415 ± 8.11	24.8 ± 0.25	47.65 ± 0.50	2.76 ± 0.06	0.504 ± 0.01	6.827 ± 0.15
UN	**NE**	394 ± 11.81	23.3 ± 0.29	46.0 ± 0.56	2.86 ± 0.07	0.584 ± 0.01	7.044 ± 0.20
	EX	385 ± 9.65	23.1 ± 0.23	45.2 ± 0.39	2.85 ± 0.12	0.507 ± 0.08	7.401 ± 0.19
Two-way ANOVA		**P-values**					
Prenatal Nutrition		***0.0005**	***0.0001**	***0.0001**	0.3088	0.1926	0.2759
Postnatal Exercise		0.1182	0.8713	0.3602	0.8429	0.5352	0.9385
Interaction		0.4655	0.4587	0.4189	0.9059	0.2180	0.052

BW = Body Weight, %BW = organ weight expressed as a percentage of total body weight at the end-point of the study. *Length (nose-anus)* = total length of subject measuring from the tip of the nose to the anus. *Length (nose-tail)* = total length of the subject measuring from the tip of the nose to the tip of the straightened tail. ***means±SEM, n=10/group**. P-values obtained with Two-Way ANOVA.

Further more, plasma measures on insulin, c-peptide and leptin, Free Fatty Acid, Urea, Glycerol, Lactate, Creatine Kinase, Triglcerides, LDL and HDL contents did not show any significant differences across the four groups, indicating normal metabolic patterns in all our subjects.

Table 3-6 Table of plasma hormone and metabolic markers I				
Condition	**Treatment**	**Insulin (mg/ml)**	**C-peptide (pM)**	**Leptin (ng/ml)**
AD	**NE**	0.99 ± 0.25	683.37 ± 110.07	3.49 ± 0.29
	EX	1.45 ± 0.61	703.67 ± 129.20	4.04 ± 0.69
UN	**NE**	1.35 ± 0.46	711.39 ± 56.04	3.42 ± 0.56
	EX	0.92 ± 0.22	696.04 ± 120.94	5.28 ± 1.16
Two-way ANOVA		**P-values**		
Prenatal Nutrition		0.847	0.927	0.438
Postnatal Exercise		0.974	0.982	0.113
Interaction		0.306	0.873	0.384

*means±SEM, n=10/group; measurements taken from extracted organs on the day of cull. Average age of rat = 138 days.

Table 3-7 Table of plasma hormone and metabolic markers II				
Condition	**Treatment**	**Glucose (mmol/l)**	**FFA (mmol/l)**	**Urea (mmol/l)**
AD	**NE**	9.11 ± 0.45	1.14 ± 0.64	5.66 ± 0.56
	EX	8.77 ± 0.58	1.42 ± 0.45	6.27 ± 1.57
UN	**NE**	9.42 ± 0.49	1.23 ± 0.64	5.70 ± 0.99
	EX	8.90 ± 0.36	1.53 ± 0.56	5.34 ± 0.69
Two-way ANOVA		P-values		
Prenatal Nutrition		0.649	0.589	0.180
Postnatal Exercise		0.373	0.131	0.703
Interaction		0.845	0.979	0.145

*means±SEM, n=10/group; measurements taken from extracted organs on the day of cull. Average age of rat = 138 days.

Table 3-8 Table of plasma hormone and metabolic markers III				
Condition	**Treatment**	**Glycerol (mmol/l)**	**Lactate (mmol/l)**	**Creatine Kinase (mmol/l)**
AD	**NE**	0.133 ± 0.024	2.314 ± 0.185	7454 ± 839
	EX	0.140 ± 0.026	2.783 ± 0.442	7740 ± 1100
UN	**NE**	0.125 ± 0.039	2.570 ± 0.258	6896 ± 929
	EX	0.156 ± 0.029	2.396 ± 0.196	8108 ± 699
Two-way ANOVA		**P-values**		
Prenatal Nutrition		0.647	0.822	0.917
Postnatal Exercise		0.056	0.613	0.413
Interaction		0.220	0.274	0.611

*means±SEM, n=10/group; measurements taken from extracted organs on the day of cull. Average age of rat = 138 days.

Table 3-9 Table of plasma hormone and metabolic markers IV				
Condition	**Treatment**	**Triglycerides (mmol/l)**	**LDL (U/L)**	**HDL (U/L)**
AD	**NE**	0.797 ± 0.058	0.192 ± 0.096	1.358 ± 0.056
	EX	0.813 ± 0.081	0.234 ± 0.030	1.443 ± 0.093
UN	**NE**	0.862 ± 0.091	0.229 ± 0.039	1.551 ± 0.051
	EX	0.797 ± 0.080	0.190 ± 0.013	1.397 ± 0.049
Two-way ANOVA		P-values		
Prenatal Nutrition		0.756	0.898	0.262
Postnatal Exercise		0.756	0.956	0.596
Interaction		0.608	0.145	0.072

*means±SEM, n=10/group; measurements taken from extracted organs on the day of cull. Average age of rat = 138 days.

4.4 Brain tissue Histology – Hippocampal Cell Estimate

Histological analysis of the hippocampus was conducted using the StereoInvestigator software, utilising the optical fractionator function to estimate the total number of neurons inside the structures of interest. The results generated by the software provide detailed breakdowns of the total cell count estimations by an unbiased approach. These figures are then tallied and analyzed with standard Two-Way ANOVA. Table 3-10 shows a summary of the cell estimation data.

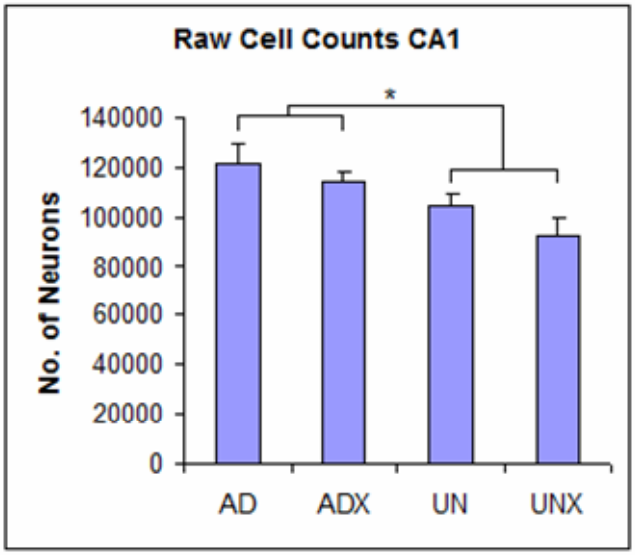

Figure 3-12 Graph of mean raw cell counts for the CA1 region. N = 7. AD/ADX > UN/UNX, P<0.01, calculated with Two-Way ANOVA.

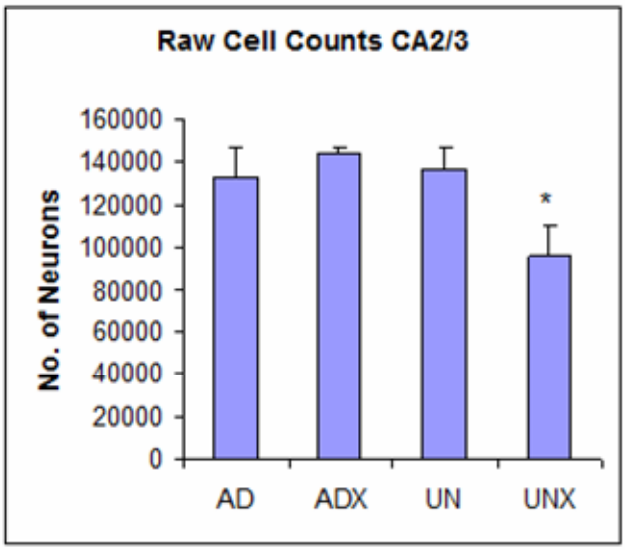

Figure 3-13 Graph of mean raw cell counts for the CA2/3 region. N = 7. UNX < ADX, P<0.05 calculated by Two-Way ANOVA

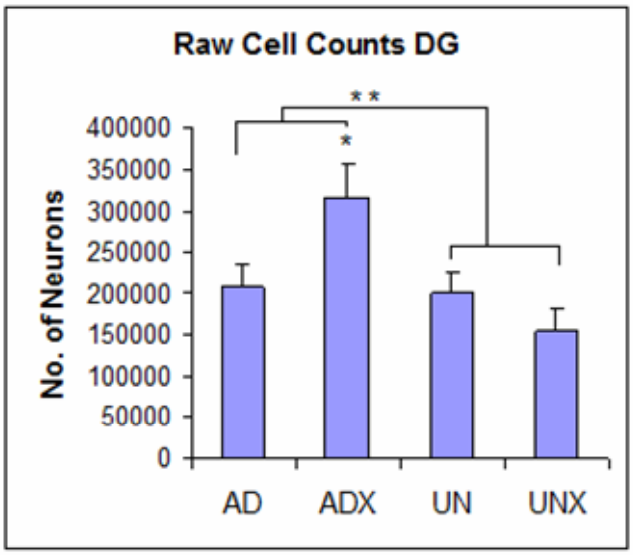

Figure 3-14 Graph of mean raw cell counts for the dentate gyrus region. N = 7. AD/ADX > UN/UNX (P=0.01); ADX > UNX ($p<0.05$), calculated with Two-Way ANOVA followed by Bonferroni Post-Test.

Table 3-10 Estimated neuron number by optical fractionator in each hippocampal region

Groups	CA1	CA2/3	DG
AD	121856 ± 7477	133239 ± 13569	206367 ± 26573
ADX	114058 ± 4414	143639 ± 2884	316302 ± 40816
UN	104650 ± 4729	136706 ± 10523	199816 ± 24545
UNX	92859 ± 7171	95081 ± 15363	154602 ± 26113
Two-way ANOVA	**p-values**		
Prenatal Nutrition	**0.004**	0.064	**0.010**
Postnatal Exercise	0.122	0.191	0.295
Interaction	0.747	**0.035**	**0.017**

*means±SEM, n=10/group; p values are calculated using Bonferroni post tests.

Bonferroni Post-Test ADX > UNX, p = 0.0103.

Detailed break down of the cell count data are shown in the following pages, with legends as follows:

- S.D.: Standard Deviation
- CE: Coefficient of Error, calculated in accordance to the new Gundersen method. (Gundersen H.J.G. et al., 1999)
- CV: Coefficient of Variation (= S.D. / mean)
- BCV: Biological Coefficient of Variation
- CE^2 ($\%CV^2$): Coefficient of Error as a percentage of CV.
- BCV^2 ($\%CV^2$): Biological variation expressed as a percentage of CV.
- Mean CE is calculated by. $\sqrt{\text{mean } \Sigma(CE^2)}$

The precision of an individual estimate can be judged by the CE, coefficient of error, which represents the intra-individual variations due to the stereological estimating process. Generally speaking a CE value < 0.1 is considered acceptable. The method used to calculate the CE in this study is what is known as the Gundersen method (Gundersen H.J.G. et al., 1999), which was covered by the StereoInvestigator program used to conduct the histology analyses. This can than be used to determine how much of the variation within the dataset was caused by biological variation or the sampling method used. In this study the majority of the variation was biological, with most groups having a BCV^2 ($\%CV^2$) reading above 90%. It is a common practice to increase the number of animals in the event where BCV^2 is more than half of CV^2 (Keuker J.I.H. et al., 2001), unfortunately that cannot be done for this study.

Table 3-11 Estimated neuron number by the optical dissector method in the CA1 region of rat hippocampal hemispheres. N = 7 in all 4 groups.

Subject	Group	CA1 (N)	CE	Subject	Group	CA1 (N)	CE
1	**AD**	141375	0.04	**7**	**ADX**	113568	0.04
3	**AD**	153126	0.06	**17**	**ADX**	110175	0.05
24	**AD**	128310	0.04	**18**	**ADX**	127335	0.04
25	**AD**	116385	0.05	**20**	**ADX**	97500	0.05
26	**AD**	108615	0.05	**36**	**ADX**	128310	0.05
31	**AD**	102115	0.06	**37**	**ADX**	102765	0.05
32	**AD**	103070	0.05	**38**	**ADX**	118755	0.05
	Mean	**121856.57**	**0.05**		**Mean**	**114058.29**	**0.05**
	SD	19783.56			**SD**	11679.59	
	CV	0.16235			**CV**	0.10240	
	CV^2	0.02636			**CV^2**	0.01049	
	CE^2	0.00250			**CE^2**	0.00222	
	BCV^2	0.02386			**BCV^2**	0.00826	
	BCV^2 (%CV^2)	**90.52%**			**BCV^2 (%CV^2)**	**78.81%**	
	CE^2 (%CV^2)	**9.48%**			**CE^2 (%CV^2)**	**21.19%**	

Subject	Group	CA1 (N)	CE	Subject	Group	CA1 (N)	CE
5	**UN**	86483	0.05	**9**	**UNX**	89535	0.06
12	**UN**	108810	0.05	**15**	**UNX**	103155	0.05
13	**UN**	102765	0.04	**28**	**UNX**	76503	0.04
21	**UN**	124995	0.04	**29**	**UNX**	102705	0.06
22	**UN**	113520	0.05	**30**	**UNX**	95597	0.04
33	**UN**	96135	0.05	**39**	**UNX**	62400	0.06
34	**UN**	99840	0.05	**40**	**UNX**	120120	0.04
	Mean	**104649.71**	**0.05**		**Mean**	**92859.29**	**0.05**
	SD	12510.90			**SD**	18973.35	
	CV	0.11955			**CV**	0.20432	
	CV^2	0.01429			**CV^2**	0.04175	
	CE^2	0.00222			**CE^2**	0.00250	
	BCV^2	0.01207			**BCV^2**	0.03925	
	BCV^2 (%CV^2)	**84.45%**			**BCV^2 (%CV^2)**	**94.01%**	
	CE^2 (%CV^2)	**15.55%**			**CE^2 (%CV^2)**	**5.99%**	

In the CA1 region, a significant reduction of mean raw cell counts was observed in the UN and UNX groups ($p<0.01$) demonstrating the adverse effects of prenatal maternal undernutrition on the hippocampal structure as the CA1 region completes its development by the time of birth, and its morphology remains unchangeable after birth under normal circumstances.

Table 3-12 Estimated neuron number by the optical dissector method in the CA2/3 region of rat hippocampal hemispheres. N = 7 in all 4 groups.

Subject	Group	CA2/3 (N)	CE	Subject	Group	CA2/3 (N)	CE
1	**AD**	177840	0.04	**7**	**ADX**	146593	0.06
3	**AD**	168795	0.05	**17**	**ADX**	132990	0.04
24	**AD**	94185	0.05	**18**	**ADX**	152685	0.04
25	**AD**	136125	0.06	**20**	**ADX**	148005	0.04
26	**AD**	149760	0.04	**36**	**ADX**	133185	0.05
31	**AD**	82465	0.06	**37**	**ADX**	144300	0.04
32	**AD**	123506	0.05	**38**	**ADX**	147713	0.05
	Mean	**133239.43**	**0.05**		**Mean**	**143638.71**	**0.05**
	SEM	13568.78			**SEM**	2884.03	
	SD	35899.62			**SD**	7630.42	
	CV	0.26944			**CV**	0.05312	
	CV^2	0.07260			**CV^2**	0.00282	
	CE^2	0.00250			**CE^2**	0.00209	
	BCV^2	0.07010			**BCV^2**	0.00073	
	BCV^2 (%CV^2)	**96.56%**			**BCV^2 (%CV^2)**	**25.95%**	
	CE^2 (%CV^2)	**3.44%**			**CE^2 (%CV^2)**	**74.05%**	

Subject	Group	CA2/3 (N)	CE	Subject	Group	CA2/3 (N)	CE
5	**UN**	123569	0.04	**9**	**UNX**	91531	0.07
12	**UN**	154830	0.05	**15**	**UNX**	88920	0.05
13	**UN**	157755	0.04	**28**	**UNX**	89312	0.04
21	**UN**	168480	0.04	**29**	**UNX**	62642	0.07
22	**UN**	135468	0.03	**30**	**UNX**	67766	0.05
33	**UN**	131820	0.05	**39**	**UNX**	81705	0.05
34	**UN**	85020	0.05	**40**	**UNX**	183690	0.04
	Mean	**136706.00**	**0.04**		**Mean**	**95080.86**	**0.05**
	SEM	10522.71			**SEM**	15362.84	
	SD	27840.49			**SD**	40646.26	
	CV	0.20365			**CV**	0.42749	
	CV^2	0.04147			**CV^2**	0.18275	
	CE^2	0.00184			**CE^2**	0.00279	
	BCV^2	0.03964			**BCV^2**	0.17996	
	BCV^2 (%CV^2)	**95.57%**			**BCV^2 (%CV)**	**98.47%**	
	CE^2 (%CV^2)	**4.43%**			**CE^2 (%CV)**	**1.53%**	

In the CA2/3 region, an interaction effect was observed whereby the effects of both prenatal maternal undernutrition and post natal exercise resulted in the overall reduction of raw cell counts in the UNX subjects compared to the other three groups ($p<0.05$). Notably,

Table 3-13 Estimated neuron number by the optical dissector method in the Dentate Gyrus of rat hippocampal hemispheres. N = 7 in all 4 groups.

Subject	Group	DG raw	CE	Subject	Group	DG raw	CE
1	**AD**	308685	0.04	**7**	**ADX**	295312	0.05
3	**AD**	286462	0.06	**17**	**ADX**	495495	0.03
24	**AD**	214110	0.04	**18**	**ADX**	285480	0.03
25	**AD**	124770	0.05	**20**	**ADX**	221715	0.03
26	**AD**	207090	0.03	**36**	**ADX**	429000	0.04
31	**AD**	158178	0.04	**37**	**ADX**	197535	0.03
32	**AD**	145275	0.04	**38**	**ADX**	289575	0.03
	Mean	**206367.14**	**0.04**		**Mean**	**316301.71**	**0.03**
	SEM	26572.96			**SEM**	40815.55	
	SD	70305.45			**SD**	107987.79	
	CV	0.34068			**CV**	0.34141	
	CV^2	0.11606			**CV^2**	0.11656	
	CE^2	0.00184			**CE^2**	0.00118	
	BCV^2	0.11423			**BCV^2**	0.11538	
	BCV^2 (%CV)	**98.42%**			**BCV^2 (%CV)**	**98.99%**	
	CE^2 (%CV)	**1.58%**			**CE^2 (%CV)**	**1.01%**	

Subject	Group	DG raw	CE	Subject	Group	DG raw	CE
5	**UN**	106159	0.06	**9**	**UNX**	195321	0.05
12	**UN**	120705	0.04	**15**	**UNX**	213915	0.04
13	**UN**	269295	0.03	**28**	**UNX**	159845	0.06
21	**UN**	259545	0.03	**29**	**UNX**	65132	0.06
22	**UN**	186512	0.04	**30**	**UNX**	49420	0.05
33	**UN**	221715	0.03	**39**	**UNX**	185640	0.04
34	**UN**	234780	0.03	**40**	**UNX**	212940	0.03
	Mean	**199815.86**	**0.04**		**Mean**	**154601.86**	**0.05**
	SEM	24545.45			**SEM**	26112.56	
	SD	64941.14			**SD**	69087.35	
	CV	0.32500			**CV**	0.44687	
	CV^2	0.10563			**CV^2**	0.19970	
	CE^2	0.00138			**CE^2**	0.00222	
	BCV^2	0.10425			**BCV^2**	0.19747	
	BCV^2 (%CV)	**98.69%**			**BCV^2 (%CV)**	**98.89%**	
	CE^2 (%CV)	**1.31%**			**CE^2 (%CV)**	**1.11%**	

And lastly, in the Dentate gyrus, the programming effect by prenatal maternal undernutrition lead to significant reductions in the neuron numbers in both the UN and UNX groups ($p=0.01$). The ADX group stood out here, demonstrating the combination of prenatal maternal ad libitum diet and postnatal exercise leading to a highly significant increase in mean raw neuron number – an indication of heightened neurogenesis ($p<0.05$).

4.5 Molecular analysis

4.5.1 Spinophilin Protein Levels

Spinophilin, a protein found within spines of connecting neurons, is often used as a marker of synaptic density. The Spinophilin levels were measured on dissected hippocampal hemispheres from each of our four study groups (n=3). From the graph it is easy to see a huge difference in expression in the UN group. Initially, none of the comparisons by a two-way ANOVA and subsequent post-hoc analyses showed any significance in the results (Table 3-14).

Table 3-14 : Spinophilin protein expression level

Groups	Spinophilin expression as % of Control	
AD	101.08 ± 12.36	
ADX	110.15 ± 2.51	
UN	55.09 ± 11.65	
UNX	117.43 ± 29.54	
Two-way ANOVA	**P-value**	**F-value**
Prenatal Nutrition	0.2901	1.283
Postnatal Exercise	0.07	4.367
Interaction	0.1576	2.431

Mean ± SEM, n=3/group;

However, a further breakdown of the statistical components revealed the variations in the data within the interaction of the two variables exceeds one of the main effects – prenatal nutrition. This prompted for the use of synergistic contract analysis on the data (ref required for Prof. Chris Trigs). This is first done by analyzing the mean effects of each group,

	AD	UN	Effect
Non-Ex	101.08	55.09	**45.99**
Ex	110.15	117.43	**-7.28**
Effect	**-9.07**	**-62.34**	

Followed by the test statistic formula, which compares the UN group against AD,ADX and UNX as a whole, in a 3:1:1:1 fashion.

$$F = \frac{(3 \times 55.09 - 101.08 - 110.15 - 117.43)^2}{3^2/3 + (-1)^2/3 + (-1)^2/3 + (-1)^2/3}$$

$$F = 6674.073$$

F value of 6674.073 with distribution (1,12) df returns a p-value of 7.50×10^{-18}
This can then be used to calculate the total variation accounted for

$$\frac{\text{Test statistic}}{\text{SS from one-way ANOVA}} = \frac{6674.073}{7076} = \mathbf{0.943199}$$

This figure means **this synergistic contrast explains 94% of the variation between the four treatment means –** that Spinophilin expression in the UN group is significantly lower than the other three groups ($p<0.001$). This also tells us that **exercise normalises** this deficit, as our UNX group demonstrated no difference between the controls in Spinophilin expression.

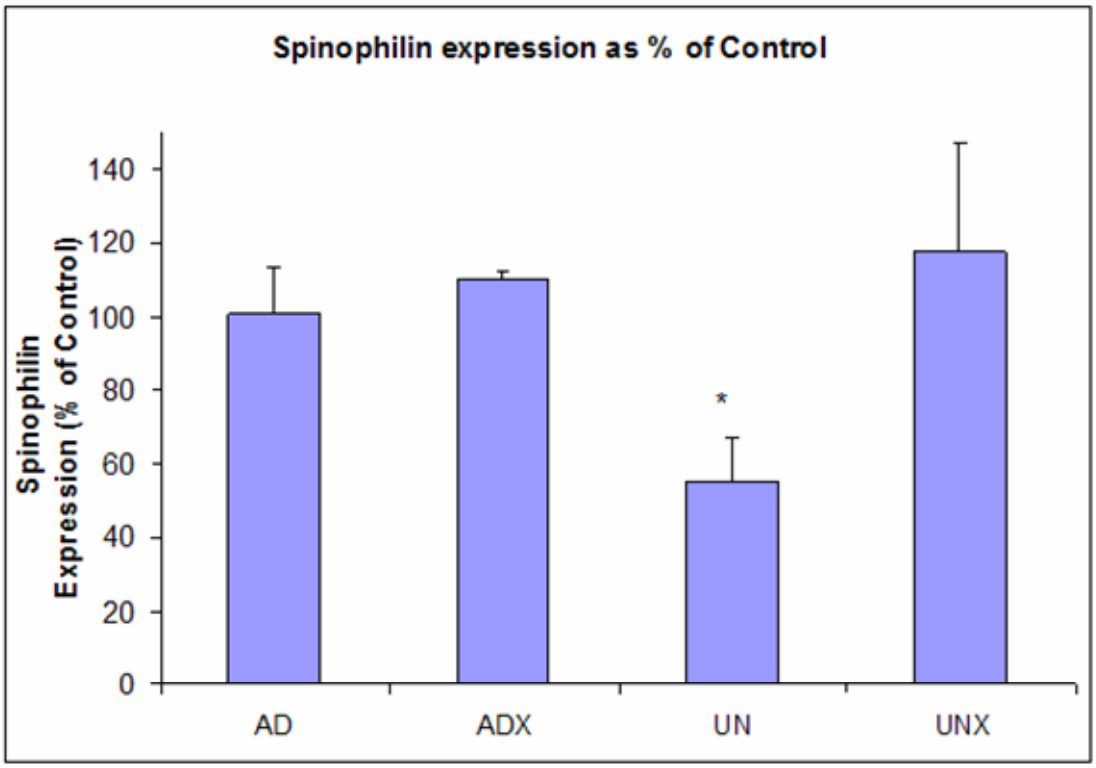

Figure 3-15 Graph showing mean levels of spinophilin in the subject's hippocampus expressed as a % of the spinophilin levels in the AD group.

4.5.2 Neurotrophin Expression

Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the mRNA expression of major neurotrophins and receptors that are known to be associated with the process of neurogenesis and survival. TrkB, the receptor of BDNF was found to be significantly increased in both exercise groups (ADX/UNX>AD/UN, $p<0.001$) and significant differences were also detected when comparing the nutritional programming effects (UN/UNX > AD/ADX, $p<0.01$).

Expression of Neurotrophin-3, a signal that promotes neuronal survival and differentiation, was also found to be significantly different via nutritional programming (AD/ADX > UN/UNX, $p<0.05$). Table 3.15 gives a detailed summary of the mRNA expression levels. Level of expression were first adjusted to cyclophilin expressions then adjusted against the AD control for each mRNA analyzed. Data are shown as mean adjusted value ± SEM, analyzed using Two-Way ANOVA with StatView statistical programme.

Table 3-15 Neurotrophin mRNA expression levels corrected to control

Groups	BDNF	TrkB	bFGF	VEGF	NT-3
AD	1.000 ± 0.081	1.000 ± 0.158	1.000 ± 0.027	1.000 ± 0.042	1.000 ± 0.101
ADX	1.171 ± 0.054	1.331 ± 0.032	0.946 ± 0.040	1.164 ± 0.010	0.840 ± 0.013
UN	1.061 ± 0.036	1.187 ± 0.040	1.057 ± 0.098	1.026 ± 0.169	0.727 ± 0.003
UNX	1.059 ± 0.092	1.618 ± 0.053	1.186 ± 0.058	1.201 ± 0.059	0.795 ± 0.016
Two-way ANOVA	**p-values**				
Prenatal Nutrition	0.726	**<0.01**	*0.068*	0.796	**0.015**
Postnatal Exercise	0.258	**<0.001**	0.600	0.195	0.398
Interaction	0.246	0.477	0.227	0.964	*0.058*

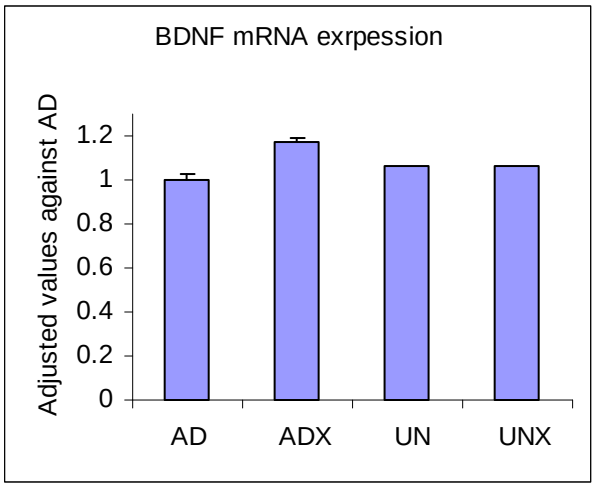

Figure 3-16 Graph showing mean expression of BDNF as a percentage of AD control. n = 3.

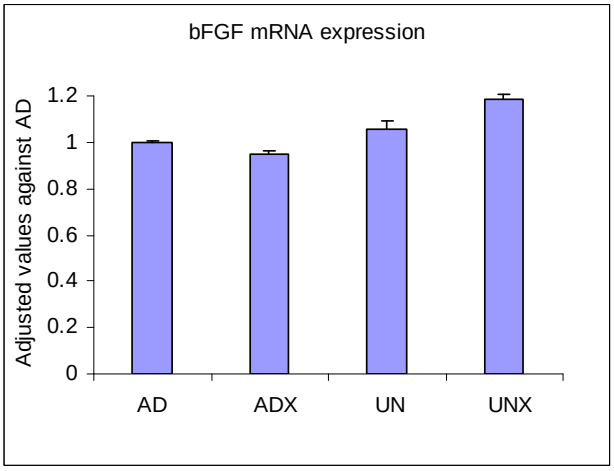

Figure 3-17 Graph showing mean expression of bFGF as a percentage of AD control. n = 3.

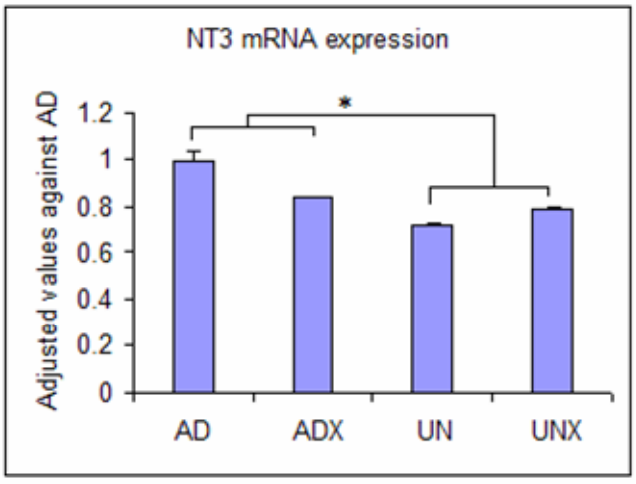

Figure 3-18 Graph showing mean expression of NT3 as a percentage of AD control. n = 3. AD/ADX > UN/UNX, $p<0.05$. Calculated using Two-Way ANOVA.

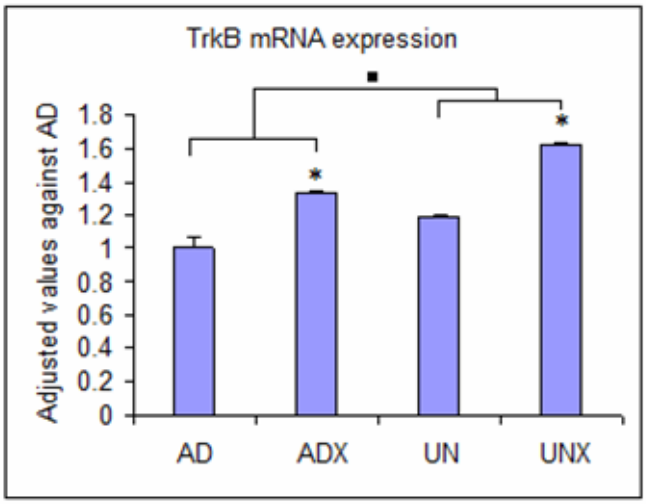

Figure 3-19 Graph showing mean expression of TrkB as a percentage of AD control. n = 3.

* indicates ADX/UNX > AD/UN, $p<0.001$, calculated with Two-Way ANOVA.

■ indicates UN/UNX > AD/ADX, $p<0.01$, calculated with Two-Way ANOVA

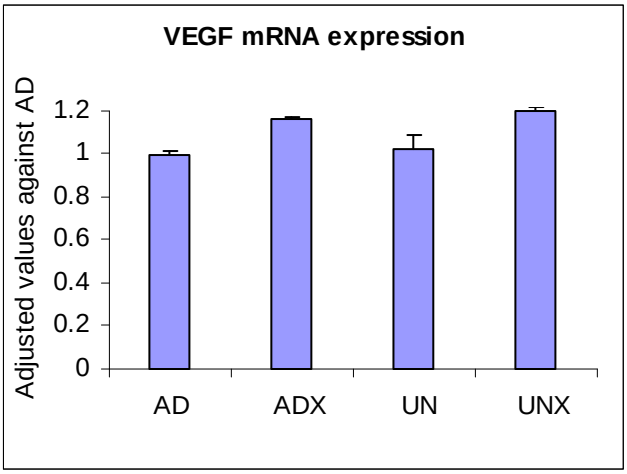

Figure 3-20 Graph showing mean expression of VEGF bFGF as a percentage of AD control. n = 3.

Although not statistically significant, the expression of bFGF and VEGF in the UNX group both appeared to be higher than that of the AD control; in the case of VEGF it approaches significance with $p = 0.058$. When viewed as a complete picture, the graphical data presents an interesting pattern in the UNX group showing a general normalizing trend when compared against its UN counterpart. This is definitely something worth investigating in future studies of a similar nature.

CHAPTER 5: DISCUSSION

5.1 Physiological Aspects

Early influences such as prenatal global maternal undernourishment have been well known to set up the developing fetus into what is called a predictive adaptive response *in utero* of which, manifestation of metabolic and endocrine abnormalities in these subjects have been well documented (Gluckman P.D. et al., 2004). The effects of undernutrition during early life leading to deficits and distortions of brain structure and function has also been reported in the past two decades (Bedi K.S., 1984, 1987).

However, the effects of this type of early nutritional influence on the process of neurodevelopment and postnatal neurogenesis in the hippocampus have not been extensively researched. Thus, it became one of the main aims of this work to look into those factors, specifically in the form of changes in neurotrophin expression and/or alterations in neuron number and morphological features of the hippocampus.

From birth, the effects of global maternal undernourishment were clearly reflected by the birth weight of the UN groups being significantly lower than their AD counterpart, and another classic sign of the early nutritional programming effect was also reflected by the size differences measured from nose to anus. The litter size was not significantly different between the dams that received *ad libitum* or the 30% diet, indicating that no other complications was present during the gestational period and all pregnant dams were able to deliver without major noticeable differences.

After weaning, the progressive changes in body weight were closely monitored. The growth curve (Figure 3-5) of all four study groups remained relatively similar, although the initial difference in size and body weight caused by the prenatal nutritional programming effect persisted throughout the study. With the average weight of AD and ADX groups being significantly higher than their UN and UNX counterparts' right from weaning till the endpoint of the study.

Notably, the differences in body weight growth in the animals was not affected by the post natal exercise regiment at all, other than on day 34 of age, which can be explained as this is roughly when the exercise regiment was first introduced to the animals, leading to a transient growth sprouting effect. Where as the difference in body weight affected by exercise on day 85 is very likely to be a chance event as it was not observed again in any other time point.

Food intake patterns were the next important aspect monitored. At first glance at the total food consumption, the UN and UNX groups initially had a significantly lower amount of food intake; however, when taken into account the fact that larger animals usually eat more while smaller animals eat less, the total food consumption per body weight demonstrated no significant differences across the spectrum throughout the study. Exercise was observed to have no effect on the consumption of food by the animals, a finding in agreement with other studies (McMaster S.B. et al., 1985). The first sign of an animal being unwell is often reflected by a sudden change in bodyweight or its food intake pattern – neither of which was observed throughout the study, indicating normal and healthy development was ensured throughout the period.

Interestingly, at the 5th major time point (average age 121 ~ 123 days), the total food intake corrected for body weight was found to be significantly higher in the UN and UNX animals in comparison to those in the AD and ADX groups. Previous studies have shown that offsprings exposed to nutritional deficiencies which subsequently result in low birth weights are more prone to developing obesity and other metabolic problems (Gluckman P.D. et al., 2004; Stocker C.J. et al., 2005), thus the difference in food intake observed here may very well be an early sign of the onset of adult obesity. Unfortunately further data on this could not be obtained as the exercise study reached an endpoint shortly after the 5th food intake measurement time point was taken, future studies could potentially investigate on this phenomenon (see 5.3 Future Study Directions).

At the end point of the study, an array of plasma analyses were also conducted on these animals in order to establish a physiological profile of their well-being and to detect for any abnormalities that are usually found to be associated with offsprings of prenatal global maternal undernourishment. Besides the general parameters such as final body length, nose-tail and nose-anus length, all other parameters measured did not show any significant differences across all four study groups. The physiological parameters investigated in depth including organ weights corrected to body weight for the animal's liver and brain, its BMI, levels of insulin, C-peptide, leptin, free fatty acid, urea, glycerol, lactate, creatine kinase, tirgycerides, LDL and HDL contents did not show any differences. These results demonstrated that aside from neurological changes, all our subjects had managed to develop normally, proportionally and were in good health, and that the only major difference that would be observed from the animals would be focused to the neuro-developmental aspects.

5.2 Morphological Aspects

The morphological changes influenced by prenatal maternal undernourishment and subsequently exposed to postnatal low-dosed routine exercise can be reflected in the total cell counts in the trisynaptic pathway – namely pyramidal cell counts of CA1 and CA2/3 region that reflects on the influences exerted on the animals prenatally, and granule cell counts in of the Dentate Gyrus region would reflect on the influences exerted on the animals postnatally.

As we know, the pyramidal neurons in the CA1 and CA2/3 area are formed and completes its development during the process of fetal development, thus the number of neurons each individual is born with cannot be altered by normal passive postnatal processes. This means that any differences observed in these areas are likely to be the result of early programming. Past studies have demonstrated the exposure to gestational undernourishment in the form of protein malnutrition can lead to adverse effects of the hippocampal development (Morgane P.J. et al., 2002), our data is in accordance to these findings, with the total neuron estimation in the CA1 region being significantly affected by the prenatal influence of a global maternal undernourishment, reflected in statistically significant reductions in total pyramidal cells in the UN and UNX groups compared to their AD and ADX counterparts.

Whilst on the other hand, postnatal exercise did not result in any detectable difference between the groups. Similar results were observed in other studies that looked into the effects of specific prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampus, in which they noted a 20% reduction in neuron numbers caused by prenatal protein malnutrition (Lister J.P. et al., 2005).

In the CA2/3 region, the difference in total pyramidal cell counts caused by prenatal global maternal undernourishment was not detected, nor did exercise create any significant differences when compared as a group. However our data showed an interaction effect, highlighting UNX as having significantly lower number of neurons with the combined effects of prenatal malnutrition and postnatal exercise. This result was rather puzzling as under normal circumstances cells in the CA2/3 region are only susceptible to prenatal influences, which was not observed as no nutritional effects were detected in the statistical analyses; and likewise no obvious exercise effects were noted either. This matter requires further attention in future studies to resolve.

The reduction in the total estimated pyramidal cell number in the CA1 region can be seen as the morphological manifestation of the adversities caused by prenatal global maternal undernourishment, similar findings was again observed in the total estimated granule cell density estimations in the Dentate Gyrus region, with UN and UNX groups containing significantly less cell compared against AD and ADX groups. This demonstrates that not only does the programming effect exert its effect prenatally during the development of the hippocampus; it also plays an impeding role in the process of postnatal neurogenesis.

Most notably, the cell counts of the exercised control (ADX) group were clearly and significantly higher than its non-exercised counterparts, and higher still than the UN and UNX groups. This is a good indication that even with a limitation of 56m per exercise period per day, the low dosage of the exercise was still able to lead to a massive increase in neurogenesis.

5.3 Molecular Aspects

In terms of key neurotrophic expressions in the hippocampus, BDNF, the most potent neurotrophin that improves neuronal survival and neurite growth was found to have no significant differences across all four groups. This is likely to be due to the nature of BDNF's effect being fast acting and transient, and if any major differences did occur in its expression, it would have faded by the time the tissues were extracted for RT-PCR analyse (see 5.2 Critical Commentary).

Both prenatal nutrition and postnatal exercise contributed to the significant differences observed in the expression of TrkB, albeit no interaction effect was observed. Both exercise groups ADX and UNX has significantly higher expression of TrkB compared to their non-exercised counterparts demonstrating postnatal exercise enhances the expression of TrkB. Interestingly, the offsprings of malnourished dams during gestation also demonstrated a significant higher expression of TrkB compared to their counterparts born from dams that received *ad libitum* diet.

Exercise-induced TrkB is well documented, and in this study it served as a clear underlying pathway that brought about the increased neurogenesis. From another perspective, past studies have also suggested the deprivation of habitual running in rats leads to the down-regulation of BDNF and TrkB in the brain (Widenfalk J. et al., 1999), further suggesting the link between exercise and its effects on the expression of BDNF and TrkB.

There was no statistical difference observed in the bFGF, argued to be a promoter in progenitor proliferation and may also act on enhancing cell migration and is generally

accepted to have neuroprotective qualities. Although the statistical analysis showed that the difference between UN/UNX and AD/ADX is only approaching significant (p=0.068), the seemingly raised levels of bFGF in the postnatal hippocampus of these UN offsprings may actually be a self-repair mechanism employed to salvage and strengthen the lowered number of neurons present due to prenatal global maternal undernourishment (See 5.2 Critical Commentaries).

NT-3 is a factor which ahs been shown to both induce differentiation of hippocampus-derived neural precursor cells and also to facilitate synaptogenesis in cultured hippocampal neurons (Vicario-Abejon C. et al., 1995; Vicario-Abejon C. et al., 2000). Our results demonstrated significantly lower levels of NT-3 expression in the offsprings of undernourished damns during gestation. This result goes directly against the current mainstream literature which suggests that voluntary exercise enhance the expression of NT-3 and its receptor TrkC, enhances the process of neurogenesis. Other studies have also suggested the increase in NT-3 following dietary restrictions (Lee J. et al., 2000), neither of which was observed in the data generated from this study.

In addition to the morphological and neuroendocrine alterations brought about by the prenatal nutritional programming, our data also demonstrated an overall reduction in synaptic density in the UN animals, with strikingly significant difference between animals of the UN group and those of the other three groups investigated in this study.

The western blot analysis conducted in this work looked at the expression of Spinophilin, a dendritic spine protein often used to assess the extent of glutamatergic synaptic connections (Feng J. et al., 2000). A significant reduction in the expression

level of spinophillin was observed in animals of the UN group when compared against animals from the AD, ADX and UNX groups. This suggests that not only does maternal undernutrition have an adverse effect on the morphological development of the hippocampus; it may also interfere with the density of synaptic terminals.

5.4 Combined Observations

In order to ensure the effects of exercise was equally exerted on all the subjects exposed to the exercise regime, a daily exercise cap of equivalent to 56m in distance ran was imposed on the animals. However, variations did occur as some of the animals do not always reach this targeted exercise cap. Nevertheless, from the analyses of the exercise data, no significant differences were detected and therefore it is safe to assume that the effects of exercise were equally exerted onto all subjects.

Having this in mind, our data suggests that the effects of postnatal exercise significantly enhanced the postnatal neurogenic process in the hippocampus, most likely through enhanced signalling of BDNF coupled with exercise-induced increase in TrkB expression. VEGF and bFGF also appears to play a role but cannot be verified by the data obtained in this study alone.

An interesting question arises when all the data from this study are combined – Why is it, with increased neurotrophin expressions, namely a marked increase in TrkB expression to enhance BDNF signalling, that the total granule cell estimation of the UNX group was not markedly increased as we saw in the ADX group?

Past studies by the DeBassio group has demonstrated a reduction in the granule cell neurogenesis in animals exposed to prenatal protein deprivation (DeBassio W.A. et al., 1994), and this work was later extended onto investigating the specific effects of prenatal malnutrition on the granule cell generation in the dentate gyrus region (DeBassio W.A. et al., 1996). Results from both of these studies suggests that by the use of prenatal protein malnutrition, not only were the animals born with a lesser number of neurons to begin with, their ability to regenerate is also significantly impeded.

Taken into context of this study, where a global undernourishment model was used instead of specific protein malnutrition, the reduction in total cell number was also reflected in both the CA1 region and the Dentate Gyrus region – this means, that even though the process of neurogenesis may have increased, due to the already low number of cells to begin with, the process of neurogenesis was only capable of replenishing the cells that were lost but could not regenerate cells beyond this point as much as the ADX group. Another possible explanation arises from a study which demonstrated that not only did running wheel exercise increased the process of neurogenesis in its animals, it also increased the rate of cell death in these subjects (Kitamura T. et al., 2006b). A very recent study also demonstrated that undernutrition during early life increased the level of apoptosis in the dentate gyrus (Jahnke S. et al., 2007).

By combining these findings, the lack of increase in granule cell number in the dentate gyrus of the UNX group can be explained. Albeit a marked exercise-induced increase evidently took place in the molecular factors that enhance neurogenesis, due

to a lower starting pool of progenitor cells caused by the adverse effects of prenatal global maternal undernourishment, combined with the nutritional programming effect that increased apoptosis, and furthermore, the exercise-induced increase in cell turnover rate, even with enhanced neurogenesis, it was not possible for the granule cells in our UNX subjects to markedly increase in number – thus resulting in the cell count result observed in this study.

Another interesting finding was observed in the analysis of spinophillin. Whilst the UN group demonstrated significantly lower expression of spinophillin, indicating that prenatal nutritional programming had a major effect that resulted in great reduction of synaptic terminals in the hippocampus, this was **not** observed in the UNX group. In fact, the expression of spinophillin in the UNX group was found to be somewhat similar to that of the AD and ADX groups – ie. Postnatal routine exercise in the animals of undernourished dams demonstrated normalization of spinophillin expression, indicating a normalised density of synaptic terminals in the hippocampus.

The results observed in the UN and UNX group demonstrated the adverse effects exerted by a prenatal nutritional deficit through maternal undernourishment. Whilst in the UNX group we observed the effects of exercise attempts to over come these programmed deficits, by increasing neurogenesis, promoting cellular survival to overcome the programming effect of increased apoptosis, and finally, increased density of synaptic terminals to restore the functions of the hippocampus.

CHAPTER 6: CONCLUSION

6.1 Context

This study was set forth to investigate the how prenatal maternal undernourishment would affect the process of neurodevelopment and postnatal morphology in the hippocampus. From the data obtained in this study, it was evident that maternal undernourishment leads to detrimental effects on the process of neurodevelopment. In particular, the pyramidal cell numbers of the CA1 region and the granule cell numbers of the Dentate Gyrus region were significantly reduced. Furthermore, the connectivity of neurons in the hippocampus was dramatically reduced as reflected by the drastically lowered levels of spinophillin expression. This is well aligned with the initial hypothesis that early life nutritional deficits through maternal undernourishment would lead to a reduction in both cell density and connectivity. However from this study alone it was not evident whether a reduction in key neurotrophic factors during gestation had played a part in causing these outcomes.

Introduction of postnatal long-term routine exercise resulted in interesting changes in the hippocampus of maternally undernourished and *ad libitum* fed offsprings in the forms of alterations in neurotrophin expressions and general morphology of the hippocampus. Notably, exercise-induced neurogenesis through enhanced BDNF signalling by increased TrkB expression resulted in a highly significant increase in the neurogenic process in the dentate gyrus, in the offspring of *ad libitum* fed dams. The hypothesized up-regulation in other key neurotrophic factors and angiogenic factors like VEGF was not observed possibly due to the transient and fast acting nature of these factors.

Whilst in the maternally undernourished offsprings, exercise-induced neurogenesis was present, as shown by the increased TrkB expression thereby enhancing the neurotrophic actions of BDNF, but an increase in granule cell number was not observed. Rather, postnatal long-term exercise resulted in the repair of cellular connectivity, reflected by the normalized levels of spinophillin.

The hypothesized re-activation of neurotrophic factors in prenatally undernourished offsprings by the use of long-term routine exercise postnatally was observed, but it was not enough to override the effects of prenatal undernourishment, as observed in the persisted lowered levels of both CA1 pyramidal cell counts and the granule cell counts of the Dentate Gyrus – the place of neurogenesis.

Overall, the effects of prenatal maternal undernourishment lead to a morphologically evident adverse effect on the neurodevelopment of the hippocampus. On the other hand, a low-dosed routine exercise postnatally, saw the increase in expression of key neurotrophic factors. Albeit never reaching the same magnitude of cell density likes their *ad libitum* offspring counterparts, it is likely that the exercise induced repair mechanism functions more at restoring the connectivity of the hippocampus rather than replenishing the reduction in neurons.

6.2 Critical Commentaries

From past studies, the adversities related to maternal undernourishment has been well identified and documented in the forms of metabolic and endocrine abnormalities. These findings promptly paved way for this study, which was aimed at identifying how a global maternal undernourished environment can lead to alterations in the hippocampal morphology and molecular pathways. Furthermore, the possibility of using low-dosed routine exercise as a post-natal modulation was examined in hopes of reversing the adverse effects caused by a global maternal undernourishment.

Firstly, the animal studies designed contained a rather big gamble. Thus far there's no clear-cut indication in this field of study as to exactly how much is the minimal amount of exercise required on a regular basis in order to trigger the onset of increased neurogenesis above normal levels. In this study, animals of the exercise group were limited to 56m per exercise period per day. Fortunately from the results of the study it is evident that even at such a mild dose of exercise, it was still enough to trigger significant increases in neurogenesis in the dentate gyrus of the hippocampus.

Secondly, the timing of the animal subjects' daily routine was also constrained due to a lack of human resource. Whilst a good staggering system was used to conduct the behavioural and exercise routines, the feeding time was fixed for all rats – this meant for rats from Run 1, after completing the running exercise at 11am they always had to wait 3 hours to be fed at 2pm. In relative comparison, subjects of Run 4 which have their exercise scheduled at 1pm daily would get their food almost immediately after exercise due to the experiment regime! Although it is unclear whether this difference in time-lag prior to feeding has had any effect on the outcome of this study, it would

be optimal if these conditions could also be set to fit across the entire group of animals being studied.

Thirdly, from the detail breakdown of results in the stereological analyses, it was evident that biological variation constituted for the majority of the variations within the data collected. This problem is generally addressed by incorporation a higher number of animals in study to smooth out the biological variations that naturally exist between animal subjects, which could not be carried out in this study. Also, estimation into the total hippocampal volume using the Cavalieri principle would provide extra information on the morphological changes brought about by prenatal nutritional influences and postnatal exercise. This information could be used to validate the effects of VEGF on the process of neurogenesis, as one would expect an increase in angiogenesis which eventually leads to an increase in total hippocampal volume.

Lastly molecular analyses on spinophilin and the main neurotrophic factor mRNA expression analyses would also likely to benefit if a larger number of animals were available for the investigations to strengthen the findings we observed. In extension, if permitted with more time and funding, mRNA expression analyses should be performed on the transiently acting neurotrophic factors immediately after exercise has taken place to clarify the differences it makes. This would generate a clearer picture to the exercise-induced pathways that leads to an increase of neurogenesis.

6.3 Future Study directions

This study demonstrated the adverse effects of global maternal undernourishment on the process of hippocampal neurogenesis, and in contrast, the beneficial effects of a low dose routine exercise postnatally. However as the molecular analyse conducted were purely based on tissue collected at the endpoint of the study, the effects of maternal undernutrition on the expression of key neurotrophic factors during the actual stages of gestation would require future studies to investigate.

It was interesting to find that even at a low dose of 56m per exercise period per day, this regime was enough to trigger the process of increased neurogenesis in the exercise groups. Thus an initial extension to this would be to investigate the minimum level of exercise required on a routine basis in order to stimulate this phenomenon of increased neurogenesis in the hippocampal region, and / or to look investigate whether the seemingly lack of neurogenesis in the UNX group is due to the offsprings of undernourished dams during gestation has an innately higher exercise threshold that needs to be reached before similar scales of neurogenesis can occur.

Also, as mentioned earlier on in this thesis, past studies of our group had demonstrated the preference of UN offsprings to exercise over feeding when given a choice. It would therefore be beneficial to approach a study design that would investigate on exactly how much extra exercise is needed for the UN offsprings to achieve the same amplitude of neurogenesis as their AD counterparts, or to determine whether or not this increase in exercise actually helps at all in recovering the deficits suffered by these UN offsprings during gestation.

Another interesting aspect to look relates to the food intake data gathered from this study. Towards the end of the study, the UN and UNX group demonstrated a higher intake in food per total body weight. By referring to the data from past studies, this may be interpreted as a sign of the onset of adult obesity; due to the nature of this study, we did not reach the stage for these symptoms to manifest. Thus this would also fuel a separate possible future study direction, by assessing the magnitude of neurogenesis at different age points of the animal subjects, comparing the influences of maternal nutrition during gestation, and the effects of post natal exercise.

Other studies have reported in noticing a drastic reduction in neurogenesis when comparing adolescent and adult rats (He J. et al., 2007). The endpoint of our study is roughly the equivalent of a young adulthood in terms of average rat life-spans, and all subsequent morphological and molecular work were conducted based on samples collected at one time-point. It would be worth looking into the different stages of neurogenesis by allowing for tissue sampling at different time points to gain better insight to the process of postnatal neurogenesis.

In a wider spectrum, while exercise has been shown in this study and many others to lead to an increase in neurogenesis and synaptogenesis, the exact role of these newly generated neurons and synapses remains unclear (Leuner B. et al., 2006). As the hippocampus is well known for its role in the process of learning and memory formation, an investigation into the direct effects of prenatal nutritional manipulations and post natal exercise on the learning process would also be worthwhile.

Whilst experimental approaches such as the Morris water maze have been widely used to assess spatial learning (D'Hooge R. et al., 2001), unbiased methods such as the operant methodology (Davison M. et al., 2000) that allows for the quantification of the conceptual parameter of behavioural learning. It would obviously be challenging but also necessary to distinguish and validate these findings from the other behavioural analyses that result in controversial or un-interpretable data.

APPENDIXES

A1. Histological Analysis

1x PBS (in 1L)

Reagent	Volume
1.9 mM Sodium Dihydrous Phosphate Monohydrate	0.23 g
8.1 mM Di-Sodium Hydrogen Phosphate anhydrous	1.15 g
Sodium Chloride	8.75 g
MilliQ Water	1 L

Cryostat Section Storage Solution (in 1L)

Reagent	Volume
Glycerol	300 ml
Ethylene Glycol	300 ml
MilliQ	300 ml
PBS	100 ml

This solution is stored at -20ºC

4% PFA (500ml)

Reagent	Volume
Paraformaldehyde powder	20g
10M NaOH	2 ~ 3 drops
PBS	500 ml

This solution requires heating at 60ºC when dissolving PFA powder.

Thereafter stored at 4ºC after filtering (Whatman, 3MM)

0.1% Cresyl Violet / 0.01% Thionin Stain (in 1L)

Reagent	Volume
Cresyl Violet	1 g
Thionin	0.1 g
PBS	1 L

10% Sucrose (with 0.01% NaN_3) in PBS

Reagent	Volume
Sucrose	10 g
NaN_3	10 mg
PBS	100 ml

This solution is stored at 4ºC

Apathy Mounting Medium

Reagent	Volume
MilliQ Water	150 ml
Sucrose	50 g
Gum Arabic	50 g
Potassium Acetate	50 g
NaCl	10 g
Thymol	0.1 g

This solution is made fresh for usage and remains in good quality for 2 month.

A2. Western Blotting

RIPA Lysis Buffer (in a final volume of 1L at pH 8.0)

Reagent	Volume
TritonX-100	10 ml
Sodium Dodecyl Sulphate	1 g
Sodium Chloride	9.77 g
Tris HCl	2.42 g
Deoxycholatic Acid	5 g
Sodium Orthovanadate	50 µl in 50ml

This solution is stored at 4ºC

Laemmli's Loading Buffer (10x SDS Buffer)

Reagent	Volume
20% Sodium Dodecyl Sulphate	6.25 ml
Dithiothreitol	770 mg
Glycerol	1.75 ml
2.5M Tris pH 6.8	1.2 ml
0.5M EDTA	0.8 ml
Bromophenol Blue	Adjust till dark blue

This solution is stored at -20ºC

PBS-Tween (in 1L)

Reagent	Volume
1x PBS	1 L
TWEEN -20	2 ml

Blocking Buffer (5x)

Reagent	Volume
Dry fat free milk	5 g
PBS-Tween	100 ml

This solution is made fresh on the day of the Western Blot

5x Running Buffer (In a final volume of 1L at pH of 8.3 – 8.6)

Reagent	Volume
Tris HCl	14.5 g
Glycine	72 g
Sodium Dodecyl Sulphate	5 g

Transfer Buffer (In a final volume of 2L)

Reagent	Volume
2.5mM Tris HCl	6.06 g
192mM Glycine	28.8 g
100% Methanol	400 ml
MilliQ Water	1600 ml

This solution is made fresh on the day of the Western Blot

8% Acrylamide Running Gel (per 2 gels)

Reagent	Volume
MilliQ Water	4.6 ml
30% Acrylamide Mix	2.7 ml
1.5M Tris HCl pH 8.8	2.5 ml
10% Sodium Dodecyl Sulphate	100 µl
10% Ammonia Persulphate	100 µl
N,N,N',N'-Tetramethylethylenediamine	6 µl

This solution is made fresh on the day of the Western Blot

5% Stacking Gel (per 2 gels)

Reagent	Volume
MilliQ Water	2.7 ml
30% Acrylamide Mix	0.67 ml
1.0M Tris HCl pH 6.8	0.5 ml
10% Sodium Dodecyl Sulphate	40 µl
10% Ammonia Persulphate	40 µl
N,N,N',N'-Tetramethylethylenediamine	4 µl

This solution is made fresh on the day of the Western Blot

10x Blocking Buffer

Reagent	Volume
Dry fat free milk	10 g
PBS-Tween	100 ml

Primary Antibody @ 1:500 (2 ml per membrane)

Reagent	Volume
Rabbit polyclonal Anti-Spinophilin IgG (Upstate Biotechnology - #06-852)	10 µl
5x Blocking Buffer	2 ml

This solution is made fresh on the day of the Western Blot

Secondary Antibody @ 1:10000 (20 ml per membrane)

Reagent	Volume
Donkey Anti-Rabbit (Santa Cruz Biotechnology – KO504)	2 µl
5x Blocking Buffer	20 ml

This solution is made fresh on the day when required.

A3. RT-PCR

6x DNA loading dye

Reagent	Volume
100% Glycerol	15 ml
1x TAE Buffer	35 ml
Bromophenol blue	Adjust till dark blue

100x TAE Buffer (final volume 500ml)

Reagent	Volume
0.1M EDTA	18.6 g
4M Tris	242 g
Acetic Acid	57 ml
MilliQ Water	Top up to 500ml

1.8% Agarose Gel

Reagent	Volume
1x TAE Buffer	100 ml
Agarose (GibcoBRL)	1.8g
Ethidium Bromide	0.1µl

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