2009

INDUCED HYPER AND HYPO AMINOACIDEMIA IN THE NEAR TERM OVINE FETUS: EFFECTS ON ELECTROCORTICAL ACTIVITY AND CEREBRAL PROTEIN SYNTHESIS

James Nakahara MacLachlan

INDUCED HYPER AND HYPO AMINOACIDEMIA IN THE NEAR TERM OVINE FETUS: EFFECTS ON ELECTROCORTICAL ACTIVITY AND CEREBRAL PROTEIN SYNTHESIS

(Spine Title: Amino Acids: Fetal Cerebral Metabolism & Protein Synthesis)

(Thesis Format: Integrated Article)

by

James Nakahara MacLachlan

Department of Physiology and Pharmacology

Submitted in partial fulfillment
of the requirements for the degree of
Master of Science

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario

ABSTRACT

We have utilized an amino acid infusate and an insulin euglycemic clamp technique in the ovine fetus near term with increases and decreases in circulating amino acids, respectively, to determine the impact on electrocortical activity and cerebral protein synthesis. Fetal sheep were studied over a 2 hour control and subsequent 6 hour experimental period, during which animals received an infusion of Primene® 10%, or a co-infusion of insulin and 10% dextrose, as well as C^{13} leucine for determining cerebral protein synthesis. With Primene infusion, basic and neutral amino acids were increased, while acidic amino acids showed little change. With insulin/dextrose infusion, basic and neutral amino acids were decreased, while acidic amino acids were again little changed. Despite changes in circulating amino acids, there was no effect upon fetal electrocortical activity or cerebral protein synthesis for either group, suggesting protective mechanisms for the fetal brain during periods of increased/decreased nutritional availability.

Keywords: fetal sheep, electrocortical activity, amino acids, insulin, cerebral protein synthesis

CO-AUTHORSHIP

The following people contributed to the manuscripts contained within this thesis in the following ways:

Dr. B Richardson:	Supervisor throughout all projects, provided grant funding to complete manuscripts, edited manuscripts
Dr. N Smith:	Supervised the development and troubleshooting of plasma and brain tissue leucine analysis using GC/MS, input into data analysis interpretation, edited manuscripts
Mr. J McCallum:	Provided technical support with troubleshooting plasma and brain tissue leucine analysis using GC/MS, input into data analysis interpretation
Mr. B Matushewski:	Provided technical assistance during all surgeries and experiments
Ms. S Hemstreet:	Provided technical assistance during all surgeries and experiments
Mr. J Homan	Provided technical assistance during all surgeries and experiments

For my family

ACKNOWLEDGEMENTS

I would like to take this moment to acknowledge the following people for their continued love, support, friendship and guidance:

To my supervisor Dr. Bryan Richardson, who has been an incredible role model and mentor throughout my studies. More than anything your leadership and patience has allowed me to succeed where others thought it impossible. For this and more, thank you.

To Dr. Norman Smith, without whom I could never have overcome the hurdles of GC/MS troubleshooting. You reminded me about the importance of first principles and I thank you for your guidance and friendship.

To my advisory committee: Dr. Victor Han, Dr. Norman Smith, Dr. Stan Leung, and Dr. Andy Watson. You have continued to support me over the extended course of my studies, and I am grateful for your combined wisdom and assistance.

To the members of the Perinatal Research Lab, especially Brad Matushewski, Jac Homan, Shannon Hemstreet and Maria Sinacori for their technical support and continued friendship.

To Dr. Tony Rupar, Mr. Martin Evers, Ina Brooks, Cindy Iacobelli and Delfina Mazzuca-Siroen for their countless hours of assistance at the bench top in their respective fields of expertise.

On a more personal note, I would like to say thank you...

To (now Doctor) Marie Czikk, my faithful sidekick (or maybe I was hers?) in the Protein Power Plan, who has since moved on to fulfill the dreams I always knew she would achieve. I am proud of you and count myself lucky to call you my friend.

To Jeremy McCallum, one of the most genuine and hardworking people I have had the good fortune to meet. Those long late nights hard at work would not have been any fun without you suffering there beside me. I have such great memories of our times both inside and out of the lab. You have always been a true friend and I look forward to the many years to come.

And finally to my family; there are no words to describe how much strength and comfort I have drawn from your love and support everyday. To my wife Morgan who has kept me sane and motivated me to achieve my potential, I love you with all my heart. To my father, my first science teacher and role model, who taught me about first principles and the value of hard work. To my mother, whose unconditional love and seemingly endless patience give me the strength to strive for greatness without fear of failure. I would not have finished this work without the continued love and support of my parents. To Gene, Phillip and Leanne who I think about everyday and miss more than words can say. And to my daughter Emma, may my efforts inspire you as you have inspired me. I dedicate this work to you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

~ – approximately

Δ – change

[^{13}C]-leucine – L-[1-^{13}C]-leucine

[^{13}C]-KIC – L-[1-^{13}C]-ketoisocaproic acid

[^{14}C]-leucine – L-[1-^{14}C]-leucine

2D – two dimensional

5-HT – 5-Hydroxytryptophan

α-KIC – alpha-ketoisocaproic acid

a – arterial

AA – amino acid

[AA_{art}] – arterial amino acid concentration

AEC – amino ethyl cystine

APE – atom percent excess

ATP – adenosine triphosphate

A-V – arterial-venous

BBB – blood brain barrier

C-14 – ^{14}C-leucine

CaO_2 – arterial oxygen content

CSF – cerebrospinal fluid

CNS – central nervous system

CV – coefficient of variation

DNA – deoxyribonucleic acid

DR_{leu} – disposal rate of leucine

ECOG – electrocorticogram

EEG – electroencephalogram

EMG – electromyogram

EOG – electrooculogram

FBM – fetal breathing movements

FBP – fetal blood pressure

FHR – fetal heart rate

FSR – fractional synthetic rate

GC/MS – gas chromatography/mass spectrometry

GLN – glutamine

GLU – glutamate

HCl – hydrochloric acid

HPLC – high performance liquid chromatography

HV – high voltage

Hz – hertz

IF – intracellular free

IGF – insulin-like growth factor

IUGR – intrauterine growth restriction

$[Leu_{art}]$ – arterial leucine concentration

LV – low voltage

MAP – mean arterial pressure

mmHG – millimeters of mercury

MPE – mole percent excess

MPE_{IF} – intracellular free enrichment

MPE_P – plasma enrichment

MPE_{PB} – protein-bound enrichment

MTBSTFA – N-Methyl-N-*tert*-butyldimethylsilytrifluoroacetamide

N – normality

NH_4OH – ammonium hydroxide

NREM – non rapid eye movement

O_2 – oxygen

PB – protein bound

pCO2 – partial pressure of carbon dioxide

pO2 – partial pressure of oxygen

r^f – infusion rate of L-[1-^{13}C]-leucine

R_0 – natural ion abundance ratio

R_s – steady-state ion abundance ratio

REM – rapid eye movement

RNA – ribonucleic acid

SEM – standard error of the mean

SSA – sulfosalicylic acid

SWS – slow wave sleep

CHAPTER ONE

LITERATURE REVIEW

1.1 BEHAVIOURAL /SLEEP STATE DEVELOPMENT

1.1.1 Adult sleep states

Sleep states in the adult can be identified and categorized according to behavioural parameters and the corresponding electrophysiological activity. The electrophysiological activity exhibits temporal patterns that are stable as well as repeating themselves in time. Identification of sleep state is determined from three primary criteria, specifically neocortical electroencephalogram (EEG), eye movements and electromyographic activity in the chin or neck (indicative of postural tone). The criteria by which sleep states are classified in the adult were developed nearly 40 years ago by Rechtschaffen and Kales (1), with sleep being divided into 2 primary types: rapid eye movement (REM) and non-rapid eye movement (NREM) sleep. REM sleep is characterized as having desynchronized EEG with increased frequency, the presence of rapid eye movements and an absence of nuchal muscle tone (1). It can be further subdivided into tonic and phasic REM, whereby phasic REM is identified from the frequent eye and muscle movements that occur during this stage, in combination with additional phasic activity that occurs within the central nervous system (2). Whereas REM sleep exists in 2 stages, NREM sleep exists in 4 sub-stages (I-IV), with a progressive loss of consciousness and decrease in EEG frequency observed during the transition from stage I to stage IV, along with a concurrent increase in synchronization and amplitude of the EEG. As a result, stages III and IV are commonly referred to as slow wave sleep (SWS). A lack of eye movements and presence of nuchal muscle tone are also defining characteristics of NREM sleep activity. The development of the sleep/wake cycle has its origins in fetal and

neonatal behavioural states, with mature sleep/wake patterns (characterized by a decrease in REM sleep and an increase in the awake state to typical adult levels) becoming clearly evident in the human from 6 years of age and onwards (3).

1.1.2 Neonatal sleep states

Sleep states in the human neonate are most often used in the assessment of neurological development (4). Although there are several ways to describe neonatal sleep states, the guidelines set out by Prechtl are the standard for neonatal sleep classification (5). Sleep states in the neonate are divided into 5 different classifications, with each one having a specific set of physiological characteristics that display a temporal link to one another. State 1 is identified as quiet sleep, which is akin to deep sleep in the adult, during which neonatal characteristics include closed eyes, normal respiration and an absence of body movement. Closed eyes with irregular respiration and the presence of infrequent body movements are typical of state 2. State 2 is often referred to as active sleep, and is akin to REM sleep in the adult. States 3-5 are all periods of wakefulness, where the neonate has its eyes open. State 3 is characterized as having few body movements, state 4 with an increase in body movements and state 5 having both frequent body movements as well as crying (5). The identification of neonatal sleep states utilizes behavioural activity to determine sleep state, thus neonatal sleep states are often referred to as behavioural states. Similar behavioural states have also been defined for the human fetus.

1.1.3 Fetal behavioural states

Fetal behavioural states bear similarities to adult and neonatal sleep states, and have been defined as the clustering of behavioural and physiological parameters that have a close temporal relationship and repeat themselves in time (4). The development of this coordinated behavioural activity before birth can be observed in those species classified as prenatal (i.e. ovine fetus) or perinatal (i.e. human fetus) brain developers, with the emergence of behavioural state activity occurring in parallel with the period of rapid neuronal growth and maturation (6). Following the criteria set out for the human neonate by Prechtl in 1974, Nijhuis developed a means to classify human fetal behavioural state activity (7). Human fetal behavioural states are classified as States 1F to 4F, and are observed by monitoring the fetus simultaneously with ultrasound and heart rate recordings. State 1F in the fetus is akin to state 1 in the neonate, with stable fetal heart rate and an absence of eye movements. State 2F is similar to state 2 in the neonate, with the presence of rapid eye movements, heart rate accelerations and frequent small body movements. States 3F and 4F are described as periods of wakefulness, with increased eye movements in 3F, and eye movements with the presence of large body movements in state 4F (7).

Since the development and emergence of behavioural state activity is similar in the human and ovine fetus, the ovine fetus has been established as the primary animal model for the study of fetal brain development and behavioural state (6), and this is the reason why I chose to use the ovine fetal model for my study. The ovine model allows for more invasive monitoring and manipulation of

the fetal environment, thus providing more accurate and specific measurement of the parameters that are used to define behavioural state activity. Behavioural states can be measured in the ovine fetus using polygraph recordings of electrocortical (ECOG) activity from the parietal cortex, electroocular (EOG) activity from the lateral canthus of the eye and electromyographic (EMG) activity from the nuchal (neck) muscle (4). From these measurements, we are able to observe three distinct states of behavioural activity. The first state of activity is characterized by low voltage (LV), high frequency ECOG activity, the presence of rapid eye movements (REM) and the absence of nuchal muscle tone, and is known as the LV/REM state. The LV/REM state is akin to REM sleep in the adult animal. The second state, known as high voltage (HV)/NREM, is akin to NREM sleep in the adult, and is characterized as having low frequency, high amplitude ECOG activity, an absence of eye movements and the presence of nuchal muscle tone. The third state of activity is comparable to postnatal wakefulness, and typically includes low voltage ECOG activity; the presence or absence of eye movements and nuchal muscle tone is present. At times it is difficult to assess the wakefulness of the ovine fetus, and as such this activity may be then defined as an indeterminate state of activity. Similarly, electrocortical activity observed during state transitions, as well as during short non-sustained epochs of LV and HV ECOG activity may also be defined as indeterminate activity. Once behavioural states are clearly delineated in the ovine fetus, it spends the majority of its time (>90%) in either the LV/REM or HV/NREM state of activity (8).

In addition to the 3 primary criteria for identifying behavioural state in the ovine fetus, other parameters are associated with behavioural state activity. One particular event frequently associated with behavioural state activity is the occurrence of fetal breathing movements (FBM), which are critical for the normal growth and development of the respiratory system in utero (9). Clearly delineated behavioural states are evident by ~120 days of gestation (term = 145 days) at which time an association is apparent between LV/REM state activity and FBM (9), since fetal breathing occurs ~30% of the time and almost exclusively during the LV/REM state, in contrast to the periods of apnea observed during HV/NREM (10). Nonetheless, due to the non-continuous nature of FBM, they cannot be used exclusively to identify behavioural state. However, the use of ECoG, EOG, nuchal EMG and FBM allows for clear identification and definition of behavioural state activity. While ECoG chart recordings can be utilized to monitor duration of high voltage and low voltage epochs, it cannot be used exclusively to determine behavioural state expression since low voltage expression is characteristic of both LV/REM as well as the awake state.

1.1.4 Ontogeny of behavioural/sleep state development

The emergence of electrocortical activity in selected areas of the ovine fetal cortex has been observed as early as 65 days of gestation (0.45 term), and over the following 2 weeks this activity spreads throughout the cortex and occurs continually throughout the remainder of the gestational period (11). Thereafter, there is a gradual delineation of the 2 primary types of electrocortical activity which will eventually be responsible for the determination and classification of

their respective behavioural state. The LV/REM state is characterized as having electrical activity <50uV in amplitude with a frequency range of ~10-20 Hz, while the electrical activity in the HV/NREM state is typified as having a larger amplitude (>100uV) but a lower frequency range (~3-12 Hz).

The appearance of fetal breathing movements occurs early in development (~40 days gestation) and occurs frequently during this early period (9). Fetal eye movements and nuchal muscle activity are in constant occurrence when measured at 95 days of gestation (10). A temporal association amongst these parameters is apparent by 105 days gestation, with initial expression of eye movements, breathing movements and nuchal tone occurring together. As gestation progresses, these associations are modified, such that postural muscle activity is suppressed during LV ECOG activity when the eye and diaphragm muscles are active (10, 12).

A temporal relationship between electrocortical activity and these behavioural parameters becomes apparent in the developing animal around 120 days of gestation (0.8 term), and as such this period is often regarded as the time at which organized behavioural state patterns begin to emerge (8). Initially, there is a larger proportion of the time spent in the low voltage REM state (50 per cent), with roughly 40 per cent of the time spent in the high voltage, NREM state. Throughout the later stages of development, there is a steady decline in the proportion of REM state activity, with REM accounting for only 40 percent of the behavioural activity at term, due to both an increase in NREM activity and an

increase in the awake state. Postnatally, this trend continues with REM incidence decreasing further in favor of increased wakefulness (4).

In addition to the ovine fetus, the guinea pig and monkey are classified as prenatal brain developers, having undergone the majority of their neuroanatomical development prior to birth and subsequently exhibiting well developed behavioural states near term (4, 13). On the contrary, animals such as the cat, rat, dog and rabbit which experience their brain growth spurt during the postnatal period and do not have well developed behavioural states until after birth are classified as postnatal brain developers (4, 14).

Comparatively, body movements in the human fetus are apparent by 8 weeks, breathing movements by 10-12 weeks and eye movements by 16-17 weeks gestation (15). The human fetus is classified as a perinatal brain developer, and as such undergoes the majority of its neuroanatomical development during the perinatal period. Accordingly, clearly delineated behavioural states become evident during the perinatal period, emerging around ~35-36 weeks, at which time the previously unsynchronized behavioural state parameters occurring during both periods of activity and quiescence begin to demonstrate temporal stability and coordinated transitions (4). Similar to the ovine fetus, the human fetus exhibits an early prominence of REM state activity (state 2F) which is present ~40% of the time, with NREM (state 1F) activity occurring ~25% of the time and brief periods of wakefulness (state 4F). As gestation progresses, the period of time spent in states 2F and 4F continue to increase while expression of 1F state activity remains unchanged (4).

1.1.5 Function of behavioural/sleep state development

1.1.5.i Function of sleep in adults

The purpose of sleep in the adult has been extensively studied both in terms of REM and NREM activity; however the majority of work in this area has focused on the function of REM sleep. Many theories exist, with one of the most well studied being the role that REM sleep may play in learning and memory. Studies have shown that regions of the human brain involved in the learning of new tasks are more active during REM sleep than those same areas in untrained individuals (16). The cycling of NREM and REM sleep activity has been suggested as the most effective mechanism of long-term memory maintenance (17), while REM sleep deprivation is associated with decreased performance on newly learned tasks in adult rats (18), further suggesting a role for REM sleep in the strengthening and consolidation of memories. The increased metabolic rate observed during REM sleep has also drawn attention to a potential thermoregulatory role in the adult, suggesting that as the temperature of the central nervous system (CNS) decreases during NREM activity, a transition to REM sleep is subsequently triggered, resulting in increased metabolic rate, thus increasing CNS temperature (19). While unique in their characteristics and activity, the deprivation of either REM or NREM sleep will result in a rebound effect, whereby the expression of the deprived state is increased during the recovery period (20). This would suggest that both REM and NREM expression are important to normal function and development.

The main hypothesis as to the function of NREM sleep is the restorative theory of sleep, suggesting that the decreased metabolic requirements observed during the NREM state promotes the restoration and repair of existing tissues while also supporting the growth and synthesis of new tissue (21). Cellular energy requirements may be in direct competition (and balance) with synthetic activities for available energy in the form of ATP. As such, the lower cellular energy requirements and decreased motor activity of the NREM state would therefore allow for the increased allocation of energy to increased synthesis.

1.1.5.ii <u>Function of behavioural/sleep state in early development</u>

Behavioural/sleep state expression can be used as an indicator of well being and normal brain development in the fetus and neonate. The emergence of clearly delineated behavioural states is delayed in the growth restricted fetus, and is an indicator of delayed or impaired brain development (4, 22). More acute brain insults can be identified through changes in behavioural state expression as well. The decrease in REM state activity following short term hypoxic episodes in the ovine fetus is an example of altered brain function (23), and if sustained over time could contribute to impaired neurological growth and function. The decrease in REM may also reflect a protective action by the fetus to decrease activity of behavioural state with high metabolic demands in order to conserve energy expenditure. The increased prevalence of the REM state during early development, including that prior to birth, led Roffwarg (22) to hypothesize that it may in fact play a developmental role for the fetus by supplying an endogenous source of stimulation in an environment relatively devoid of such. The lack of

exogenous stimulation in utero and shortly after birth would be compensated for by the increased REM expression (4), therefore providing the necessary stimuli for proper neuronal development.

The early prominence of the REM sleep state has raised many questions as to its potential role in fetal and neonatal development. The selective deprivation of REM, through both pharmacological and behavioural means, has been utilized in various studies in the hopes of elucidating its importance in neuronal development. A study by Mirmiran (24) used clonidine to suppress REM activity in newborn rats in order to compare their neural development with control animals. When both groups are exposed to an enriched environment, the normal rats show an increase in cortical size while the REM deprived rats display no change in brain size. This evidence supports a role for REM sleep in brain growth, as well as serving to increase its adaptability to environmental stimuli. Another study utilized the combined effects of impaired REM sleep and monocular deprivation in kittens to elucidate the role that sleep may play in neuronal development within the feline visual system, specifically the lateral geniculate nucleus or LGN (25). Monocular deprivation alone impairs development of the LGN due to the decreased optical stimulus, and when combined with REM sleep deprivation, the resulting damage is further amplified. Animals attempt to compensate for the decrease in REM with elevated periods of wakefulness, but this did not compensate for the increased damage observed to the LGN. The linkage between anatomical brain development and neurophysiological development, as observed between both prenatal and

postnatal brain developing species, supports a role for the REM state in early brain development.

1.1.6 Control of Behavioural/Sleep States

The mechanisms that control behavioural state expression have not yet been determined, although many possibilities exist. Changes in behavioural state expression are linked to changes in protein synthesis in the adult animal. In studies of the adult rat and cat brain, a positive correlation exists between cerebral protein synthesis and REM sleep (26, 27). However, more recent evidence suggests that the cerebral protein synthetic rate is more closely linked to NREM state expression in the adult rat (28) and monkey (29). While these results might appear to conflict with one another, there is evidence suggesting a link between REM and NREM expression in rats, whereby NREM sleep duration is positively correlated with the length of previous REM-sleep episodes (30). This theory would add support to the restorative theory of sleep, whereby increased expression of the metabolically active REM state may trigger a transition to the NREM state, with the reduced energy requirement and decreased activity then allowing for increased provision of energy for synthetic processes.

Neurotransmitter expression is also linked to the control of behavioural states, specifically circulating concentrations of serotonin in the fetal brain. Low voltage electrocortical expression is decreased and a reduction in fetal breathing movements is observed in the ovine fetus following maternal hypoglycemia (31). These changes in electrocortical expression may be due in part to changes in brain serotonin concentrations resulting from decreases in amino acid precursors

for serotonin synthesis. Similar changes in low voltage REM-sleep expression are observed in rats fed a tryptophan-free amino acid diet (32). When fed a modified formula containing tryptophan, human neonates enter quiet (NREM) sleep and active (REM) sleep sooner than infants not receiving tryptophan, and spend more time in REM sleep and less time awake (33). These studies suggest that both increased (hyper) and decreased (hypo) amino acid availability will impact upon neurotransmitter synthesis, ultimately affecting behavioural/sleep state expression.

Many theories exist as to the purpose, function and control of sleep, but as of yet there has been no definitive work that clearly elucidates a role for sleep and behavioural state in terms of growth and development. Undoubtedly the function of sleep states is constantly changing throughout the various stages of development, and research is required to identify their role in fetal growth.

1.2 CEREBRAL PROTEIN SYNTHESIS

1.2.1 Brain growth and development

The fetal and neonatal period is a time of rapid growth and development in the brain. The majority of studies in fetal brain metabolism have been carried out in the ovine fetal model, with postnatal studies in humans and rats. The sheep is relatively mature from a neuroanatomical and physiological standpoint at birth, having undergone the majority of its development prenatally, thus it is classified as a prenatal brain developer. Other animals classified as prenatal developers include the monkey and guinea pig. Perinatal brain developers, such as the pig

and human, undergo the majority of their brain growth in the period surrounding birth, while animals such as the dog, cat and rat are classified as postnatal brain developers as they experience their brain growth spurt postnatally (13, 34). The ovine fetal brain growth spurt is biphasic, with the first major period of growth occurring around 40-80 days gestation, representing an increase in neurogenesis characterized by an increase in DNA synthesis and an increase in brain weight. The second phase of the growth spurt occurs later in gestation, around 95-130 days, during which glial proliferation and myelination occurs and cells increase in size and protein content (13).

1.2.2 Cerebral substrate metabolism

Fetal cerebral metabolism has been studied in the ovine fetus using Fick methodology to monitor changes in substrate uptake and utilization by the brain, which states that uptake of a substrate across an organ (in this case the brain) is related to the arterial-venous difference in substrate concentration across that organ. Measurements of oxygen, glucose, lactate and amino acids uptake across the brain can be determined using this method (31, 35-39).

From the standpoint of the brain, glucose uptake can account for 100% of the aerobic substrate requirements for cerebral metabolism in the ovine fetus (39, 40). This is measured by determining the arterial-venous (A-V) differences in glucose and oxygen concentrations across the brain, and then calculating the glucose/oxygen quotient using the following equation:

$$\text{Glucose}/O_2 \text{ quotient} = \frac{6 \times \Delta \text{ glucose (mM)}}{\Delta\ O_2 \text{ (mM)}}$$

The glucose/oxygen quotient is the fraction of oxygen consumed by the fetus required to completely oxidize the glucose consumed by the fetus (41). With a cerebral glucose/oxygen quotient of ~1, glucose can account for all cerebral oxidative metabolism in the ovine fetus. Using 2-deoxyglucose autoradiography to monitor cerebral glucose utilization, it was discovered that over the last 7 weeks of gestation a threefold increase occurs, with an additional 50% increase during the early neonatal period (42). This is a direct reflection of the increased metabolic demands of the fetal brain during this critical period of development.

Isotopically labeled amino acids can also be used to measure fetal metabolism in both the whole body and the brain. Initial studies of amino acid metabolism utilized radiolabeled tracer molecules; however more recent studies have demonstrated the safety, reliability and accuracy of using stable isotope tracer molecules in the measurement of amino acid concentration in fetal plasma (43, 44), as well as whole body protein synthesis in the adult sheep (45, 46) and muscle protein synthesis in the dog (47) . The use of steady state infusion of radiolabeled tracer molecules has been utilized to measure amino acid incorporation into cerebral proteins during the period of the brain growth spurt in the ovine fetus (36, 48). By quantifying tracer incorporation into cerebral proteins, the tissue fractional synthetic rate (FSR), which is the percentage of total brain protein newly synthesized per day, can be calculated. The ovine fetus has a high cerebral FSR (~20%/day), and cerebral leucine uptake in these studies was found to be two to three fold greater than rates observed in the adult

sheep (49), further demonstrating the elevated metabolic requirements of the developing brain.

1.2.3 Neonatal/fetal cerebral protein synthesis

Protein synthesis is a measurement of the movement of amino acids into protein, and can be defined as the unidirectional flux of amino acids into protein, while protein breakdown is the process whereby amino acids are liberated with protein remodeling. The accretion of protein by the brain is the difference between protein synthesis (the production of new proteins) and protein breakdown (the degradation of existing proteins) (50). The fetal and early neonatal period of rapid cerebral growth and development, commonly referred to as the brain growth spurt (13) is characterized by a constant remodeling of the brain involving high rates of protein synthesis coupled with high rates of protein degradation. Brain protein synthesis has been studied from both a prenatal and postnatal standpoint in the sheep (36, 48, 51) and rat (52) respectively. In the ovine fetus near term, studies utilizing steady state infusion of tracer amino acids observed fractional protein synthetic rates for the brain of approximately 20%/day (36, 48). The rate of absolute synthesis in the fetal brain, which is the product of the brain weight and fractional synthetic rate, was close to 1g protein/day in both studies, which is in sharp contrast to rates of cerebral protein accretion calculated from data published by McIntosh (13) for the adult sheep (~24mg/day).

These high rates of protein synthesis and breakdown are critical to the maturation of the brain (52) and require a continual supply of energy and nutrients to ensure proper development. As a result, the fetus may be more

susceptible to insults that affect its ability to effectively synthesize or breakdown proteins, since protein synthesis is an energy requiring process that can be down regulated when energy supplies become limited. While there have been few studies of the fetal brain, some evidence does exist with regards to whole body response to nutrient deficit as well as for postnatal brain development. Prolonged periods of maternal nutrient deprivation in sheep results in decreases in fetal glucose, leucine and insulin concentrations, with an increase in fetal protein breakdown (53). This in turn affects protein accretion in the fetus, ultimately resulting in a decrease of newly synthesized protein and decreased fetal growth. Similarly, induced dietary protein restriction early in pregnancy decreases fetal amino acid supply in the rat, ultimately decreasing brain growth within the resulting offspring (54). Nutrient precursor availability to the fetus, specifically amino acids, during this period of rapid brain growth and constant remodeling is of critical importance for the maintenance of normal brain development.

1.2.4 Developmental changes in cerebral protein synthesis

Studies of developmental changes in protein synthesis have been carried out prenatally using the fetal sheep brain and postnatally in the rat. The rate of leucine incorporation into brain protein of the ovine fetus has been measured over the last quarter of pregnancy using leucine autoradiography, and increases steadily from ~118 days gestation until birth (~145 days). The rate of incorporation varies somewhat depending on the tissue type, with the highest rates observed in the pineal body, brain stem and hypothalamic nuclei, and an overall average rate of leucine incorporation into brain protein of ~5nmol/g/min

(51). The observed increases in protein synthesis over this period were accompanied by high turnover of the leucyl-tRNA pool, which is indicative of increased protein degradation. Similar studies in the postnatal rat by Dunlop (55) determined that while protein is synthesized at a rapid rate by the brains of these animals, there is an almost equally high rate of protein breakdown, resulting in a much smaller accretion of protein than one might anticipate based on such high rates of synthesis. These high rates of cerebral protein synthesis increase from birth until 10 days postnatally, at which point they decrease to adult levels by approximately 60 days postnatal (52, 56). The level of protein breakdown in the rat brain is highest at birth, and decreases over time until it becomes equal to rates of synthesis by 30 days postnatally (55). It is apparent that the brain undergoes constant remodeling, with recently synthesized proteins having a shorter life span. It is possible these short-lived proteins are more active in the generation of transient structures (i.e. synapses), and that their labile nature allows the growing brain to rapidly change structured proteins by breaking them down in favour of newly required proteins. Studies in the neocortex of the macaque have shown that during the perinatal period surrounding birth, upwards of 40 000 new synapses are formed every second in the striate cortex (57), reflecting the rapid rates of protein synthesis ongoing in the developing brain during the period of the brain growth spurt. Identifying proteins with high turnover rates during the period of the brain growth spurt would allow us to identify those proteins critical to early neurodevelopment. A recent study utilizing 2-D gel electrophoresis discovered that 22 proteins were expressed in higher amounts in

the neonatal rat brain when compared with protein expression in the brains of adult animals (58). Some of the elevated neonatal proteins, such as dihydropyrimidinase-related protein 2, are likely involved in neuronal migration and may play a role in neuronal repair in the adult.

1.2.5 Behavioural/sleep states and cerebral protein synthesis

The emergence of clearly delineated behavioural states occurs around 120 days gestation in the ovine fetus (8) and parallels the period of the brain growth spurt. The early prominence of the REM state as well as the increased metabolic rate of the brain observed during the REM period led to further investigation into the role REM activity might play in relation to cerebral protein synthesis. The use of pharmacological agents to decrease REM activity in rat pups (59) results in impaired neurodevelopment and decreased brain size, indicating that REM deprivation may directly alter protein synthetic rates. Additionally, the use of cycloheximide or chloramphenicol to block protein synthesis in cats (60, 61) results in decreased REM expression, supporting a link between REM activity and protein synthesis.

While REM expression may impact upon cerebral protein synthesis, more recent studies have discovered a positive correlation between protein synthetic rates and NREM activity. Studying the adult rat, Ramm and Smith (28) determined that leucine incorporation into brain tissue is higher during SWS as compared to REM or wakefulness. Similarly in the adult monkey, protein synthesis is increased during deep sleep as compared to rates observed when the animal is awake or in a period of light sleep (29). Recent studies in the ovine

fetus utilizing radiolabeled C-14 Leucine tracer technology have demonstrated that leucine uptake is increased during NREM periods of activity when compared with uptake during REM activity (36). This implies that the decreased metabolic demands of the NREM period are conducive to cerebral protein synthesis due to the increased availability of energy resources, thus supporting the restorative theory of sleep as proposed by Adam (21). With REM state activity providing endogenous stimulation for the fetal brain and protein synthesis being increased during NREM epochs, it is apparent that both REM and NREM activity are necessary for normal brain growth and development. Furthermore, it is likely that the two are reliant upon each other in their function, as suggested by linkage studies in the adult rat (30) and sheep fetus (62). The function of behavioural state activity has not yet been determined, but these recent findings reveal a strong correlation with cerebral protein synthesis, suggesting an important role for behavioural state expression in the growth and development of the fetal brain.

1.3 SUBSTRATE AVAILABILITY AND UTILIZATION

1.3.1 Neonatal/fetal substrate utilization

1.3.1.i Glucose

In the well fed animal, glucose is an important substrate for metabolism in the ovine fetus (63). The fetus derives its glucose supply from the mother, and as such is subject to changes in glucose concentration within the maternal circulation. Under normal conditions, the glucose concentration in the maternal circulation is higher than that within the fetus. This concentration gradient, in

conjunction with a facilitated diffusion across the placenta is the means by which glucose enters the fetal circulation (35, 63). Approximately half of the glucose taken up by the placenta is delivered to the fetus, since the placenta does utilize maternal glucose to fulfill its own energy requirements. Once reaching the fetus, glucose accounts for approximately half of the oxidative metabolism at the whole body level, with amino acids accounting for ~25% and lactate ~20% (35, 41, 64). The importance of glucose to fetal whole body metabolism becomes apparent when glucose levels are decreased either through maternal nutritional restriction or hyperinsulinemia. Chronic maternal dietary restriction resulting in fetal hypoglycemia decreases fetal body weight (65, 66), ultimately resulting in significant growth retardation of the fetus. This growth restriction occurs despite attempts by the fetus to maintain normal glucose concentrations by decreasing glucose uptake to less than 40% of normal fed values and increasing fetal gluconeogenesis (41).

From the standpoint of the brain, the glucose/oxygen quotient for the ovine fetus is ~1, suggesting that glucose can account for 100% of the aerobic substrate requirements for fetal cerebral metabolism (39, 40). However, previous studies in the ovine fetus have found that oxygen uptake by the ovine fetal brain during the HV/NREM state is higher than that accounted for by glucose oxidation alone, suggesting that alternative substrates such as lactate or amino acids are metabolized by the brain as well (67).

1.3.1.ii Amino acids

Amino acids are essential for fetal growth and metabolism, and are particularly important as precursor molecules for protein synthesis. In addition to their primary role, amino acids are utilized for other purposes including the regulation of hormone expression, as signaling molecules, and as energy sources for fetal metabolism (68). The importance of amino acids for proper fetal development is supported by the observations that amino acid levels are higher in the fetal circulation than maternal concentrations in both the human (69) and ovine fetus (68). Furthermore, the amino acid concentrations in the umbilical circulation of intrauterine growth restricted (IUGR) newborns are significantly reduced versus those measured in healthy neonates (70). Developmental changes in amino acid concentrations occur throughout gestation in fetal fluids, becoming higher as gestation proceeds (68) in both ovine fetal plasma and ovine allantoic fluid.

When considering the uptake and transport of amino acids from the maternal circulation to the fetus, the placenta plays a vital role. The placenta facilitates the transfer of neutral and basic amino acids from maternal to fetal circulation, but there is limited or no transport of acidic amino acids from the mother to the fetus. In fact there is a net uptake of glutamate and aspartate by the placenta from the fetus, indicating that the fetus is responsible for its own glutamate production (35). Nonetheless, the fetus acquires most amino acids from the maternal circulation via the placenta, and impaired placental amino acid transfer to the fetus will result in fetal growth restriction (71). Since the maternal

circulation is the primary source for the fetus to acquire amino acids, the effect of maternal nutrition on fetal nutrient supply has been studied in pregnant rats. A carbohydrate/fat meal produces an increase in tryptophan and serotonin concentrations in the fetal brain, while a casein diet will decrease tryptophan and serotonin in the brain (72). Furthermore, maternal protein restriction early in pregnancy decreases fetal amino acid supply, with detrimental effects on brain growth of the resulting offspring (54). Not only is there an acute retardation of fetal brain growth, but fetal undernutrition ultimately leads to chronic health problems, including the early onset of adult diseases such as coronary heart disease, stroke and non-insulin dependant diabetes (73, 74). Maternal protein restriction in the pregnant pig decreases fetal concentrations of most amino acids, impairs placental transfer of amino acids, and ultimately results in fetal growth retardation (75), demonstrating the cascade of events resulting from a deficiency in amino acid availability.

In addition to their role in tissue accretion, amino acids are key energy substrates for the fetus. During maternal fasting in the sheep the majority of amino acids in the fetal umbilical circulation either increase or remain unchanged in concentration, likely to be catabolized by the fetus due to decreased glucose concentrations (76). Measurements of amino acid metabolism in the ovine fetal hindlimb during periods of maternal fasting indicate that the fetus further adapts to the decreased glucose availability by liberating amino acids from protein stores so as to use them either directly as sources for oxidation or as precursors for hepatic gluconeogenesis (35, 77).

1.3.1.iii Insulin

Insulin is a hormone secreted by islet cells of the pancreas, and is regarded as one of the most important hormones regulating metabolism in humans and animals. Insulin performs many different roles at the whole body level including:

1.) Stimulation of lipogenesis and inhibition of lipolysis

2.) Inhibition of gluconeogenesis and glucose release from the liver

3.) Stimulation of uptake and utilization of glucose by peripheral tissues

4.) Stimulation of uptake and incorporation of amino acids into protein

5.) Inhibition of proteolysis (78)

Insulin is an important factor in fetal growth. Chronic fetal intrauterine growth restriction can arise as a result of pancreotomy, as observed in studies of human newborns having a non functional pancreas (79), and in the ovine fetus having undergone pancreas removal (80, 81). This growth restriction is thought to be caused by the resulting hypoinsulinemia.

In the human, maternal derived insulin crosses the placenta with the amount transferred from mother to fetus equivalent to 33% of the insulin utilized by the normal fetus at term (82). This amount is also equal to 33% of the insulin synthesized by the normal fetus at term. Insulin immunoreactive cells are observed as early as 40-45 days gestation in the ovine fetal pancreas (83). In the fetal sheep, plasma insulin concentrations increase in response to a glucose

infusion at 110 days gestation (84), where after the concentration of insulin in fetal plasma increases throughout gestation and into the neonatal period.

In addition to its effects on glucose metabolism (37, 41, 78, 81, 85, 86), insulin plays a role in amino acid utilization by the fetus and neonate. Insulin stimulates amino acid utilization in the ovine fetus (87, 88) and the neonatal pig (89), with this response to exogenous amino acids decreasing over the neonatal period (90).

Whether alone or in combination with glucose, insulin markedly increases amino acid uptake into peripheral tissues of adult sheep, while at the same time decreasing protein catabolism, thus increasing net protein accretion (78). Insulin infusion decreases intracellular free amino acid concentrations in liver, skeletal muscle and heart tissues, but not the brain of the ovine fetus (91). The decrease in amino acids in that study was accompanied by no change in protein synthesis in any of the tissues studied, however protein breakdown was reduced in skeletal and cardiac muscle. As such, there is an overall increase in protein accretion in these tissues, since insulin acts to reduce protein breakdown while having no measurable effect on protein synthesis. In sheep with reduced maternal substrate intake, hyperinsulinemia results in increased non-oxidative disposal of leucine, indicative of an increase in protein synthesis (92). No change in protein breakdown was observed in that study; therefore the constant availability of amino acids provides the fetus with the necessary precursor substrates to promote an increase in synthesis. However, in well fed sheep, insulin infusion does not affect fetal leucine oxidation. The importance of insulin as a regulator of

metabolism is apparent, but it is unclear as to whether insulin affects protein accretion directly by increasing/decreasing protein synthesis and breakdown or indirectly through changes in substrate availability. A study by Milley (93) in the ovine fetus examined the effect on the whole body of an insulin infusion either with co-infusion of leucine to maintain leucine euaminoacidemia, or without leucine infusion thus resulting in a hypoaminoacidemic environment. The hyperinsulinemic hypoaminoacidemic environment results in decreased protein synthesis and decreased protein breakdown, while the hyperinsulinemic euaminoacidemic condition produced a decrease in protein breakdown, but no change in protein synthesis as seen in the earlier study for skeletal and cardiac muscle. Thus, when amino acid levels are maintained, protein accretion in peripheral tissues is increased with insulin infusion through altered substrate availability and changes in fetal amino acid oxidation.

Insulin also controls substrate availability of amino acids. This subsequently alters protein synthesis, for which amino acids are a direct precursor. In the fasted rat, insulin lowers plasma concentrations of all amino acids through the facilitation of uptake into skeletal muscle, with the notable exception of tryptophan which increases in concentration in response to insulin in the rat (94). Amino acid uptake by the ovine fetus is similarly affected by insulin, whereby whole body uptake of amino acids is increased during hyperinsulinemia, independent of glucose concentration (88). The ovine fetal hind limb, which is representative of non-visceral tissues, increases uptake of the amino acids alanine, asparagine, glycine, isoleucine, methionine and tyrosine in response to

insulin infusion (95), further demonstrating the role of insulin in the control of amino acid availability.

The role that insulin may play in terms of fetal cerebral protein synthesis remains to be defined, but one must consider the possible role insulin could play either by acting directly upon cerebral cellular metabolism or indirectly by affecting substrate transport across the fetal blood brain barrier. It has long been thought that the brain is an insulin insensitive organ; however, studies suggest that insulin is in fact present in cerebrospinal fluid (CSF) of rats and baboons (96). There is evidence that CSF insulin concentrations affect feeding behavior and body weight while insulin itself may be a neuromodulator, indicating that insulin has a direct influence upon metabolism and neurotransmission within the brain (97). While the role of insulin in the fetal brain is unknown, these findings suggest insulin could regulate fetal cerebral metabolism either directly or indirectly.

1.3.2 Neonatal/Fetal substrate availability and utilization

The uptake of substrates from the umbilical circulation by the fetus has primarily one of two fates; fetal oxidative metabolism or fetal protein synthesis. While glucose is the primary substrate for oxidative metabolism in the fetus under normal conditions, when faced with periods of global nutrient restriction the fetus will respond by modifying its metabolic requirements, including altering the availability and utilization of substrates, in an effort to maintain normal metabolic activity. Reduction of dietary protein intake during pregnancy in the rat results in a decreased fetal amino acid supply, which in turn is detrimental to brain growth

during the neonatal period (54). Prolonged periods of maternal nutrient deprivation in sheep results in decreases in fetal glucose, leucine and insulin concentrations, with an increase in fetal protein breakdown (53). This serves to slow the rate of protein accretion in the fetus, ultimately resulting in a decrease in newly synthesized protein and decreased fetal growth. Like maternal fasting, insulin has been used to study the effects of nutrient deprivation, especially changes in glucose availability, upon fetal metabolism. Insulin infusion with hypoglycemia decreases amino acid concentrations in liver, skeletal muscle and heart tissues, but not the brain of the ovine fetus, with no observed effects on protein synthesis in any of the tissues studied (91).

The effect of hyperglycemia on ovine fetal metabolism further demonstrates the impact of circulating glucose concentration upon fetal metabolism. With the maternal/fetal glucose concentration gradient promoting glucose uptake by the fetus, it follows that an infusion of glucose to the fetus decreases fetal umbilical glucose uptake, while increasing fetal metabolic rate and insulin secretion (98). From the standpoint of the developing brain, glucose infusion (following hypoglycemia induced by maternal fasting) produces fetal hyperglycemia, and is characterized by increases in cerebral blood flow, oxygen consumption and glucose consumption (31). At the whole body level in the fetal sheep, glucose accounts for approximately 50% of requirements for aerobic metabolism, demonstrating that other substrates such as amino acids and lactate are important elements of fetal oxidative metabolism as well (41). In the ovine fetal brain however, while glucose would seem to account for 100% of cerebral

aerobic metabolism, oxygen uptake during HV/NREM activity is greater than that accounted for by glucose alone, suggesting that alternative substrates, such as lactate and amino acids, are utilized for aerobic metabolism by the brain as well (67).

The effect of increased amino acid availability, or hyperaminoacidemia, on protein synthesis in early fetal and neonatal development has been studied primarily in systemic tissues or at the whole body level. Protein synthesis can be directly affected by amino acid concentration as demonstrated in the neonatal pig, where an amino acid infusion increasing systemic amino acid levels increases protein synthesis in both skeletal and liver tissues (89). Of interest is that fact that while the skeletal muscle response is thought to be insulin mediated, the stimulation of protein synthesis in the liver is thought to be governed by amino acid concentration independent of insulin, indicating that protein synthesis is not uniformly regulated in all tissue types, and that availability of amino acids may directly influence rates of protein accretion. Studies in the ovine fetus have focused mainly on whole body protein synthesis, utilizing both an infusion of mixed amino acids as well as arginine alone to measure amino acid effects on protein synthesis (99, 100). Protein synthesis in both control and growth restricted fetuses, as measured from the whole body and hind limb, is increased with the mixed amino acid infusion while arginine alone inhibited protein synthesis, indicating that a mixed amino acid infusion is an effective way to stimulate protein accretion in the fetal animal (99).

While the effect of hyperaminoacidemia on fetal and neonatal protein synthesis is well studied in the whole body, there is very limited evidence as to what effect it may have upon rates of synthesis in the brain. A study in adult rats that elevated dietary leucine and norleucine showed an increase in plasma amino acid levels not only for leucine, but for other amino acids as well (101). As well, the increased plasma amino acids produced significantly increased rates of protein synthesis in adipose tissue, skeletal muscle and liver, with no measurable effect on protein synthesis in the brain. Similarly, the amino acid valine was infused into adult rats and protein synthesis was monitored. Protein synthesis was increased in the liver, while rates were unchanged in the brain, heart and skeletal muscle (102). The effects of hyperaminoacidemia upon cerebral protein synthetic rates in the developing fetus remain to be determined. With an increased cerebral metabolic rate during the prenatal period and increased uptake of amino acids versus adult sheep (36, 49), changes in the nutritional milieu are likely to have a greater impact upon cerebral metabolism in the ovine fetus than those observed in the adult animal.

Both maternal protein restriction and insulin infusion have been utilized to study the effects of decreased amino acid concentration on fetal metabolism. It is important to note that studies of nutritional deprivation involving insulin must control for resulting changes in glucose. The use of a concurrent euglycemic clamp to maintain normal glucose concentrations provides a method to study insulin's effects on protein synthesis while eliminating any potential role of hypoglycemia. Originally developed by DeFronzo et al. (103), this technique has

been used successfully to examine insulin's effects on the regulation of amino acids and protein metabolism/synthesis in both the neonate and the fetus. The hyperinsulinemic-euglycemic clamp technique is employed in the ovine fetus to maintain normal glucose concentrations while increasing circulating insulin levels (95). Use of the hyperinsulinemic euglycemic clamp technique in newborn pigs has demonstrated the role that insulin plays in increasing uptake of amino acids into peripheral tissues, as well as directly increasing skeletal muscle protein synthetic rates (89). Similar studies in the ovine fetus have determined that hyperinsulinemia decreases fetal protein synthesis and degradation, resulting in a decrease in protein accretion; however when glucose and amino acid concentrations are maintained at control levels, only protein degradation is decreased, resulting in an overall accretion of protein at the whole body level (93). Similarly, when glucose and amino acid concentrations are held at control levels, insulin infusion increases fetal protein synthesis at the whole body level (87). While the effect of insulin upon protein synthesis in the neonate and fetus is apparent, little is known as to what effect insulin induced hypoaminoacidemia might have upon cerebral protein synthesis.

1.3.3 Behavioural/sleep states and substrate availability

A relationship between substrate availability and behavioural state must be considered, as previous studies have demonstrated a link between the two. In an effort to establish what role nutritional substrates might play in the regulation of sleep state activity in rats, Danguir and Nicolaidis (104) took fasted-rats and infused them with glucose, lipids, amino acids or a composite solution containing

all three in order to monitor their effect on sleep state expression. While the infusion of glucose and lipids had no apparent effect on sleep state expression, the infusion of amino acids produced a marked increase in REM sleep activity, while NREM activity was unchanged. As studied in cats, the concentration of proteins in brain perfusates vary in a cyclic fashion with behavioural/sleep activity, and are higher during rapid eye movement sleep than during the waking state (26). Furthermore, the inhibition of protein synthesis in cats with chloramphenicol subsequently decreased REM sleep activity (60), suggesting an important link between amino acids, protein synthesis and electrocortical expression. The impact of altered substrate availability upon fetal behavioural state activity has also been studied in the ovine fetus. Using insulin infusion to induce fetal hypoglycemia reduces the incidence of fetal breathing movements, but does not affect behavioural state expression (39). However, with hypoglycemia induced by maternal fasting, REM activity and FBM in the fetal sheep are significantly reduced, although these decreases in REM activity return to euglycemic levels with subsequent glucose infusion (31). The mechanism by which this hypoinsulinemic hypoglycemic insult is thought to affect electrocortical expression is through altered substrate uptake, thereby affecting synthesis of neurotransmitters.

Neurotransmitters such as serotonin also play an important role in the control of behavioural state expression through the modulation of brain centers responsible for generation of the sleep-waking cycle (105). As mentioned in the previous paragraph, the decrease in low voltage electrocortical expression and

fetal breathing movements (FBM) that results from maternal fast-induced hypoglycemia may be caused by a change in brain serotonin levels (31). An increase in maternal availability of tryptophan, the amino acid precursor for serotonin, will alter FBM (106), while intravenous infusion of 5-hydroxytryptophan to the fetus leads to an increased incidence of high voltage electrocortical activity, in addition to an increase in FBM (107, 108). This suggests a role for tryptophan, and subsequently serotonin in the control and expression of fetal behavioural state activity.

Studies in the adult have demonstrated similar effects of serotonin and serotonin precursors upon adult sleep, but the resulting effect varies depending upon the concentration of 5-Hydroxytryptophan (5-HT) used. In the adult rat, low doses of 5-HT during sleep results in a delayed increase in NREM activity, possibly through increased serotonin activity (109). However, the injection of high doses of 5-HT produces the opposite effect whereby NREM activity is inhibited, while the direct injection of the amino acid L-tryptophan had no affect whatsoever upon sleep state expression. While the effects of biochemical molecules such as serotonin and 5-HT in the adult brain are well studied, there are no definitive answers as to how they might influence fetal behavioural state activity. The potential role for other amino acids must be examined, as they may play a role in the control and expression of behavioural state activity.

1.3.4 Cerebral protein synthesis and substrate availability

The impact of individual amino acid availability within the brain upon biosynthetic processes has been studied to some degree in the adult rat, with

limited study in the fetus. Utilizing an infusion of valine in adult rats to increase circulating amino acid concentration results in an increase in protein synthetic rates in fetal liver, while having no direct effect on protein synthesis in the brain or heart and producing decreased rates in adrenal tissue (102). Contrary to the potentially beneficial effects observed with amino acid supplementation, a decrease in fetal amino acid availability, such as that achieved through maternal protein restriction during pregnancy, will alter early brain development in rat neonates by affecting astrocytogenesis, extra cellular matrix production, neuronal differentiation and programmed cellular death (54). Just altering the quality of food provided to pregnant rats can significantly alter neurotransmitter concentrations in the fetal brain, whereby a carbohydrate/fat meal produces an increase in tryptophan and serotonin in the fetal brain, while a casein diet decreases cerebral tryptophan and serotonin concentrations (72). With effects akin to altering maternal diet, insulin infusion has been utilized to induce changes to the fetal nutritional milieu, with insulin induced hypoglycaemia producing a fall in brain tissue concentrations of glutamine and alanine in the postnatal rat (110).

When considering the relationship between substrate availability and cerebral metabolism, it is important to consider the role played by the capillary endothelial wall of the brain, commonly referred to as the blood brain barrier (BBB). Movement of amino acids across fetal capillary endothelial membranes, such as the placenta and BBB, have been studied both in vivo and in vitro (71, 111, 112). Amino acid uptake at these sites is mediated by transport systems

specific to cationic (basic), zwitterionic (neutral), and anionic (acidic) amino acids (71).

Amino acid movement across the BBB is transport mediated, with several neutral amino acids utilizing the same transport sites (111). For example, neutral amino acids leucine, isoleucine, valine, tryptophan, phenylalanine, methionine, tyrosine and glutamine all utilize amino acid transport System L (71). When the concentration of an individual amino acid is significantly elevated in the plasma, its uptake by the brain is increased at the expense of other amino acids that utilize the same transport mechanism, creating an imbalance. For example in the adult rat, a three fold increase in plasma phenylalanine concentration to 200 μM increases phenylalanine uptake by the brain, producing a decreased influx of competing neutral amino acids into the brain and thus decreasing overall brain protein accretion (112). This likely occurs through a decrease in cerebral protein synthesis or an increase in cerebral proteolysis in an attempt to offset the imbalance in amino acids resulting from their decreased influx into the brain. Uptake of glucose by the brain is similarly mediated through BBB mediated transporters (113), as demonstrated during periods of chronic hyperglycemia in the adult rat, during which the number of high-affinity hexose carrying molecules at the BBB are decreased, resulting in a decreased flux of glucose into the brain (114). While physiological levels of hyperinsulinemia have no effect on net cerebral glucose metabolism or glucose transport across the BBB in the adult human (115), it is unknown what role insulin might play in cerebral amino acid uptake at the BBB. In vitro studies of amino acid transporters in cultured

umbilical endothelial cells suggest that basal rates of arginine and lysine uptake are increased with exposure to insulin, with no change in transport of leucine, serine or cystine (116), suggesting that insulin may affect transport of specific amino acids across endothelial membranes, such as the BBB, in vivo. The relationship between fetal metabolism and altered substrate accessibility has been examined in the whole body, and while altered amino acid availability has been studied in many tissues in the fetal rat, postnatal piglet and the ovine fetus, the potential effects on cerebral protein synthesis and thereby brain development in the near term fetus remains to be investigated.

1.4 SUMMARY

A relationship is established between substrate availability, protein synthesis and behavioural state in the ovine fetus near term. The application of Fick methodology to monitor amino acid uptake by the brain, and radiolabeled tracer technology to measure the incorporation of tracer amino acids into cerebral protein, has allowed for monitoring of the ongoing changes in the fetal brain. While earlier studies suggested a link between REM activity and cerebral protein synthesis, more recent work has demonstrated a positive correlation between the NREM behavioural state and rates of protein synthesis in the fetal brain. Whole body protein synthesis is altered in the fetus by changes in the nutritional milieu, whereby an increase/decrease in amino acid availability will result in an increase/decrease in protein synthesis, respectively. The use of an amino acid infusion to increase, and an insulin infusion to decrease circulating fetal amino acid concentrations, in combination with steady state tracer methodology, would allow for measurement of cerebral protein synthesis in the ovine fetus as it relates to nutritional substrate availability and fetal behavioural state activity.

1.5 REFERENCES

1. Rechtschaffen A, Kales A. A manual of standardized terminology, techniques and scoring system for sleep stages of human sleep subjects. Washington DC: U.S. Government Printing Office; 1968.

2. McGinty DJ, Drucker-Colin RR. Sleep mechanisms: Biology and control of REM sleep. Int Rev Neurobiol. 1982;23:391-436.

3. Curzi-Dascalova L, Challamel M. Neurophysiological basis of sleep development. In: Loughlin GM, Carroll JL, Marcus CL, editors. Sleep and Breathing in Children: A Developmental Approach. Lung Biology in Health and Disease Vol 147. New York: Marcel Dekker Inc.; 2000. p. 3-37.

4. Richardson BS. Ontogeny of behavioural states in the fetus. In: Thorburn G, Harding R, editors. *Textbook of Fetal Physiology*. New York, NY: Oxford University Press; 1994. p. 322-8.

5. Prechtl HF. The behavioural states of the newborn infant (a review). Brain Res. 1974 Aug 16;76(2):185-212.

6. Richardson BS. Metabolism of the fetal brain: Biological and pathological development. In: Hanson MA, editor. The Fetal and Neonatal Brain Stem. Cambridge, UK: Cambridge University Press; 1991. p. 87-105.

7. Nijhuis JG. Behavioural states: Concomitants, clinical implications and the assessment of the condition of the nervous system. Eur J Obstet Gynecol Reprod Biol. 1986 May;21(5-6):301-8.

8. Szeto HH, Hinman DJ. Prenatal development of sleep-wake patterns in sheep. Sleep. 1985 Dec;8(4):347-55.

9. Dawes GS, Fox HE, Leduc BM, Liggins GC, Richards RT. Respiratory movements and rapid eye movement sleep in the foetal lamb. J Physiol. 1972 Jan;220(1):119-43.

10. Clewlow F, Dawes GS, Johnston BM, Walker DW. Changes in breathing, electrocortical and muscle activity in unanaesthetized fetal lambs with age. J Physiol. 1983 Aug;341:463-76.

11. Gluckman PD, Williams CE, Gunn AJ. Brain stem and cerebral function in the fetus: Its assessment and the impact of asphyxia. In: Hanson MA, editor. The Fetal and Neonatal Brain Stem. Cambridge: Cambridge University Press; 1991. p. 185-210.

12. Walker DW. Brain mechanisms, hypoxia and fetal breathing. J Dev Physiol. 1984 Jun;6(3):225-36.

13. McIntosh GH, Baghurst KI, Potter BJ, Hetzel BS. Foetal brain development in the sheep. Neuropathol Appl Neurobiol. 1979 Mar-Apr;5(2):103-14.

14. Jouvet-Mounier D, Astic L, Lacote D. Ontogenesis of the states of sleep in rat, cat, and guinea pig during the first postnatal month. Dev Psychobiol. 1970;2(4):216-39.

15. Nijhuis JG, Tas BAPJ. Physiological and clinical aspects of the development of fetal behaviour. In: Hanson MA, editor. The Fetal and Neonatal Brain Stem. Cambridge: Cambridge University Press; 1991. p. 268-80.

16. Maquet P, Laureys S, Peigneux P, Fuchs S, Petiau C, Phillips C, et al. Experience-dependent changes in cerebral activation during human REM sleep. Nat Neurosci. 2000 Aug;3(8):831-6.

17. Kavanau JL. REM and NREM sleep as natural accompaniments of the evolution of warm-bloodedness. Neurosci Biobehav Rev. 2002 Dec;26(8):889-906.

18. Karni A, Tanne D, Rubenstein BS, Askenasy JJ, Sagi D. Dependence on REM sleep of overnight improvement of a perceptual skill. Science. 1994 Jul 29;265(5172):679-82.

19. Wehr TA. A brain-warming function for REM sleep. Neurosci Biobehav Rev. 1992 Fall;16(3):379-97.

20. Anch AM, Browman CP, Mitler MM, Walsh JK. Sleep: A scientific perspective. New Jersey: Prentice Hall; 1988.

21. Adam K. Sleep as a restorative process and a theory to explain why. Prog Brain Res. 1980;53:289-305.

22. Roffwarg HP, Muzio JN, and Dement WC. Ontogenic development of the human sleep-dream cycle. Science. 1966;152:604-19.

23. Kawagoe Y, Green L, White S, Richardson B. Intermittent umbilical cord occlusion in the ovine fetus near term: Effects on behavioral state activity. Am J Obstet Gynecol. 1999 Dec;181(6):1520-9.

24. Mirmiran M, Uylings HB. The environmental enrichment effect upon cortical growth is neutralized by concomitant pharmacological suppression of active sleep in female rats. Brain Res. 1983 Feb 21;261(2):331-4.

25. Marks GA, Shaffery JP, Oksenberg A, Speciale SG, Roffwarg HP. A functional role for REM sleep in brain maturation. Behav Brain Res. 1995 Jul-Aug;69(1-2):1-11.

26. Drucker-Colin RR, Spanis CW, Cotman CW, McGaugh JL. Changes in protein levels in perfusates of freely moving cats: Relation to behavioral state. Science. 1975 Mar 14;187(4180):963-5.

27. Shapiro C, Girdwood P. Protein synthesis in rat brain during sleep. Neuropharmacology. 1981 May;20(5):457-60.

28. Ramm P, Smith CT. Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. Physiol Behav. 1990 Nov;48(5):749-53.

29. Nakanishi H, Sun Y, Nakamura RK, Mori K, Ito M, Suda S, et al. Positive correlations between cerebral protein synthesis rates and deep sleep in macaca mulatta. Eur J Neurosci. 1997 Feb;9(2):271-9.

30. Benington JH, Heller HC. REM-sleep timing is controlled homeostatically by accumulation of REM-sleep propensity in non-REM sleep. Am J Physiol. 1994 Jun;266(6 Pt 2):R1992-2000.

31. Richardson BS, Hohimer AR, Bissonnette JM, Machida CM. Cerebral metabolism in hypoglycemic and hyperglycemic fetal lambs. Am J Physiol. 1983 Nov;245(5 Pt 1):R730-6.

32. Moja EA, Mendelson WB, Stoff DM, Gillin JC, Wyatt RJ. Reduction of REM sleep by a tryptophan-free amino acid diet. Life Sci. 1979 Apr 16;24(16):1467-70.

33. Yogman MW, Zeisel SH, Roberts C. Assessing effects of serotonin precursors on newborn behavior. J Psychiatr Res. 1982 -83;17(2):123-33.

34. Pond WG, Boleman SL, Fiorotto ML, Ho H, Knabe DA, Mersmann HJ, et al. Perinatal ontogeny of brain growth in the domestic pig. Proc Soc Exp Biol Med. 2000 Jan;223(1):102-8.

35. Battaglia FC, Meschia G. Fetal nutrition. Annu Rev Nutr. 1988;8:43-61.

36. Czikk MJ, Sweeley JC, Homan JH, Milley JR, Richardson BS. Cerebral leucine uptake and protein synthesis in the near-term ovine fetus: Relation to fetal behavioral state. Am J Physiol Regul Integr Comp Physiol. 2003 Jan;284(1):R200-7.

37. Hay WW,Jr, Meznarich HK, Sparks JW, Battaglia FC, Meschia G. Effect of insulin on glucose uptake in near-term fetal lambs. Proc Soc Exp Biol Med. 1985 Apr;178(4):557-64.

38. Milley JR. Ovine fetal leucine kinetics and protein metabolism during decreased oxygen availability. Am J Physiol. 1998 Apr;274(4 Pt 1):E618-26.

39. Richardson BS, Hohimer AR, Bissonnette JM, Machida CM. Insulin hypoglycemia, cerebral metabolism, and neural function in fetal lambs. Am J Physiol. 1985 Jan;248(1 Pt 2):R72-7.

40. Makowski EL, Schneider JM, Tsoulos NG, Colwill JR, Battaglia FC, Meschia G. Cerebral blood flow, oxygen consumption, and glucose utilization of fetal lambs in utero. Am J Obstet Gynecol. 1972 Oct 1;114(3):292-303.

41. Hay WW,Jr. Fetal glucose metabolism. Semin Perinatol. 1979 Apr;3(2):157-76.

42. Abrams RM, Ito M, Frisinger JE, Patlak CS, Pettigrew KD, Kennedy C. Local cerebral glucose utilization in fetal and neonatal sheep. Am J Physiol. 1984 Apr;246(4 Pt 2):R608-18.

43. Loy GL, Quick AN,Jr, Teng CC, Hay WW,Jr, Fennessey PV. Versatile stable isotope technique for the measurement of amino acids and keto acids:

Comparison with radioactive isotope and its use in measuring in vivo disposal rates. Anal Biochem. 1990 Feb 15;185(1):1-9.

44. Ross ,J.C., Fennessey PV, Wilkening RB, Battaglia FC, Meschia G. Placental transport and fetal utilization of leucine in a model of fetal growth retardation. Am J Physiol. 1996 Mar;270(3 Pt 1):E491-503.

45. Sano H, Kajita M, Fujita T. Effect of dietary protein intake on plasma leucine flux, protein synthesis, and degradation in sheep. Comp Biochem Physiol B Biochem Mol Biol. 2004 Oct;139(2):163-8.

46. Wester TJ, Lobley GE, Birnie LM, Crompton LA, Brown S, Buchan V, et al. Effect of plasma insulin and branched-chain amino acids on skeletal muscle protein synthesis in fasted lambs. Br J Nutr. 2004 Sep;92(3):401-9.

47. Patterson BW, Zhang XJ, Chen Y, Klein S, Wolfe RR. Measurement of very low stable isotope enrichments by gas chromatography/mass spectrometry: Application to measurement of muscle protein synthesis. Metabolism. 1997 Aug;46(8):943-8.

48. Schaefer AL, Krishnamurti CR. Whole body and tissue fractional protein synthesis in the ovine fetus in utero. Br J Nutr. 1984 Sep;52(2):359-69.

49. Pell JM, Bergman EN. Cerebral metabolism of amino acids and glucose in fed and fasted sheep. Am J Physiol. 1983 Mar;244(3):E282-9.

50. Wolfe RR. Radioactive and stable isotope tracers in biomedicine: Principles and practice of kinetic analysis. New York: Wiley-Liss Inc.; 1992.

51. Abrams RM, Burchfield DJ, Sun Y, Smith CB. Rates of local cerebral protein synthesis in fetal and neonatal sheep. Am J Physiol. 1997 Apr;272(4 Pt 2):R1235-44.

52. Sun Y, Deibler GE, Jehle J, Macedonia J, Dumont I, Dang T, et al. Rates of local cerebral protein synthesis in the rat during normal postnatal development. Am J Physiol. 1995 Feb;268(2 Pt 2):R549-61.

53. Carver TD, Quick AA, Teng CC, Pike AW, Fennessey PV, Hay WW,Jr. Leucine metabolism in chronically hypoglycemic hypoinsulinemic growth-restricted fetal sheep. Am J Physiol. 1997 Jan;272(1 Pt 1):E107-17.

54. Gressens P, Muaku SM, Besse L, Nsegbe E, Gallego J, Delpech B, et al. Maternal protein restriction early in rat pregnancy alters brain development in the progeny. Brain Res Dev Brain Res. 1997 Oct 20;103(1):21-35.

55. Dunlop DS, Elden WV, Lajtha A. Protein degradation rates in regions of the central nervous system in vivo during development. Biochem J. 1978 Mar 15;170(3):637-42.

56. Goldspink DF. Protein turnover and growth of the rat brain from the foetus to old age. J Neurochem. 1988 May;50(5):1364-8.

57. Bourgeois JP. Synaptogenesis, heterochrony and epigenesis in the mammalian neocortex. Acta Paediatr Suppl. 1997 Jul;422:27-33.

58. Fountoulakis M, Hardmaier R, Schuller E, Lubec G. Differences in protein level between neonatal and adult brain. Electrophoresis. 2000 Feb;21(3):673-8.

59. Mirmiran M, Uylings HB, Corner MA. Pharmacological suppression of REM sleep prior to weaning counteracts the effectiveness of subsequent

environmental enrichment on cortical growth in rats. Brain Res. 1983 Mar;283(1):102-5.

60. Drucker-Colin R, Bowersox SS, McGinty DJ. Sleep and medial reticular unit responses to protein synthesis inhibitors: Effects of chloramphenicol and thiamphenicol. Brain Res. 1982 Dec 2;252(1):117-27.

61. Stern WC, Morgane PJ, Panksepp J, Zolovick AJ, Jalowiec JE. Elevation of REM sleep following inhibition of protein synthesis. Brain Res. 1972 Nov 27;47(1):254-8.

62. Rao N, Keen A, Czikk M, Frasch M, Richardson BS. Behavioural state linkage in the ovine fetus near term. Brain Res. 2009 Jan 23;1250:149-56.

63. Battaglia FC, Meschia G. Principal substrates of fetal metabolism. Physiol Rev. 1978 Apr;58(2):499-527.

64. Bell AW, Kennaugh JM, Battaglia FC, Makowski EL, Meschia G. Metabolic and circulatory studies of fetal lamb at midgestation. Am J Physiol. 1986 May;250(5 Pt 1):E538-44.

65. Ahokas RA, Lahaye EB, Anderson GD, Lipshitz J. Effect of maternal dietary restriction on fetal growth and placental transfer of alpha-amino isobutyric acid in rats. J Nutr. 1981 Dec;111(12):2052-8.

66. Scheaffer AN, Caton JS, Redmer DA, Reynolds LP. The effect of dietary restriction, pregnancy, and fetal type in different ewe types on fetal weight, maternal body weight, and visceral organ mass in ewes. J Anim Sci. 2004 Jun;82(6):1826-38.

67. Chao CR, Hohimer AR, Bissonnette JM. The effect of electrocortical state on cerebral carbohydrate metabolism in fetal sheep. Brain Res Dev Brain Res. 1989 Sep 1;49(1):1-5.

68. Kwon H, Spencer TE, Bazer FW, Wu G. Developmental changes of amino acids in ovine fetal fluids. Biol Reprod. 2003 May;68(5):1813-20.

69. Crumpler HR, Dent CE, Lindan O. The amino-acid pattern in human foetal and maternal plasma at delivery. Biochem J. 1950 Aug;47(2):223-7.

70. Cetin I, Marconi AM, Bozzetti P, Sereni LP, Corbetta C, Pardi G, et al. Umbilical amino acid concentrations in appropriate and small for gestational age infants: A biochemical difference present in utero. Am J Obstet Gynecol. 1988 Jan;158(1):120-6.

71. Regnault TR, de Vrijer B, Battaglia FC. Transport and metabolism of amino acids in placenta. Endocrine. 2002 Oct;19(1):23-41.

72. Nelson MF, Picon HF, Lytle LD. Maternal meal quality alters fetal rat brain serotonin concentrations. Life Sci. 1981 Jan 19;28(3):231-7.

73. Barker DJ, Clark PM. Fetal undernutrition and disease in later life. Rev Reprod. 1997 May;2(2):105-12.

74. Rees WD, Hay SM, Buchan V, Antipatis C, Palmer RM. The effects of maternal protein restriction on the growth of the rat fetus and its amino acid supply. Br J Nutr. 1999 Mar;81(3):243-50.

75. Wu G, Pond WG, Ott T, Bazer FW. Maternal dietary protein deficiency decreases amino acid concentrations in fetal plasma and allantoic fluid of pigs. J Nutr. 1998 May;128(5):894-902.

76. Lemons JA, Schreiner RL. Amino acid metabolism in the ovine fetus. Am J Physiol. 1983 May;244(5):E459-66.

77. Liechty EA, Lemons JA. Changes in ovine fetal hindlimb amino acid metabolism during maternal fasting. Am J Physiol. 1984 May;246(5 Pt 1):E430-5.

78. Prior RL, Smith SB. Hormonal effects on partitioning of nutrients for tissue growth: Role of insulin. Fed Proc. 1982 Jul;41(9):2545-9.

79. Lemons JA, Ridenour R, Orsini EN. Congenital absence of the pancreas and intrauterine growth retardation. Pediatrics. 1979 Aug;64(2):255-7.

80. Fowden AL, Comline RS. The effects of pancreatectomy on the sheep fetus in utero. Q J Exp Physiol. 1984 Apr;69(2):319-30.

81. Fowden AL, Hughes P, Comline RS. The effects of insulin on the growth rate of the sheep fetus during late gestation. Q J Exp Physiol. 1989 Sep;74(5):703-14.

82. Gitlin D, Kumate J, Morales C. On the transport of insulin across the human placenta. Pediatrics. 1965 Jan;35:65-9.

83. Reddy S, Bibby NJ, Elliott RB. An immunofluorescent study of insulin-, glucagon-, pancreatic polypeptide- and somatostatin-containing cells in the early ovine fetal pancreas. Q J Exp Physiol. 1988 Mar;73(2):225-32.

84. Philipps AF, Carson BS, Meschia G, Battaglia FC. Insulin secretion in fetal and newborn sheep. Am J Physiol. 1978 Nov;235(5):E467-74.

85. Hay WW,Jr, Sparks JW, Gilbert M, Battaglia FC, Meschia G. Effect of insulin on glucose uptake by the maternal hindlimb and uterus, and by the fetus in conscious pregnant sheep. J Endocrinol. 1984 Jan;100(1):119-24.

86. Hill DE. Effect of insulin on fetal growth. Semin Perinatol. 1978 Oct;2(4):319-28.

87. Shen W, Wisniowski P, Ahmed L, Boyle DW, Denne SC, Liechty EA. Protein anabolic effects of insulin and IGF-I in the ovine fetus. Am J Physiol Endocrinol Metab. 2003 Apr;284(4):E748-56.

88. Thureen PJ, Scheer B, Anderson SM, Tooze JA, Young DA, Hay WW,Jr. Effect of hyperinsulinemia on amino acid utilization in the ovine fetus. Am J Physiol Endocrinol Metab. 2000 Dec;279(6):E1294-304.

89. Davis TA, Burrin DG, Fiorotto ML, Reeds PJ, Jahoor F. Roles of insulin and amino acids in the regulation of protein synthesis in the neonate. J Nutr. 1998 Feb;128(2 Suppl):347S-50S.

90. Wray-Cahen D, Nguyen HV, Burrin DG, Beckett PR, Fiorotto ML, Reeds PJ, et al. Response of skeletal muscle protein synthesis to insulin in suckling pigs decreases with development. Am J Physiol. 1998 Oct;275(4 Pt 1):E602-9.

91. Chrystie S, Horn J, Sloan I, Stern M, Noakes D, Young M. Effect of insulin on protein turnover in foetal limbs. Proc Nutr Soc. 1977 Dec;36(3):118A.

92. Liechty EA, Boyle DW, Moorehead H, Liu YM, Denne SC. Effect of hyperinsulinemia on ovine fetal leucine kinetics during prolonged maternal fasting. Am J Physiol. 1992 Oct;263(4 Pt 1):E696-702.

93. Milley JR. Effects of insulin on ovine fetal leucine kinetics and protein metabolism. J Clin Invest. 1994 Apr;93(4):1616-24.

94. Wurtman RJ, Fernstrom JD. Control of brain neurotransmitter synthesis by precursor availability and nutritional state. Biochem Pharmacol. 1976 Aug 1;25(15):1691-6.

95. Wilkening RB, Boyle DW, Teng C, Meschia G, Battaglia FC. Amino acid uptake by the fetal ovine hindlimb under normal and euglycemic hyperinsulinemic states. Am J Physiol. 1994 Jan;266(1 Pt 1):E72-8.

96. Woods SC, Porte D,Jr, Bobbioni E, Ionescu E, Sauter JF, Rohner-Jeanrenaud F, et al. Insulin: Its relationship to the central nervous system and to the control of food intake and body weight. Am J Clin Nutr. 1985 Nov;42(5 Suppl):1063-71.

97. Baskin DG, Figlewicz DP, Woods SC, Porte D,Jr, Dorsa DM. Insulin in the brain. Annu Rev Physiol. 1987;49:335-47.

98. Philipps AF, Rosenkrantz TS, Porte PJ, Raye JR. The effects of chronic fetal hyperglycemia on substrate uptake by the ovine fetus and conceptus. Pediatr Res. 1985 Jul;19(7):659-66.

99. de Boo HA, van Zijl PL, Smith DE, Kulik W, Lafeber HN, Harding JE. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr Res. 2005 Aug;58(2):270-7.

100. Liechty EA, Boyle DW, Moorehead H, Auble L, Denne SC. Aromatic amino acids are utilized and protein synthesis is stimulated during amino acid infusion in the ovine fetus. J Nutr. 1999 Jun;129(6):1161-6.

101. Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC. Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats. Am J Physiol Endocrinol Metab. 2002 Oct;283(4):E824-35.

102. Smith CB, Sun Y. Influence of valine flooding on channeling of valine into tissue pools and on protein synthesis. Am J Physiol. 1995 Apr;268(4 Pt 1):E735-44.

103. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: A method for quantifying insulin secretion and resistance. Am J Physiol. 1979 Sep;237(3):E214-23.

104. Danguir J, Nicolaidis S. Dependence of sleep on nutrients' availability. Physiol Behav. 1979 Apr;22(4):735-40.

105. Ursin R. Serotonin and sleep. Sleep Med Rev. 2002 Feb;6(1):55-69.

106. Devoe LD, Castillo RA, Searle NS, Searle JR. Maternal dietary substrates and human fetal biophysical activity. I. the effects of tryptophan and glucose on fetal breathing movements. Am J Obstet Gynecol. 1986 Jul;155(1):135-9.

107. Fletcher DJ, Hanson MA, Moore PJ, Nijhuis JG, Parkes MJ. Stimulation of breathing movements by L-5-hydroxytryptophan in fetal sheep during normoxia and hypoxia. J Physiol. 1988 Oct;404:575-89.

108. Quilligan EJ, Clewlow F, Johnston BM, Walker DW. Effect of 5-hydroxytryptophan on electrocortical activity and breathing movements of fetal sheep. Am J Obstet Gynecol. 1981 Oct 1;141(3):271-5.

109. Imeri L, Mancia M, Bianchi S, Opp MR. 5-hydroxytryptophan, but not L-tryptophan, alters sleep and brain temperature in rats. Neuroscience. 2000;95(2):445-52.

110. Lewis LD, Ljunggren B, Norberg K, Siesjo BK. Changes in carbohydrate substrates, amino acids and ammonia in the brain during insulin-induced hypoglycemia. J Neurochem. 1974 Oct;23(4):659-71.

111. Omidi Y, Barar J, Ahmadian S, Heidari HR, Gumbleton M. Characterization and astrocytic modulation of system L transporters in brain microvasculature endothelial cells. Cell Biochem Funct. 2008 Apr;26(3):381-91.

112. Pardridge WM. Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. Neurochem Res. 1998 May;23(5):635-44.

113. McCall AL. Cerebral glucose metabolism in diabetes mellitus. Eur J Pharmacol. 2004 Apr 19;490(1-3):147-58.

114. McCall AL, Millington WR, Wurtman RJ. Metabolic fuel and amino acid transport into the brain in experimental diabetes mellitus. Proc Natl Acad Sci U S A. 1982 Sep;79(17):5406-10.

115. Hasselbalch SG, Knudsen GM, Videbaek C, Pinborg LH, Schmidt JF, Holm S, et al. No effect of insulin on glucose blood-brain barrier transport and cerebral metabolism in humans. Diabetes. 1999 Oct;48(10):1915-21.

116. Mann GE, Yudilevich DL, Sobrevia L. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. Physiol Rev. 2003 Jan;83(1):183-252.

CHAPTER TWO

RATIONALE, HYPOTHESIS AND RESEARCH OBJECTIVES

2.1 RATIONALE

In the ovine fetus, well-differentiated electrocortical (ECOG) patterns are evident from 120 days gestation (term = 145 days), with a temporal relationship to episodic muscle and breathing activity, indicative of behavioural states which are analogous to postnatal sleep states (1). There is initially a high proportion of time in the low-voltage ECOG state with rapid eye movements (REM) at >50%, with approximately 40% of the time spent in the high-voltage ECOG NREM state and only brief periods of wakefulness. There are developmental changes to the proportioning of these behavioural state activities and metabolic associations with an increase in cerebral metabolic rate during the low-voltage REM state (2) and conversely, an increase in cerebral protein synthesis during the high-voltage NREM state (3), supporting a role for these activities in the brain's growth and development. While the relationship of circulating amino acids to biosynthetic processes within the fetal brain and behavioural state activity remains unclear, earlier studies in adult animals have shown intravenous infusion of amino acids (4) and exposure to various protein synthesis inhibitors (5, 6) results in increased and decreased low-voltage ECOG or REM sleep, respectively.

While glucose is the major metabolic substrate of the ovine fetal brain near term and can account for all oxidative metabolism (7, 8) amino acids are also taken up for protein accretion and possibly oxidative needs (3) as observed in the adult rat (9)(10). Substrate consumption is well studied in the chronically catheterized ovine fetus over the latter half of gestation using Fick methodology with measurements of umbilical or cotyledonary blood flow and the venoarterial

substrate difference across the placenta (11, 12). That for amino acids indicates a substantial uptake, exceeding the requirements for growth needs alone and is consistent with other data supporting a role for amino acids as substrates for energy production during fetal life (13). As such, alterations in the availability of amino acids likely impact on metabolic and/or growth processes both globally as well as differentially for given fetal tissues.

Availability of amino acids within the fetus will impact upon protein synthetic processes. The use of amino acid infusion to increase (14, 15), and insulin infusion to decrease (16, 17) circulating amino acid concentrations in the ovine fetus demonstrates that protein synthesis can be altered at the whole body level, and is dependant to a degree upon substrate availability. From the standpoint of the brain, cerebral protein synthesis has been studied postnatally in the rat (18), with additional work being carried out in the ovine fetus both prenatally (3, 19) and postnatally (20) utilizing radioactive amino acid tracers to determine rates of synthesis.

Using a steady state tracer amino acid infusion, my study investigated the measurement of amino acid flux into the brain, as well as cerebral protein synthesis. Used in conjunction with an amino acid infusion to increase and insulin infusion to decrease fetal plasma amino acid concentrations, my study investigated the importance of nutritional substrate availability upon fetal cerebral protein metabolism. Fetal ECOG was monitored to test the hypothesis that alterations in circulating amino acids will affect low-voltage ECOG state activity.

2.2 HYPOTHESES

1. Increases/decreases in circulating amino acids will increase/decrease low-voltage ECOG state activity
2. Amino acid infusion to the ovine fetus will increase circulating amino acid concentrations, and will result in elevated rates of cerebral fractional protein synthesis when compared with those observed with insulin infusion.
3. Insulin infusion to the ovine fetus will decrease circulating amino acid concentrations, and will result in reduced rates of cerebral protein synthesis when compared with those observed with amino acid infusion.
4. There will be a linkage between behavioural state expression and cerebral protein synthesis as a result of the induced changes to fetal substrate availability.

2.3 OBJECTIVES

1. To determine the effect that Increases/decreases in circulating amino acids will have upon fetal ECOG and ultimately behavioural state activity
2. To determine changes in circulating fetal amino acid concentration in relation to amino acid infusion.
3. To determine changes in circulating fetal amino acid concentration in relation to insulin infusion.

4. To determine the rate of fractional protein synthesis and unidirectional flux of leucine into the ovine fetal brain using ^{13}C-labelled leucine tracer methodology during altered amino acid availability, as it relates to fetal behavioural state expression

2.4 REFERENCES

1. Szeto HH, Hinman DJ. Prenatal development of sleep-wake patterns in sheep. Sleep. 1985 Dec;8(4):347-55.

2. Richardson BS, Patrick JE, Abduljabbar H. Cerebral oxidative metabolism in the fetal lamb: Relationship to electrocortical state. Am J Obstet Gynecol. 1985 Oct 15;153(4):426-31.

3. Czikk MJ, Sweeley JC, Homan JH, Milley JR, Richardson BS. Cerebral leucine uptake and protein synthesis in the near-term ovine fetus: Relation to fetal behavioral state. Am J Physiol Regul Integr Comp Physiol. 2003 Jan;284(1):R200-7.

4. Danguir J, Nicolaidis S. Intravenous infusions of nutrients and sleep in the rat: An ischymetric sleep regulation hypothesis. Am J Physiol. 1980 Apr;238(4):E307-12.

5. Rojas-Ramirez JA, Aguilar-Jimenez E, Posadas-Andrews A, Bernal-Pedraza JG, Drucker-Colin RR. The effects of various protein synthesis inhibitors on the sleep-wake cycle of rats. Psychopharmacology (Berl). 1977 Jul 18;53(2):147-50.

6. Prospero-Garcia O, Jimenez-Anguiano A, Drucker-Colin R. Chloramphenicol prevents carbachol-induced REM sleep in cats. Neurosci Lett. 1993 May 14;154(1-2):168-70.

7. Makowski EL, Schneider JM, Tsoulos NG, Colwill JR, Battaglia FC, Meschia G. Cerebral blood flow, oxygen consumption, and glucose utilization of fetal lambs in utero. *Am J Obstet Gynecol. 1972 Oct 1;114(3):292-303.*

8. Richardson BS. Metabolism of the fetal brain: Biological and pathological development. In: Hanson MA, editor. The Fetal and Neonatal Brain Stem. Cambridge, UK: Cambridge University Press; 1991. p. 87-105.

9. Govinatzki MT, Velleda LS, Trindade VM, Nagel FM, Bueno D, Perry ML. Amino acid metabolism in rat hippocampus during the period of brain growth spurt. Neurochem Res. 1997 Jan;22(1):23-6.

10. Keen RE, Barrio JR, Huang SC, Hawkins RA, Phelps ME. In vivo cerebral protein synthesis rates with leucyl-transfer RNA used as a precursor pool: Determination of biochemical parameters to structure tracer kinetic models for positron emission tomography. J Cereb Blood Flow Metab. 1989 Aug;9(4):429-45.

11. Battaglia FC. New concepts in fetal and placental amino acid metabolism. J Anim Sci. 1992 Oct;70(10):3258-63.

12. Lemons JA, Adcock EW,3rd, Jones MD,Jr, Naughton MA, Meschia G, Battaglia FC. Umbilical uptake of amino acids in the unstressed fetal lamb. J Clin Invest. 1976 Dec;58(6):1428-34.

13. Battaglia FC, Meschia G. Fetal nutrition. Annu Rev Nutr. 1988;8:43-61.

14. de Boo HA, van Zijl PL, Smith DE, Kulik W, Lafeber HN, Harding JE. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr Res. 2005 Aug;58(2):270-7.

15. Liechty EA, Boyle DW, Moorehead H, Auble L, Denne SC. Aromatic amino acids are utilized and protein synthesis is stimulated during amino acid infusion in the ovine fetus. J Nutr. 1999 Jun;129(6):1161-6.

16. Thureen PJ, Scheer B, Anderson SM, Tooze JA, Young DA, Hay WW,Jr. Effect of hyperinsulinemia on amino acid utilization in the ovine fetus. Am J Physiol Endocrinol Metab. 2000 Dec;279(6):E1294-304.

17. Wilkening RB, Boyle DW, Teng C, Meschia G, Battaglia FC. Amino acid uptake by the fetal ovine hindlimb under normal and euglycemic hyperinsulinemic states. Am J Physiol. 1994 Jan;266(1 Pt 1):E72-8.

18. Sun Y, Deibler GE, Jehle J, Macedonia J, Dumont I, Dang T, et al. Rates of local cerebral protein synthesis in the rat during normal postnatal development. Am J Physiol. 1995 Feb;268(2 Pt 2):R549-61.

19. Schaefer AL, Krishnamurti CR. Whole body and tissue fractional protein synthesis in the ovine fetus in utero. Br J Nutr. 1984 Sep;52(2):359-69.

20. Abrams RM, Burchfield DJ, Sun Y, Smith CB. Rates of local cerebral protein synthesis in fetal and neonatal sheep. Am J Physiol. 1997 Apr;272(4 Pt 2):R1235-44.

CHAPTER THREE

INDUCED HYPER AND HYPO AMINO ACIDEMIA IN THE OVINE FETUS NEAR TERM: EFFECTS ON ELECTROCORTICAL ACTIVITY [1]

---

[1]A version of this chapter has been published: MacLachlan JN, Hemstreet S, Matushewski B, McCallum JD, Richardson BS. *Induced hyper and hypo amino acidemia in the ovine fetus near term: Effects on electrocortical activity.* Reproductive Sciences. September 2008;15(7):710-19.

3.1 INTRODUCTION

Substrate consumption is well studied in the chronically catheterized ovine fetus over the latter half of gestation using Fick methodology with measurements of umbilical or cotyledonary blood flow and the venoarterial substrate difference across the placenta (1, 2). That for amino acids indicates a substantial uptake, exceeding the requirements for growth needs alone, and is consistent with data supporting a role for amino acids as substrates for energy production during fetal life (3). Studies in the human fetus at the time of elective cesarean section or vaginal delivery show a similar pattern for amino acid venoarterial differences across the placenta (4, 5), again supporting their role as important substrates for fetal energy production and growth. As such, alterations in the availability of amino acids might then impact on metabolic and/or growth processes both globally as well as differentially for given fetal tissues. In this regard, there has been limited study in the ovine fetus near term using the infusion of mixed amino acids (6-8) and the induction of euglycemic hyperinsulinemic states (9, 10) which increase and decrease circulating amino acid levels respectively, in relation to measures of protein metabolism.

Measurements of brain metabolism in the unanaesthetized fetus are again largely limited to sheep, an animal that is relatively mature neurophysiologically at birth leading to their classification as prenatal brain developers (11). While glucose is the major metabolic substrate of the ovine fetal brain near term and can account for all oxidative metabolism (12) amino acids are also taken up for protein accretion and possibly oxidative needs (13) as observed in the adult rat

(14, 15). The precursor availability of amino acids within the brain might then impact upon protein biosynthetic processes either generally or for specific proteins. For example in the rat fetus, the synthetic rate of the neurotransmitter noradrenalin is regulated by tyrosine brain concentration and therefore maternal intake of tyrosine (16).

For the ovine fetus, well-differentiated electrocortical (ECOG) patterns are evident from 120 days gestation (term = 145 days), with a temporal relationship to episodic muscle and breathing activity, indicative of behavioural states (17). There is initially a high proportion of time in the low-voltage ECOG state with rapid eye movements (REM) at >50%, with approximately 40% of the time spent in the high-voltage ECOG NREM state and only brief periods of wakefulness. A behavioural state effect on cerebral metabolic rate is evident for the near term ovine fetus with an increase during the low-voltage REM state which likely reflects increased neuronal activity (18). Conversely, cerebral protein synthesis and degradation, i.e. turnover, appear to be increased during the high-voltage NREM state (13) as seen in adult animals (19, 20), indicating that the decrease in the brain's metabolic demand during NREM sleep does not result from a decrease in biosynthetic activity and may, in fact, favour the synthesis of new proteins. These findings support the restorative theory of sleep (21) and a role for sleep state activity in the brain's development with REM providing a degree of endogenous stimulation through neuronal activity, leading to increased protein synthesis which subsequently occurs during the following NREM period when energy needs for neuronal activity are lower. A metabolic linkage for behavioural

state activity is further supported by the recent finding in the near term ovine fetus within which high-voltage NREM episode duration is positively correlated with the duration of the previous low-voltage REM episode (22) in keeping with a homeostatic model of REM-NREM sleep control as proposed for the adult rat (23).

In the present study we have utilized a mixed amino acid infusate and an insulin euglycemic clamp technique in the chronically catheterized ovine fetus near term to establish a research model of moderate increases and decreases in circulating amino acid levels, respectively. Fetal ECOG activity was additionally monitored to determine the impact of changing amino acid levels on the incidence and linkage of low and high-voltage activities which might be altered through related changes in cerebral protein synthesis and/or neurotransmitter levels, for example serotonin, given their putative role in sleep state control (24).

3.2 MATERIALS AND METHODS

3.2.1 Surgical Procedure

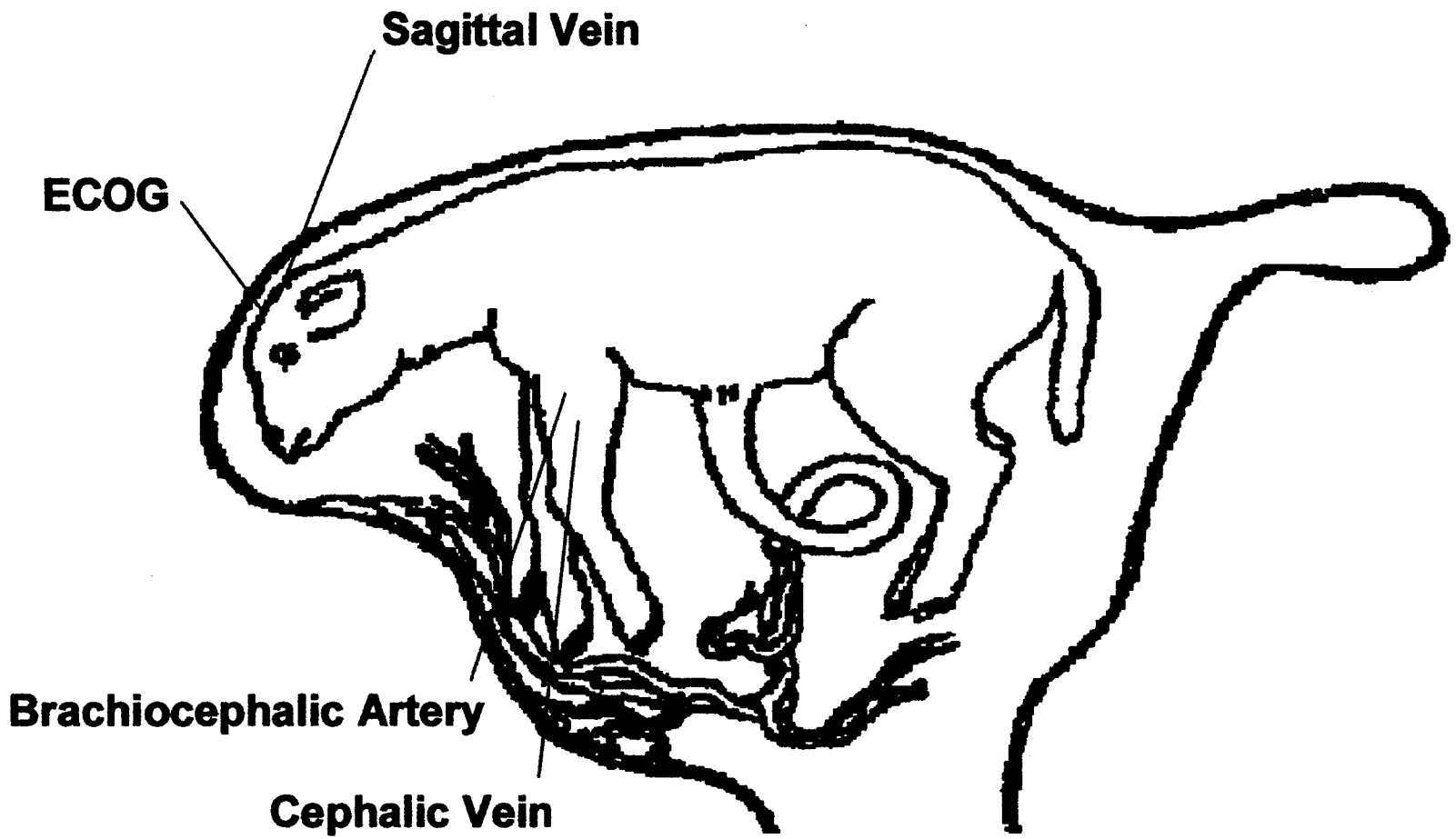
Twenty fetal sheep were surgically prepared at ~129 days gestation; Hyper amino acid (AA) group N=11, Hypo AA group N = 9. Anesthesia was induced using an intravenous injection of Thiotal (Vetoquinol, Lavaltrie, PQ) and was maintained throughout surgery with 1-1.5% halothane in oxygen (Halocarbon Laboratories, Hackensack, NJ). A polyvinyl catheter (V11, Bolab, Lake Havasu City, AZ) was placed in the maternal femoral vein for infusion of fluids throughout the surgery (1000 mL 0.9% saline solution). Utilizing sterile technique, a midline

incision was made in the ewe's lower abdominal wall and the uterus was palpated to ascertain fetal position. Once determined, an incision was made in the uterus and the head and upper body of the fetus were exteriorized. Polyvinyl catheters (V4, Bolab) were placed in the brachiocephalic artery for blood sampling and pressure recording, and the cephalic vein for infusion of insulin, dextrose, amino acids (Primene®), ^{13}C-labelled leucine and administration of antibiotics (Figure 3.1). A catheter was also placed in the amniotic cavity for pressure recording as well as intra amniotic administration of antibiotics. Small burr holes were drilled biparietally to the dura of the skull, and electrodes of Teflon coated stainless steel wire (Cooner Wire, Chatsworth, CA) were inserted for ECOG recording. A small window of bone was removed along the midline between the coronal and lambdoid sutures, and the sagittal vein was then catheterized (V4, Bolab) for cerebral venous blood sampling. Following placement of all catheters and electrodes, the uterus and abdomen were closed in layers. Catheters and electrodes were exteriorized through the flank of the ewe and secured to its back in a plastic pouch. Post surgery, the ewes were given a long acting, broad spectrum antibiotic intramuscularly (1,000,000 IU sodium penicillin G; Ayerst, Montreal, PQ).

3.2.2 Post-operative care

Immediately following surgery, ewes were returned to their metabolic cages and administered an analgesic (1.5 mL Banamine, Schering-Plough, Point Claire, PQ). For 3 days post-operatively, antibiotics were administered to both the fetus (1,000,000 IU sodium penicillin G, intravenous) and to the amniotic

Figure 3.1 Surgical preparation of the ovine fetus at ~129 days gestation (term = 145 days). Catheters were placed in the brachiocephalic artery (blood sampling), cephalic vein (Primene/insulin + dextrose infusion) and sagittal vein (blood sampling); electrocortical (ECOG) electrodes were inserted for monitoring of fetal behavioural state. All catheters and electrodes were exteriorized to the flank of the ewe. Animals were given 4 days recovery prior to initiation of experiments.


Sagittal Vein
ECOG
Brachiocephalic Artery
Cephalic Vein

cavity (1,000,000 IU sodium penicillin G). Animals were allowed 4 days of post-operative recovery, during which time all maternal and fetal catheters were flushed daily with heparinized saline and fetal arterial blood was collected for blood gas analysis. The fetal sagittal vein catheter was placed on an infusion pump with continuous infusion of heparinized saline (~1.5 mL/hr) to maintain patency. Ewes were allowed food and water ad libitum, and all surgical, post-operative and experimental procedures followed the guidelines provided by the Canadian Council on Animal Care and the University of Western Ontario Council on Animal Care.

3.2.3 Physiological Measurements

On the day of experimental study, strain gauge transducers (Statham model, P-2310, Gould, Oxnard CA), a physiograph recorder (model 78D, Grass Instrument Co., Quincy MA) and a PowerLab recording unit (model ML795, AdiInstruments, Colorado Springs, CO) were used to record all physiological data, and included measurements of fetal arterial blood pressure, fetal heart rate (obtained from the pulse pressure wave) and amniotic pressure. Fetal ECOG potentials were passed through a passive band-pass filter, 0.3 to 30 Hz on the preamplifier, and then further processed by means of a frequency integrator with the ECOG frequency shift displayed separately on the chart recorder.

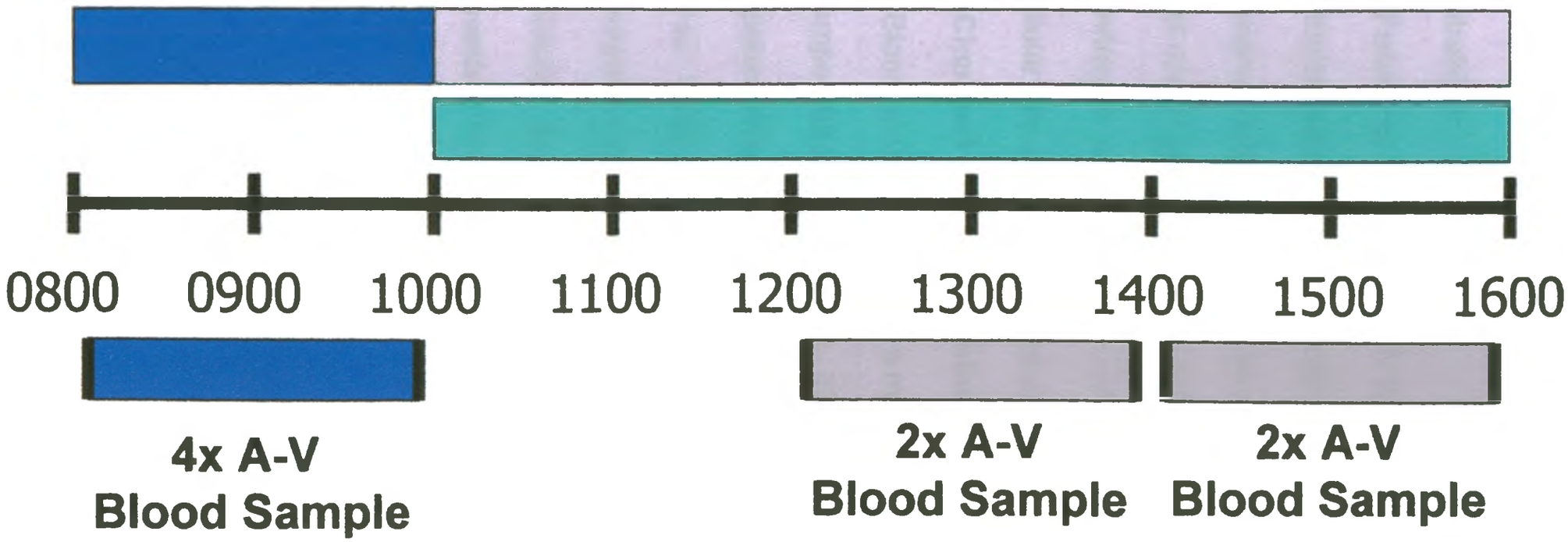
Animals were studied over an 8 hour period from ~8am until 4pm comprised of a 2 hour control period and a subsequent 6 hour experimental period. Following initiation of the control period recording, a baseline fetal arterial blood sample was taken and analyzed for pH, pO2, pCO2, hemoglobin, oxygen

saturation, glucose and lactate. As well during the control period, changes in electrocortical activity were monitored and 2 low voltage and 2 high voltage epochs were studied, with cerebral arterial-venous (A-V) blood samples collected from the brachiocephalic artery (1.5 mL) and the sagittal vein (1.5 mL) at least 3 minutes into each state epoch. Electrocortical activity was determined from the ECOG frequency and amplitude. The 2 hour control period was followed by a 6 hour experimental period, during which all animals received L-[1-^{13}C] leucine in sterile normal saline initially as a 5 mL bolus injection of 16.8 μmol/mL followed by a continuous infusion at a rate of 0.53μmol/min/kg estimated fetal weight. Additionally, animals in the Hyper AA group received an infusion of a mixed amino acid solution (Primene® 10%; Baxter Corporation; Toronto, Ontario; 10μM/min/kg estimated fetal wt; specific composition in Table 3.1) while animals in the Hypo AA group received a co-infusion of insulin (4mU/min/kg estimated fetal wt.) and 10% dextrose (initial rate of ~12mL/hr with infusion rates adjusted to maintain control plasma glucose concentrations). Arterial blood samples were drawn at 20, 40 and 80 minutes after the initiation of the infusions to monitor hemoglobin, oxygen saturation, glucose and lactate, as well as plasma amino acid concentrations and atom percent excess (APE) of the L-[1-^{13}C] leucine to ensure that these variables were achieving steady state levels. During the last 4 hours of the experimental period, cerebral A-V blood samples were taken during each of 2 low voltage and 2 high voltage epochs (Figure 3.2). All A-V blood samples were analyzed for oxygen saturation, hemoglobin, glucose and lactate. The remaining whole blood was then spun (4°C, 5 minutes at 10000*g), the

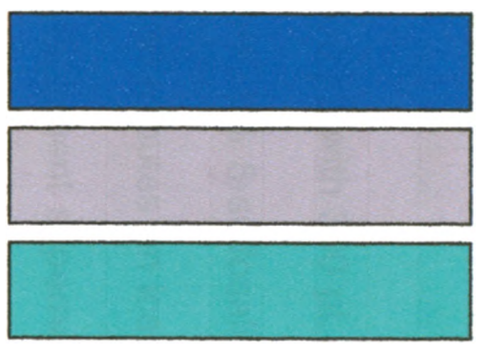
Table 3.1 Composition of the amino acid infusate. Values are those stated by the manufacturer (Primene® 10%; Baxter Corporation; Toronto, Ontario).

Amino Acid	g/L	µmol/L
Alanine	8.0	90.0
Arginine	8.4	48.0
Aspartic Acid	6.0	45.0
Cysteine	1.9	16.5
Glutamic Acid	10.0	67.5
Glycine	4.0	54.0
Histidine	3.8	24.0
Isoleucine	6.7	51.0
Leucine	10.0	76.5
Lysine	11.0	75.0
Methionine	2.4	16.5
Ornithine	3.2	24.0
Phenylalanine	4.2	25.5
Proline	3.0	25.5
Serine	4.0	37.5
Taurine	0.6	4.5
Threonine	3.7	31.5
Tryptophan	2.0	10.5
Tyrosine	0.5	3.0
Valine	7.6	64.5

Figure 3.2 Timeline of the 8 hour experiment, consisting of a 2 hour control period followed by a 6 hour experimental period. Following attainment of steady state plasma L-[1-^{13}C]-leucine enrichment there were 2 blood sampling periods between 1200-1400h and 1400-1600h. Paired blood samples from the brachiocephalic artery and sagittal sinus were collected at least 3 minutes into 2 LV/REM and 2 HV/NREM epochs during both the control and experimental period. All samples were analyzed for oxygen saturation, hemoglobin, glucose and lactate.



Continuous monitoring of FHR, FBP, behavioural state, amniotic pressure



Control period

Steady state infusion of insulin/dextrose or Primene

Steady state infusion of C13-Leucine tracer

plasma carefully titrated off and divided into 2 equal aliquots. The plasma aliquots were then frozen at -80°C for subsequent analysis of amino acid concentrations.

Following completion of the 2 hour control and subsequent 6 hour experimental period, the ewe and fetus were immediately sacrificed. The fetus was weighed and the brain then rapidly removed, weighed and dissected into cerebral cortex and cerebellum fractions. Dissected tissues were flash frozen in liquid nitrogen and stored at -80°C for later analysis of protein-bound and intracellular free L-[1-^{13}C] leucine activity.

3.2.4 Chemical Analysis: Blood gases, pH and Glucose/Lactate

Blood gases and pH were measured using an ABL-500 blood gas analyzer with temperature corrected to 39.5°C (Radiometer, Copenhagen, Denmark). Hemoglobin levels and blood oxygen saturation were measured in duplicate using an OSM-3 Hemoximeter (Radiometer). Blood glucose and lactate measurements were made in duplicate with membrane bound glucose oxidase and D-lactate dehydrogenase, respectively (YSI 2300 Stat Plus; Yellow Springs Instruments, Yellow Springs, OH).

3.2.5 Amino Acid Analysis using High Pressure Liquid Chromatography (HPLC)

Samples and standards were prepared by mixing 200 µL with 300 µL of a cold SSA/AEC Solution (16.7 µL S-(2-aminoethly)-L-cystine, 5% 5-sulfosalicylic acid, 0.13 M tri-lithium citrate, pH 2.6). After standing for 30 minutes on ice, the samples were spun at 16000*g for 10 minutes and the subsequent supernatant

was then filtered through a 0.22 μm filter, collected, and analyzed for amino acid content.

Amino acid analysis was performed on a Beckman System Gold Nouveau system upgraded with 32-Karat software, using post column ninhydrin reaction and a 10 cm lithium column. The analysis was done using Beckman buffers (LiA (338063), LiB (338064), LiC (338065), Li-R (338086), Nin-Rx (338069). The instrument was calibrated using Beckman standards Std (338088), AN+(338156), and B+ (338157), supplemented with allo-isoleucine, glutamine, and homocitrulline.

3.2.6 Electrocortical Analysis

The onset of state epochs was determined by visual analysis of chart recordings. A period of ECOG activity maintained for a minimum of 3 minutes and with an electrocorticogram < 50 μV and high frequency activity met the criteria for a low voltage ECOG epoch, while that with an electrocorticogram of 100-200 μV and low frequency activity met the criteria for a high voltage ECOG epoch. A period of ECOG activity with an electrocorticogram of 50-100 μV or low and high voltage periods of less than 3 minutes duration was defined as indeterminate voltage ECOG activity. When a low or high voltage state epoch was interrupted by an indeterminate voltage period of less than 3 minutes duration, then the ECOG state epoch duration was calculated as the total duration of low or high voltage ECOG activity of both segments.

3.2.7 Data Analysis

Since there were no evident changes in fetal arterial blood gases and pH, oxygen saturation and hemoglobin, glucose and lactate, and amino acid concentrations in relation to low and high voltage ECOG activity, all of these measurements during the control period were averaged to obtain a mean control value for each animal for each of these parameters. The 6 hour experimental period was divided into 2 hour time blocks, and as for the control period, all arterial blood measurements within each of these 2 hour periods then averaged to obtain mean values for each animal for each of the blood parameters. ECOG activity presented as percent time low voltage, high voltage and indeterminate voltage and duration of low and high voltage ECOG epochs, and mean arterial blood pressure and fetal heart rate, were likewise averaged to obtain mean values for each animal for the control period and each of the three 2 hour experimental period time blocks. Data for these blood and biophysical measurements are presented as grouped means ± SEM for the Hyper and Hypo AA group animals with significance determined by analysis of variance for repeated measures, followed by post hoc Dunnett's t-test if a significant F ratio was obtained ($p < 0.05$) (SAS/STAT Statistical Software, SAS Canada, Toronto, ON). Not all measurements were obtained for each animal due to catheter or electrode failure (see Tables 3.2 and 3.3, and Figures 3.3 and 3.4). The cerebral A-V amino acid data and thereby amino acid flux, and ^{13}C leucine analysis and thereby protein synthesis, are reported in Chapter 4.

3.3 RESULTS

3.3.1 Fetal Arterial Amino Acid Concentrations

Fetal arterial concentrations for acidic, basic, and neutral amino acids (determined by the Beckman System Gold Nouveau System), was measured for the 2 hour control period and subsequent 6 hour experimental period for both the Hyper AA group with Primene infusion and the Hypo AA group with insulin/dextrose infusion (Table 3.2). With the Primene infusion, the basic amino acids underwent the highest increase (on average by 43%), while the neutral amino acids were more variably increased (on average by 25%), with the majority of the increase attained within the initial 2 hours of the experimental period. Conversely, the acidic amino acids showed little change during the experimental period when compared to control period values. With the insulin/dextrose infusion, the basic amino acids displayed the greatest decrease (on average by 48%), while the neutral amino acids were decreased only on average by 30%, with the majority of the decrease attained by 2 to 4 hours of the experimental period. Again, the acidic amino acids showed little change.

Table 3.2 Arterial plasma amino acid measurements (μmol/L). Data presented as means ± SEM. Significance within groups based on 1-way ANOVA with post-hoc Dunnett's t-test, *$p<0.05$ versus control values; percent change from control values indicated in brackets when significant. Significance between groups based on non-paired t-test, $^{+}p<0.01$ between Primene and insulin infusion values.

	Control Period	Experimental Period 0 – 2 hrs	2 – 4 hrs	4 – 6 hrs
Primene Infusion n = 11				
Acidic AA				
Aspartate	33 ± 2	33 ± 2	33 ± 2	34 ± 3
Glutamate	71 ± 10[+]	74 ± 10	76 ± 11	75 ± 10
Basic AA				
Arginine	101 ± 6	131 ± 9*(30)	128 ± 8*(27)	122 ± 9*(21)
Histidine	35 ± 4	48 ± 4*(39)	50 ± 3*(44)	51 ± 3*(48)
Lysine	95 ± 12	143 ± 17*(51)	149 ± 16*(58)	152 ± 16*(61)
Neutral AA				
Alanine	169 ± 14	211 ± 20*(24)	237 ± 29*(40)	257 ± 35*(52)
Glutamine	354 ± 28[+]	383 ± 29*(8)	392 ± 28*(10)	402 ± 32*(14)
Glycine	452 ± 56	481 ± 57*(5)	475 ± 54	479 ± 61*(6)
Isoleucine	89 ± 6	115 ± 9*(29)	119 ± 9*(34)	123 ± 9*(38)
Leucine	171 ± 14	234 ± 21*(36)	241 ± 19*(42)	249 ± 19*(47)
Phenylalanine	92 ± 4	108 ± 5*(17)	112 ± 6*(22)	116 ± 6*(27)
Serine	582 ± 58	606 ± 61*(3)	613 ± 63*(5)	617 ± 67*(6)
Threonine	329 ± 38	355 ± 39*(7)	356 ± 40*(8)	356 ± 39*(8)
Tyrosine	132 ± 12	132 ± 11	131 ± 12	130 ± 11
Valine	387 ± 26	445 ± 31*(14)	462 ± 31*(19)	478 ± 29*(24)
Insulin Infusion n = 9				
Acidic AA				
Aspartate	34 ± 7	36 ± 7	30 ± 6	29 ± 6
Glutamate	144 ± 7[+]	143 ± 10	144 ± 9	145 ± 12
Basic AA				
Arginine	104 ± 17	79 ± 12*(-24)	49 ± 6*(-53)	39 ± 4*(-63)
Histidine	34 ± 4	31 ± 3	26 ± 3*(-24)	24 ± 3*(-28)
Lysine	104 ± 12	82 ± 9*(-20)	57 ± 5*(-45)	51 ± 3*(-52)
Neutral AA				
Alanine	187 ± 13	160 ± 8*(-13)	131 ± 9*(-30)	129 ± 9*(-31)
Glutamine	205 ± 13[+]	169 ± 9*(-15)	141 ± 13*(-31)	128 ± 10*(-36)
Glycine	666 ± 72	593 ± 54*(-10)	501 ± 47*(-24)	463 ± 38*(-31)
Isoleucine	85 ± 5	68 ± 5*(-19)	58 ± 4*(-32)	58 ± 4*(-32)
Leucine	156 ± 12	147 ± 17	121 ± 7*(-23)	119 ± 9*(-24)
Phenylalanine	90 ± 8	79 ± 8*(-11)	71 ± 7*(-21)	72 ± 8*(-20)
Serine	762 ± 66	702 ± 48*(-7)	608 ± 53*(-20)	571 ± 52*(-25)
Threonine	360 ± 56	329 ± 60*(-8)	265 ± 54*(-26)	250 ± 51*(-31)
Tyrosine	103 ± 11	85 ± 10*(-17)	68 ± 8*(-34)	68 ± 8*(-34)
Valine	352 ± 38	310 ± 37*(-11)	256 ± 29*(-27)	244 ± 27*(-31)

3.3.2 Fetal Cardiovascular Measurements

Fetal mean arterial blood pressure (MAP) and fetal heart rate (FHR), which were monitored throughout the control and experimental periods in both Hyper and Hypo AA groups, are shown in Table 3.3. During the control period MAP averaged 43 ± 3 mmHg, with an average FHR of 157 ± 6 beats/min. There were no changes observed in MAP for either the Hyper or Hypo AA group during the experimental period; however, fetal heart rate in both the Hyper AA and Hypo AA animals increased significantly from control values over the course of both experiments.

3.3.3 Fetal Blood Metabolites

Fetal arterial blood gases and pH were measured throughout the control period, with an average pO2 value of 19.8 ± 1.0 mmHg, pCO2 of 51.9 ± 0.8 mmHg and pH of 7.35± 0.01 for the Hyper AA group with Primene infusion. The Hypo AA group with insulin infusion had an average pO2 of 23.0 ± 1.0 mmHg, pCO2 of 51.6 ± 0.6 mmHg and pH of 7.36 ± 0.01. These values indicate that all animals were in good health at the time of the experiments. Fetal oxygen saturation levels decreased from control values with both Hyper and Hypo AA, but hemoglobin levels were unchanged over the course of the experiment in both groups (Table 3.3).

3.3.4 Glucose and Lactate Metabolism

Arterial glucose concentration was 0.9 ± 0.1 mmol/L and 0.8 ± 0.1 mmol/L during the control period for the Hyper and Hypo AA groups respectively (Table 3.3). Glucose levels remained unchanged during the experimental period for

Table 3.3 Cardiovascular and arterial blood measurements. Data presented as means ± SEM. Significance within groups based on 1 way ANOVA with post-hoc Dunnett's t-test, *$p<0.05$ versus control values. bpm = beats per minute

	Control Period	Experimental Period 0 – 2 hrs	2 – 4 hrs	4 – 6 hrs
Primene Infusion n=11				
O2 sat (%)	54 ± 1	51 ± 1*	49 ± 1*	46 ± 2*
Hb (gm/100mL)	10.1 ± 0.1	10.1 ± 0.1	9.9 ± 0.1	9.6 ± 0.1
Glucose (mmol/L)	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Lactate (mmol/L)	1.3 ± 0.1	1.4 ± 0.1	1.6 ± 0.2	2.1 ± 0.3*
FHR (bpm)	158 ± 4	170 ± 6*	170 ± 5*	166 ± 5*
MAP (mmHg)	45 ± 1	45 ± 2	46 ± 2	46 ± 2
Insulin Infusion n = 9				
O2 sat (%)	60 ± 1	63 ± 1	54 ± 1	52 ± 1
Hb (gm/100mL)	9.4 ± 0.1	9.5 ± 0.1	9.4 ± 0.1	9.2 ± 0.1
Glucose (mmol/L)	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Lactate (mmol/L)	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
FHR (bpm)	157 ± 8	172 ± 10*	186 ± 5*	179 ± 6*
MAP (mmHg)	41 ± 4	41 ± 4	39 ± 4	39 ± 4

both groups. Arterial lactate concentration increased slightly with the amino acid infusion, but was unchanged in the group receiving the insulin infusion (Table 3.3).

3.3.5 Fetal Electrocortical Activity

The percent time low-voltage, high-voltage, and indeterminate voltage ECOG activity for both the Hyper AA and Hypo AA groups were similar during the control period of study averaging 58 ± 2%, 36 ± 3%, and 6 ± 2%, respectively (Figure 3.3). The percent time for these ECOG activities showed little change for either group during the subsequent six hours of experimental study. The mean duration of low and high-voltage ECOG epochs for both animal groups were likewise similar during the control period of study averaging 18 ± 2 mins and 12 ± 1 mins, respectively (Figure 3.4). Again, the mean duration for these ECOG activities showed no significant change for either group during the experimental period.

Figure 3.3 Changes in fetal electrocortical activity during the control and infusion periods with data presented as 2 hour means ± SEM. Hyper AA, N=9; Hypo AA, N=8. Significance within/between groups based on 1 way ANOVA with post-hoc Dunnett's t-test, *$p<0.05$ versus control values.

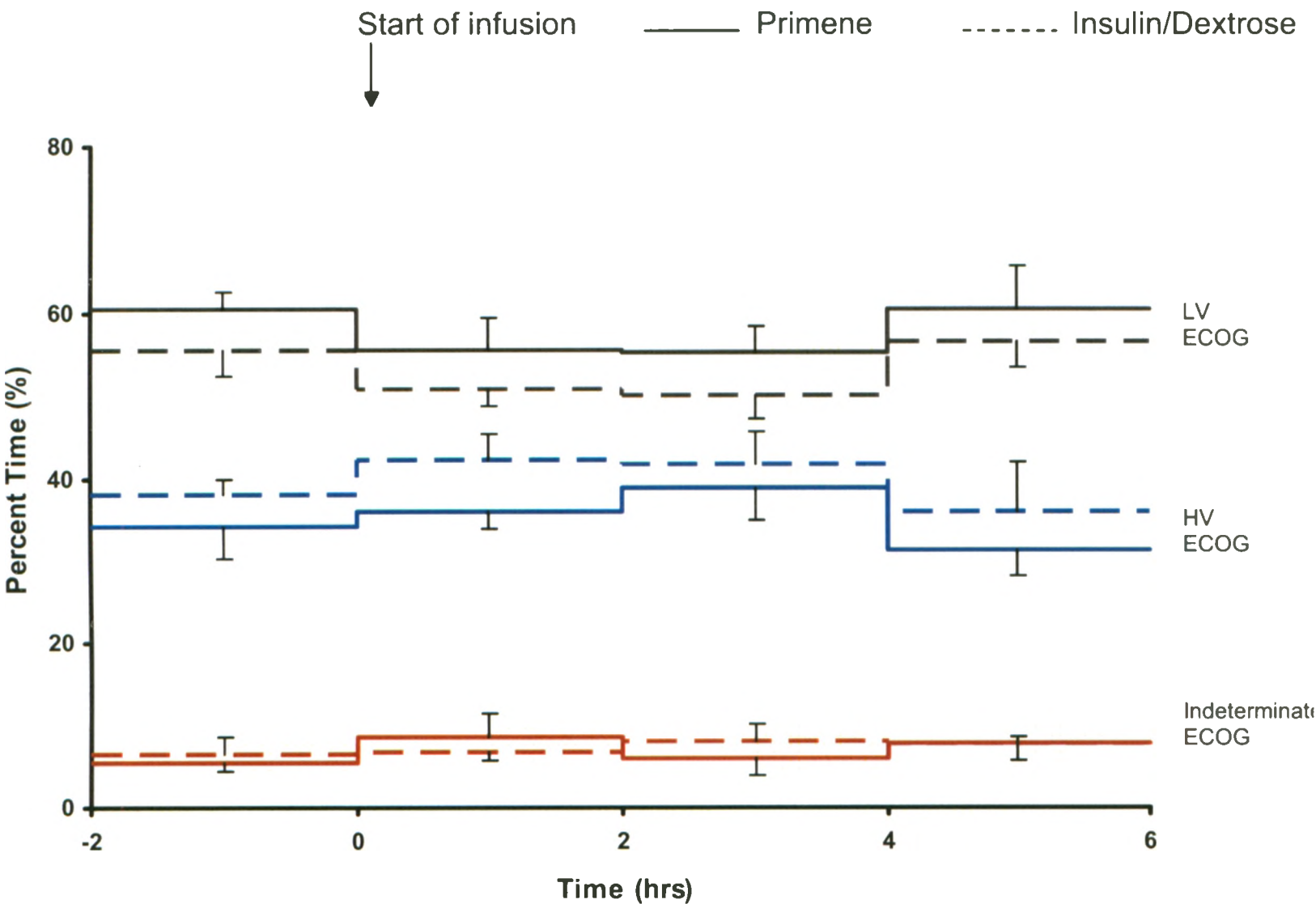
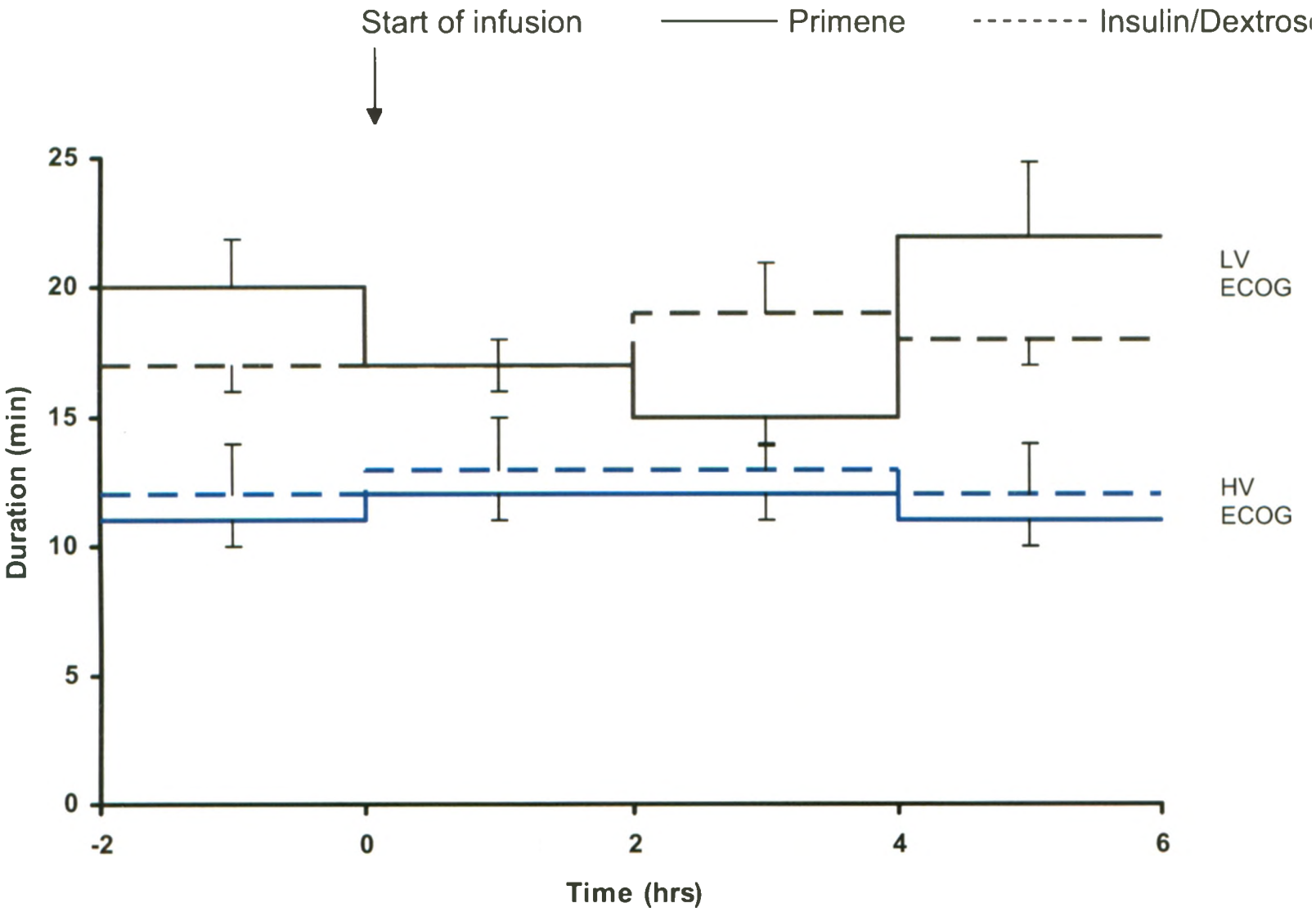

Start of infusion
Primene
Insulin/Dextrose
80
60
40
20
0
Percent Time (%)
LV ECOG
HV ECOG
Indeterminate ECOG
-2
0
2
4
6
Time (hrs)

Figure 3.4 Duration of low and high voltage ECOG epochs during the control and experimental periods with data presented as 2 hour means ± SEM (see Electrocortical Analysis for defining ECOG epochs). Significance within groups based on 1 way ANOVA with post-hoc Dunnett's t-test, *$p<0.05$ versus control values. LV=low voltage HV=high voltage


Start of infusion
Primene
Insulin/Dextros
Duration (min)
25
20
15
10
5
0
-2
0
2
4
6
Time (hrs)
LV
ECOG
HV
ECOG

3.4 DISCUSSION

My objective in the present study was to produce a modest increase and decrease in the levels of circulating amino acids in the ovine fetus near term and determine the impact on electrocortical activity. For the Hyper AA group, we used a continuous infusion of 10% Primene, an amino acid mixture commonly used in neonatal intensive care units, at 4mL/hr. This is similar to the infusion rate used by De Boo et al (6) but less than that used by Liechty et al (7) for comparable amino acid mixtures given to the ovine fetus near term and approximates 50% of estimated uptake assuming a fetal weight of 3 kg at this gestational age (2) albeit with considerable variance for individual amino acids (see Table 3.1). Accordingly, the increase in amino acid levels was similar to that reported by De Boo et al (5), but somewhat less than that reported by Liechty et al (7), as a function of the rate of infusion and composition of the infusate, and the continuing disposal of amino acids within the fetal compartment, including protein accretion, amino acid oxidation and the loss of amino acids back to and/or across the placenta (1, 2). It is of note that the basic amino acids were increased the most with the Primene infusion which for lysine at ~60% above control period values may be accounted for by the high infusate rate at 5.0 μmol/min relative to the estimated lysine uptake at 4.4 μmol/min for a 3 kg weight fetus (2). However, this increase may also reflect differential effects on amino acid transport and/or metabolism which increases with amino acid supplementation (6, 7) and would limit the increase in amino acid levels, but has not been well studied for individual amino acids. Conversely, tyrosine levels changed little with the Primene infusion

which is likely due to the low tyrosine levels in the infusate. Of interest, aspartate and glutamate levels also changed very little despite their adequate supply which may instead be attributed to their shared transporter system and rapid clearance through fetal and/or placental oxidation (1, 2, 25).

For the Hypo AA group, we used a hyperinsulinemic euglycemic clamp technique which is a well established method (9, 10) that decreases circulating amino acids while maintaining glucose levels. The rate of insulin/dextrose infusion was similar to that used by Wilkening et al (10) in fetal sheep near term and accordingly, the decrease in amino acid levels was likewise similar. This can be attributed to an insulin-mediated increase in the utilization of amino acids by the fetus and placenta which likely involves both amino acid oxidation and protein synthesis (6, 7, 9, 10), but again has not been well studied for individual amino acids. It is thus of interest that the basic amino acids were now decreased the most which may reflect differential effects of insulin on amino acid transport and/or metabolism. Such an effect, if maximally increasing the disposal of a given amino acid with the insulin infusion (thereby maximizing the decrease in that amino acid), might be expected to maximally increase the disposal of that same amino acid with the mixed amino acid infusion with insulin again increased (6) (thereby now minimizing the increase in that amino acid). This may also depend upon the extent to which insulin is increased (6, 7, 9, 10) being considerably greater with the insulin infusion (6, 10). The acidic amino acids aspartate and glutamate were again little changed despite their apparent oxidative clearance with the mixed amino acid infusion, and might instead

indicate a tight regulation of these amino acids as gluconeogenic precursors to protect glucose levels with their release from peripheral tissues (26).

The mixed amino acid and insulin/dextrose infusions resulted in arterial blood and cardiovascular changes which are well described by others (6, 7, 9, 10, 27-29). Both the Hyper AA and the Hypo AA groups showed a marginal decrease in oxygen levels which can be attributed to an insulin-mediated increase in oxygen consumption and thereby O_2 fractional extraction by the fetus (28). Both animal groups also showed a modest increase in FHR which can likewise be attributed to the insulin-mediated increase in fetal metabolic rate and an associated increase in cardiac output (29). Of interest, the decrease in oxygenation and increase in FHR was similar for the two groups although insulin levels were presumably much higher with the insulin/dextrose infusion (6, 10), suggesting a threshold effect with these insulin-mediated metabolic effects in the fetus. Somewhat surprising was the increase in lactate with the mixed amino acid infusion which may relate in part to the interconversions between amino acids and the products of carbohydrate catabolism, but was largely contributed to by one animal which otherwise appeared normal.

During the control period of study, low-voltage and high-voltage ECOG activity as measured were evident 58% and 36% of the time, respectively, which is similar to that we and others have previously reported for the ovine fetus near term (17, 27, 30). Likewise, the mean duration of the low and high-voltage ECOG epochs at 18 mins and 12 mins, respectively, is similar in agreement with what has been previously reported (31). However, despite the modest overall

increase and decrease in circulating amino acids with the Primene and insulin/dextrose infusions, respectively, we observed no significant change in fetal ECOG activity over the six hours of experimental study. While the reasons are not readily apparent for the lack of a behavioural state effect in the ovine fetus as herein studied in contrast to the sleep state effects reported in adult animals with amino acid alterations (32-34) possible explanations can be offered. The transport of amino acids across the blood-brain barrier (BBB) is an important control point for the regulation of metabolic processes within the brain, including protein synthesis and neurotransmitter production, and is affected by the transport capacity of the various amino acid BBB transporters, amino acid affinity for these transporters, and amino acid plasma concentrations (35). While the increases and decreases in plasma amino acids as studied would be expected to have corresponding effects on cerebral protein synthesis and precursor-related neurotransmitter production, it is possible that the amino acid changes were of insufficient duration, or degree, given the low k_m (high affinity) values reported for BBB amino acid transport such that the neutral and basic amino acid transporters are normally highly saturated at physiologic concentrations of plasma amino acids (35). In fact, the cerebral protein synthesis rates differed little between the Hyper AA and Hypo AA animal groups (discussed in Chapter 4) consistent with their similar and unchanging behavioural state activity, to the extent that cerebral protein synthesis does impact on REM state regulation (21) and thereby affects REM state incidence and/or duration. Moreover, given the low k_m values for the BBB transporters, a selective increase or decrease in the plasma concentration

of a single amino acid is more likely to effect the brain availability of that amino acid as well as the other amino acids competing for the shared BBB carrier system, than a generalized increase or decrease in all amino acids (35) as in the present study.

Previous studies in fetal sheep near term have shown that fasting hypoglycaemia results in decreased low-voltage ECOG (36), which may involve decreases in the blood tryptophan-to-neutral amino acid ratio and thereby brain tryptophan and its metabolic product serotonin (37) as a regulator of REM sleep activity (38, 39). However, glucose infusion to fasted animals only increased low-voltage ECOG to control levels (36) and direct infusion of insulin to the fetus failed to increase low-voltage ECOG further (27), indicating that if increases in the blood tryptophan-to-neutral amino acid ratio and thereby in brain tryptophan and serotonin are now occurring, there is a limit to the associated increase in REM generation. The lack of behavioural state change in the present study with modest increases and decreases in circulating amino acids indicates that those amino acid dependent neurotransmitters involved with sleep state modulation are unlikely to be changed or at their limit for such modulation. The lack of behavioural state change also indicates that the administration of amino acid supplements as often required for infants in neonatal intensive care units, is unlikely to impact on the brain's growth and development to the extent that behavioural state activity has a role and reflects such (13, 30, 31).

3.5 REFERENCES

1. Battaglia FC. New concepts in fetal and placental amino acid metabolism. J Anim Sci. 1992 Oct;70(10):3258-63.

2. Lemons JA, Adcock EW,3rd, Jones MD,Jr, Naughton MA, Meschia G, Battaglia FC. Umbilical uptake of amino acids in the unstressed fetal lamb. J Clin Invest. 1976 Dec;58(6):1428-34.

3. Battaglia FC, Meschia G. Fetal nutrition. Annu Rev Nutr. 1988;8:43-61.

4. Cetin I, Marconi AM, Bozzetti P, Sereni LP, Corbetta C, Pardi G, et al. Umbilical amino acid concentrations in appropriate and small for gestational age infants: A biochemical difference present in utero. Am J Obstet Gynecol. 1988 Jan;158(1):120-6.

5. Ronzoni S, Marconi AM, Cetin I, Paolini CL, Teng C, Pardi G, et al. Umbilical amino acid uptake at increasing maternal amino acid concentrations: Effect of a maternal amino acid infusate. Am J Obstet Gynecol. 1999 Aug;181(2):477-83.

6. de Boo HA, van Zijl PL, Smith DE, Kulik W, Lafeber HN, Harding JE. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr Res. 2005 Aug;58(2):270-7.

7. Liechty EA, Boyle DW, Moorehead H, Auble L, Denne SC. Aromatic amino acids are utilized and protein synthesis is stimulated during amino acid infusion in the ovine fetus. J Nutr. 1999 Jun;129(6):1161-6.

8. Oliver MH, Harding JE, Breier BH, Evans PC, Gluckman PD. Glucose but not a mixed amino acid infusion regulates plasma insulin-like growth factor-I concentrations in fetal sheep. Pediatr Res. 1993 Jul;34(1):62-5.

9. Thureen PJ, Scheer B, Anderson SM, Tooze JA, Young DA, Hay WW,Jr. Effect of hyperinsulinemia on amino acid utilization in the ovine fetus. Am J Physiol Endocrinol Metab. 2000 Dec;279(6):E1294-304.

10. Wilkening RB, Boyle DW, Teng C, Meschia G, Battaglia FC. Amino acid uptake by the fetal ovine hindlimb under normal and euglycemic hyperinsulinemic states. Am J Physiol. 1994 Jan;266(1 Pt 1):E72-8.

11. McIntosh GH, Baghurst KI, Potter BJ, Hetzel BS. Foetal brain development in the sheep. Neuropathol Appl Neurobiol. 1979 Mar-Apr;5(2):103-14.

12. Makowski EL, Schneider JM, Tsoulos NG, Colwill JR, Battaglia FC, Meschia G. Cerebral blood flow, oxygen consumption, and glucose utilization of fetal lambs in utero. Am J Obstet Gynecol. 1972 Oct 1;114(3):292-303.

13. Czikk MJ, Sweeley JC, Homan JH, Milley JR, Richardson BS. Cerebral leucine uptake and protein synthesis in the near-term ovine fetus: Relation to fetal behavioral state. Am J Physiol Regul Integr Comp Physiol. 2003 Jan;284(1):R200-7.

14. Govinatzki MT, Velleda LS, Trindade VM, Nagel FM, Bueno D, Perry ML. Amino acid metabolism in rat hippocampus during the period of brain growth spurt. Neurochem Res. 1997 Jan;22(1):23-6.

15. Keen RE, Barrio JR, Huang SC, Hawkins RA, Phelps ME. In vivo cerebral protein synthesis rates with leucyl-transfer RNA used as a precursor pool: Determination of biochemical parameters to structure tracer kinetic models for positron emission tomography. J Cereb Blood Flow Metab. 1989 Aug;9(4):429-45.

16. Garabal MV, Arevalo RM, Diaz-Palarea MD, Castro R, Rodriguez M. Tyrosine availability and brain noradrenaline synthesis in the fetus: Control by maternal tyrosine ingestion. Brain Res. 1988 Aug 9;457(2):330-7.

17. Szeto HH, Hinman DJ. Prenatal development of sleep-wake patterns in sheep. Sleep. 1985 Dec;8(4):347-55.

18. Richardson BS. The effect of behavioral state on fetal metabolism and blood flow circulation. Semin Perinatol. 1992 Aug;16(4):227-33.

19. Nakanishi H, Sun Y, Nakamura RK, Mori K, Ito M, Suda S, et al. Positive correlations between cerebral protein synthesis rates and deep sleep in macaca mulatta. Eur J Neurosci. 1997 Feb;9(2):271-9.

20. Ramm P, Smith CT. Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. Physiol Behav. 1990 Nov;48(5):749-53.

21. Adam K. Sleep as a restorative process and a theory to explain why. Prog Brain Res. 1980;53:289-305.

22. Rao N, Keen A, Czikk M, Frasch M, Richardson BS. Behavioural state linkage in the ovine fetus near term. Brain Res. 2009 Jan 23;1250:149-56.

23. Benington JH, Heller HC. REM-sleep timing is controlled homeostatically by accumulation of REM-sleep propensity in non-REM sleep. Am J Physiol. 1994 Jun;266(6 Pt 2):R1992-2000.

24. Monnier M, Gaillard JM. Biochemical regulation of sleep. Experientia. 1980 Jan 15;36(1):21-4.

25. Moores RR,Jr, Vaughn PR, Battaglia FC, Fennessey PV, Wilkening RB, Meschia G. Glutamate metabolism in fetus and placenta of late-gestation sheep. Am J Physiol. 1994 Jul;267(1 Pt 2):R89-96.

26. Battezzati A, Benedini S, Fattorini A, Piceni Sereni L, Luzi L. Effect of hypoglycemia on amino acid and protein metabolism in healthy humans. Diabetes. 2000 Sep;49(9):1543-51.

27. Richardson BS, Hohimer AR, Bissonnette JM, Machida CM. Insulin hypoglycemia, cerebral metabolism, and neural function in fetal lambs. Am J Physiol. 1985 Jan;248(1 Pt 2):R72-7.

28. Milley JR, Papacostas JS, Tabata BK. Effect of insulin on uptake of metabolic substrates by the sheep fetus. Am J Physiol. 1986 Sep;251(3 Pt 1):E349-56.

29. Milley JR. Effect of insulin on the distribution of cardiac output in the fetal lamb. Pediatr Res. 1987 Aug;22(2):168-72.

30. Richardson BS. Metabolism of the fetal brain: Biological and pathological development. In: Hanson MA, editor. The Fetal and Neonatal Brain Stem. Cambridge, UK: Cambridge University Press; 1991. p. 87-105.

31. Richardson BS, Patrick JE, Abduljabbar H. Cerebral oxidative metabolism in the fetal lamb: Relationship to electrocortical state. Am J Obstet Gynecol. 1985 Oct 15;153(4):426-31.

32. Danguir J, Nicolaidis S. Intravenous infusions of nutrients and sleep in the rat: An ischymetric sleep regulation hypothesis. Am J Physiol. 1980 Apr;238(4):E307-12.

33. Rojas-Ramirez JA, Aguilar-Jimenez E, Posadas-Andrews A, Bernal-Pedraza JG, Drucker-Colin RR. The effects of various protein synthesis inhibitors on the sleep-wake cycle of rats. Psychopharmacology (Berl). 1977 Jul 18;53(2):147-50.

34. Moja EA, Mendelson WB, Stoff DM, Gillin JC, Wyatt RJ. Reduction of REM sleep by a tryptophan-free amino acid diet. Life Sci. 1979 Apr 16;24(16):1467-70.

35. Pardridge WM. Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. Neurochem Res. 1998 May;23(5):635-44.

36. Richardson BS, Hohimer AR, Bissonnette JM, Machida CM. Cerebral metabolism in hypoglycemic and hyperglycemic fetal lambs. Am J Physiol. 1983 Nov;245(5 Pt 1):R730-6.

37. Wurtman RJ, Fernstrom JD. Control of brain neurotransmitter synthesis by precursor availability and nutritional state. Biochem Pharmacol. 1976 Aug 1;25(15):1691-6.

38. Mendelson WB, Gillin JC, Wyatt RJ. Human sleep and its disorders. Plenum. 1977.

39. McCarley RW. Neurobiology of REM and NREM sleep. Sleep Med. 2007 Jun;8(4):302-30.

CHAPTER FOUR

CEREBRAL PROTEIN SYNTHESIS IN THE OVINE FETUS NEAR TERM WITH INDUCED HYPER AND HYPO AMINO ACIDEMIA [2]

---

[2] A version of this chapter has been submitted for publication: MacLachlan JN, McCallum JD, Smith N, Matushewski B, Richardson BS. *Cerebral protein synthesis in the ovine fetus near term with induced hyper and hypo amino acidemia.* Neonatology, *submitted July 2009.*

4.1 INTRODUCTION

Protein synthesis during brain growth and development has been studied both pre- and postnatally in sheep (1-3) and postnatally in rats (4), since proteins are fundamental components of all tissue elements and with protein metabolism directly related to maturational events (4). Initial study in the near-term ovine fetus by Schaefer and Krishnamurti (1) using a tyrosine isotopic-dilution technique reported high rates of cerebral protein synthesis with a protein fractional synthetic rate (FSR) between 14 and 37%/day, ie., the percentage of brain protein that is newly synthesized per day. In a more recent study using a continuous tracer infusion of L-[1-^{14}C]-leucine, we (3) showed a cerebral protein FSR of ~20%/day for the near-term ovine fetal brain, which was similar when measured regionally in the cerebral cortex and cerebellum. Postnatal studies in rats have also shown high rates of brain protein synthesis during early development, with peak values occurring shortly after birth and gradually decreasing thereafter (4, 5). While high rates of cerebral protein synthesis are thus evident during early life in both sheep and rats in support of the brain's growth and development, these rates are considerably greater than anticipated protein accretion, indicating that protein degradation must also play an important role in the growth and development of the brain (3, 5).

The transport of amino acids across the blood brain barrier (BBB) is an important control point for the regulation of metabolic processes within the brain, including protein synthesis and is affected by the transport capability of the various amino acid BBB transporters, amino acid affinity for these transporters

and amino acid plasma concentrations (6). The precursor availability of amino acids within the brain might then impact on protein synthesis and/or degradation, ie., protein turnover, thereby impacting growth processes. While the effects of increasing and decreasing amino acid availability on protein turnover in the developing brain have yet to be determined, postnatal studies as well as that in the whole fetus would suggest that alterations in protein synthesis and/or degradation should be considered. Studies in both animals and humans with acute or chronic amino acid supplementation report a protein-anabolic effect at the whole-body level and for select organ tissues (7-12). Similarly, studies in the ovine fetus with a mixed amino acid infusion report protein accretion to be increased at the whole-body level (13, 14). These protein effects can be attributed in part to related increases in insulin (14) which promotes protein accretion when given to the ovine fetus (15-17), although this effect has not been studied in the brain. Conversely, hemodialysis induced reduction in plasma amino acids in adult pigs leads to an inhibition of protein synthesis (18). Furthermore, maternal protein restriction during pregnancy in the rat and a presumed reduction in essential amino acid availability alters growth processes in the brain during early development (19).

In the present study we used the chronically catheterized ovine fetus at ~0.85 of gestation to determine the effects of hyper and hypo amino acidemia on cerebral protein synthesis during early brain development using non-radioactive L-[1-^{13}C]-leucine tracer technology. A mixed amino acid infusate and an insulin euglycemic clamp technique were used to induce increases and decreases in

circulating amino acid levels of ~30 to 40% on average, respectively, as we (20) and others (13, 14, 16, 17) have previously described. Protein FSR values within the brain were calculated from the enrichment of protein-bound [^{13}C]-leucine as measures of cerebral protein synthesis. Cerebral flux for leucine and leucine enrichment during continuous tracer infusion with L-[1-^{13}C]-leucine have been determined as additional measures of amino acid metabolism. Leucine disposal rates (DR_{leu}) were calculated from the plasma [^{13}C]-leucine enrichment values at steady state as measures of fetal leucine utilization for protein synthesis and oxidation in relation to the altered amino acid levels.

4.2 MATERIALS AND METHODS

The surgical and experimental procedures, tissue collection, and plasma leucine measurement and analysis are reported in chapter 3.

4.2.1 Plasma Leucine Enrichment

Methods for measurement of plasma leucine enrichment are modified from that previously described by Loy et al (21). Plasma samples (0.1 ml) were acidified with 0.2 ml of 8.5N acetic acid and combined with 0.4 ml of HPLC-grade water and 0.02 µmol norleucine in 0.1 ml of 0.01N HCl as the internal standard. The combined solution of 0.8 ml was placed onto an ion-exchange column (Poly-Prep Chromatography Columns; Bio-Rad Laboratories, Hercules CA) conditioned for the removal of contaminants from both keto acids and amino acid fractions, and allowed to flow through under the influence of gravity. The column was washed twice (2 x 2 ml) with deionized water and the amino acids then eluted

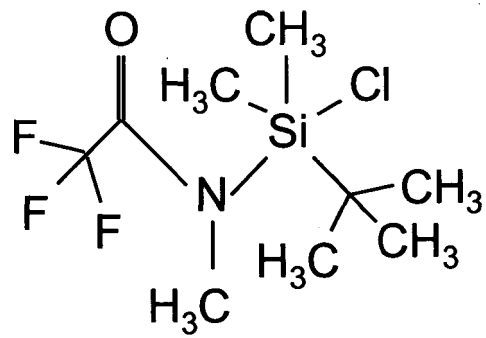
with 1.8 ml 10 N NH_4OH and the eluate dried overnight under vacuum. To the dried amino acid sample, 75 µL of the solvent dimethylformamide, 50 µL of the derivatizing reagent N-(tert-butyldimethylsilyl)-N-trifluoroacetamide + tert-butyldimethylchlorosilane (99:1) (Sigma-Aldrich, Oakville, ON), and 5 µL of the catalyst triethylamine were added. The tubes were then capped, vortexed-mixed and allowed to react at room temperature for 15 minutes (see Figure 4.1 for derivatization reaction). Derivatized samples (2 µL) were injected into a split/splitless injector of an Agilent 6890 gas chromatograph/ mass selective detector MS5973 (Sao Paolo, CA). The gas chromatographic conditions that were used are as follows: column: 30 m, SPB-5 (5% phenyl, 95% methylsilicone), 0.250 mm i.d. with 0.25 µm film thickness (Supelco) with helium as the carrier gas. The split/splitless injector was at 250°C with a split ratio of 60:1. The interface temperature was set at 280°C. The column oven was programmed to run from an initial temperature of 145°C to 220°C at 10°C/min, then to 300°C at 50°C/min with a final hold time of 5 minutes. Total run time was 14.2 minutes. Under these conditions the tert-butyldimethylsilyl derivative of leucine and norleucine were baseline resolved from each other and other amino acids with similar retention times.

4.2.2 Tissue Leucine Measurements

Tissue concentrations for leucine and [^{13}C]-leucine enrichment were measured using a modification described by Horber et al (22) and Czikk et al (3). Frozen tissue samples from the cerebral cortex and cerebellum were weighed (~0.3 – 1.0 g) and 4 mL of 6% sulfosalicylic acid (SSA) was added. Samples

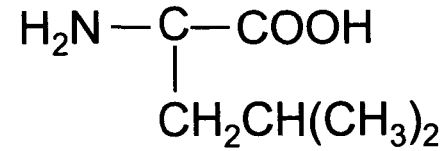
Figure 4.1 Derivatization reaction scheme for leucine. MTBSTFA = N-(tert-butyldimethylsilyl)-tri-fluoroacetamide.

2

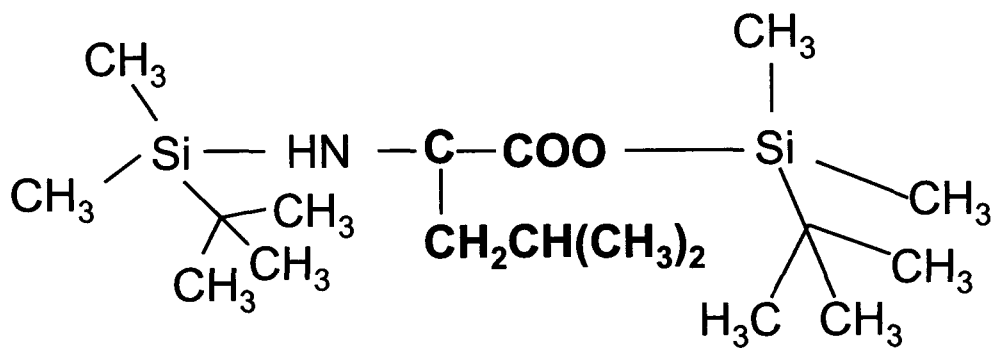


MTBSTFA

+



Leucine



Derivatized Leucine

were homogenized with a high-speed power homogenizer on ice for ~45 seconds and then centrifuged for 40 minutes at 1500*g and 4°C. The protein-free supernatant (containing the intracellular free leucine fraction) was decanted and kept cold in a separate tube, with this process repeated two more times adding 4 mL 6% SSA each time. To the pooled supernatant 250 µL (26.2mg/100ml) of norleucine internal standard was added prior to freezing at –80°C. The pellet (containing the protein-bound leucine) was dried overnight in a drying oven at 65°C. The dried pellet was weighed and 250 µL of norleucine internal standard was added prior to adding 4 mL of 6N HCl and purging with nitrogen. Hydrolysis was carried out in a metal heating block (110°C) for 30 hours. Following hydrolysis, the HCl was removed via evaporation using a ThermoSavant SpeedVac. When nearly dry, 4 mL water was added, and evaporation continued as above, and the process repeated again until all HCl was removed. The dried residue was then re-suspended in 4 mL of water.

Ion exchange columns were prepared as described for the plasma leucine analysis. The frozen supernatant samples were thawed and along with a set of leucine standards then applied to individual ion exchange columns. Columns were washed with 4 x 2 mL 0.01N HCl, drained to waste, washed with 4 mL water, and again drained to waste. The intracellular free leucine samples were then eluted off the columns using 1 x 1.75 mL 10N NH_4OH and 200 µL of the eluate taken to dryness in the SpeedVac with the subsequent dry sample derivatized and run on the GC/MS as described for the plasma samples. The protein bound samples were placed on 3 mL resin bed ion-exchange columns,

washed 4 x 2 mL 0.01N HCl, drained to waste, washed with 4 mL water, drained to waste, with the leucine fraction collected by eluting with 6 mL 10N NH_4OH. 50 µL of the eluate was then taken to dryness and the subsequent dry sample was then derivatized and run on the GC/MS as described above.

4.2.3 Protein Synthesis Measurements

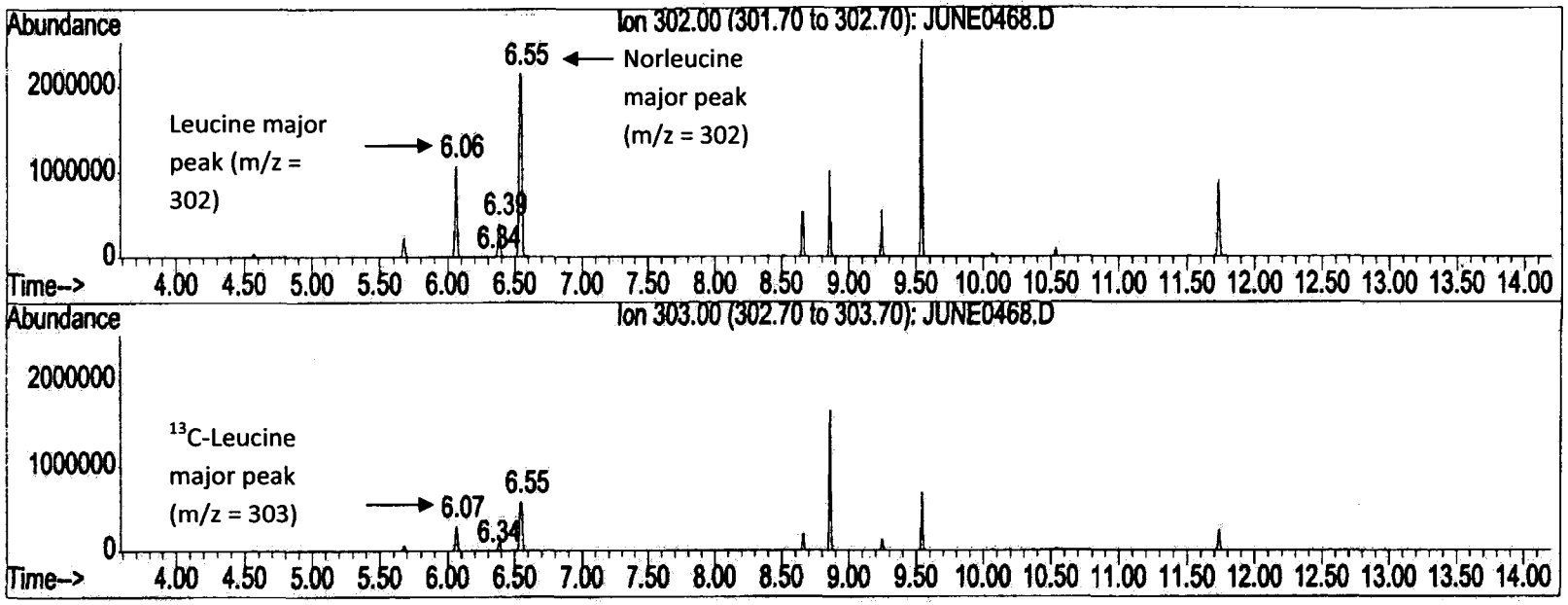
Leucine enrichment expressed as mole percent excess (MPE) during the infusion of L-[1-^{13}C]-leucine was calculated from ion abundance ratios (R) of the major fragments. Selected ion monitoring of the leucine peak was carried out at m/z 303 and 302 (Figure 4.2). The natural ratio of peak areas at 303 versus 302 (303/302 = R_0) was 0.2654 ± .0005 (n=15). The ratio at steady-state enrichment for the arterial plasma measurements and at animal necropsy for the tissue measurements (303/302 = R_S) was calculated as MPE using the following formula (21):

$$MPE = 100 (R_S - R_0) / [1 + (R_S - R_0]$$

The enrichment of [^{13}C]-leucine within arterial plasma (MPE_P) was determined using the steady-state measurements obtained over the last 4 hours of experimental study for both the Hyper and Hypo AA groups during which time the changes in plasma leucine values had largely stabilized (20). The enrichment of [^{13}C]-leucine within brain tissue protein-bound (MPE_{PB}) and intracellular free (MPE_{IF}) were determined using the tissue measurements obtained from the pellet and supernatant, respectively.

Cerebral protein FSR is the percentage of brain protein that is newly synthesized per day. It can be calculated for each brain region using respective

Figure 4.2 Representative GC/MS chromatogram of leucine, ^{13}C-leucine and norleucine. Selected ion monitoring of the leucine peak was carried out at a mass/charge ratio (m/z) 303 and 302.


Abundance
Ion 302.00 (301.70 to 302.70): JUNE0468.D
2000000
1000000
0
6.55
Norleucine
major peak
(m/z = 302)
Leucine major
peak (m/z =
302)
6.06
6.39
6.84
Time-->
4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50 8.00 8.50 9.00 9.50 10.00 10.50 11.00 11.50 12.00 12.50 13.00 13.50 14.00
Abundance
Ion 303.00 (302.70 to 303.70): JUNE0468.D
2000000
1000000
0
13C-Leucine
major peak
(m/z = 303)
6.07
6.55
6.34
Time-->
4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50 8.00 8.50 9.00 9.50 10.00 10.50 11.00 11.50 12.00 12.50 13.00 13.50 14.00

MPE_{PB} and MPE_{IF} or MPE_P values, depending on which is considered to be more representative of the precursor pool, and the total infusion time (days) in the following formulae (3):

$$FSR\ (\%/day) = ([MPE_{PB} / MPE_{IF}] / \text{infusion time}) \times 100\%$$

or

$$FSR\ (\%/day) = ([MPE_{PB} / MPE_P] / \text{infusion time}) \times 100\%$$

4.2.4 Amino acid flux measurements

Fractional extraction of leucine and [^{13}C]-leucine was calculated as leucine or [^{13}C]-leucine uptake divided by leucine or [^{13}C]-leucine delivery, respectively:

$$\text{Leucine fractional extraction} = \frac{\text{relative Leu uptake}}{\text{relative Leu delivery}} \times 100\%$$

$$\text{Leucine fractional extraction} = \frac{[Leu]_a - [Leu]_v}{[Leu]_a} \times 100\%$$

4.2.5 Systemic [^{13}C]-leucine disposal rate measurements

The disposal rate of [^{13}C]-leucine (DR_{leu}) from the fetal plasma leucine pool was calculated from the rate of tracer leucine infusion into the fetal circulation (r^f) and the enrichment (MPE) of leucine in fetal arterial plasma in the following formula (21):

$$DR_{leu} = (100 \times [r^f / MPE_P]) - r^f$$

4.2.6 Data Analysis

Due to the absence of changes in fetal arterial oxygen saturation and hemoglobin, glucose and lactate, and amino acid concentrations in relation to low and high-voltage ECOG activity, all of these measurements during the control

period were averaged to obtain a mean control value for each animal for each of these parameters. The 6 h experimental period was divided into 2 h time blocks, and as for the control period, all arterial blood measurements within each of these 2 h periods then averaged to obtain mean values for each animal for each of the blood parameters.

For each animal studied, four separate tissue samples within the cerebral cortex and cerebellum were analyzed. MPE_{PB} and $_{IF}$ and respective leucine content values from the homogenates of each of the four separate tissue samples from the two brain regions were then averaged to obtain a single mean value for each brain region for each animal for each of these parameters. Arterial plasma leucine enrichment measurements were conducted in duplicate with values then averaged to obtain a mean value for each blood sample.

Data for these blood and brain tissue measurements are presented as grouped means ± SEM for the Hyper and Hypo AA group animals. Within group differences were determined by analysis of variance (ANOVA) for repeated measures, followed by post hoc Dunnett's *t* test if a significant F ratio was obtained ($p<0.05$) (SAS/STAT) Statistical Software, SAS Canada, Toronto, ON) or by paired *t* test analysis. Between group differences were analyzed by a non-paired *t* test with significance again taken at $p<0.05$. Not all measurements were obtained for each animal (see Table 4.1).

4.3 RESULTS

The fetal arterial blood gas, pH, glucose and amino acid data for the Hyper and Hypo AA group animals are presented in chapter 3.

4.3.1 Fetal Arterial Plasma and Cerebral Tissue Leucine Enrichment

Leucine enrichment in the arterial plasma achieved steady state values by 40 minutes of infusion. Figure 4.3 demonstrates mean leucine enrichment for both animal groups at time 0, 20, 40, and 80 minutes of the L-[1-^{13}C]-leucine infusion and thereafter over the last 4 hours of experimental study illustrating the attainment of the leucine enrichment plateau values. At this time the leucine enrichment values averaged 5.09 ± .45 and 4.88 ± .54 MPE for the Hyper and Hypo AA groups, respectively, which were not significantly different from one another (Table 4.1).

Cerebral tissue leucine enrichment was determined for both the intracellular-free and protein-bound components of the cerebral cortex and cerebellum. Intracellular-free leucine enrichment was similar for both regions of the brain and showed no differences between the two animal groups, with mean enrichment values of ~3.98 MPE for the Hyper AA and ~3.04 MPE for the Hypo AA animals (Table 4.1). However, protein-bound leucine enrichment was higher in the cerebral cortex than the cerebellum for both the Hyper and Hypo AA groups, 0.52 ± .06 vs 0.29 ± .03 MPE and 0.43 ± .04 vs 0.31 ± .04 MPE, respectively (both $p<.05$), although again there were no differences between the two animal groups (Table 4.1).

4.3.2 Cerebral Tissue Leucine Content and Protein Synthesis

Cerebral tissue leucine content was also determined for both the intracellular-free and protein-bound components of the cerebral cortex and cerebellum. Intracellular-free leucine content was similar for both regions of the

Figure 4.3. Arterial plasma L-[1-^{13}C]-leucine enrichment as measured throughout the infusion period. Leucine enrichment in the arterial plasma achieved steady state values by 40 minutes of infusion.

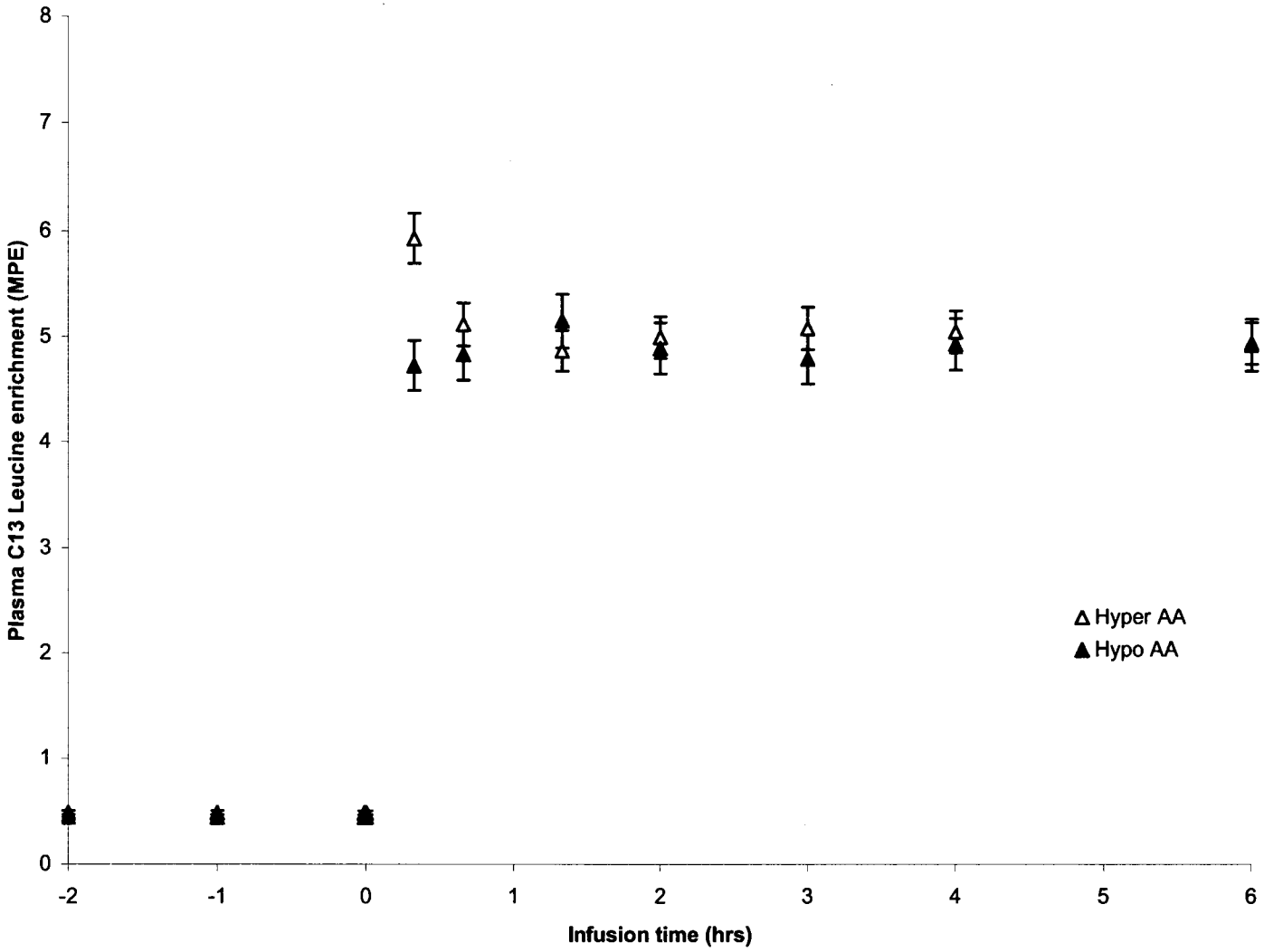

Plasma C13 Leucine enrichment (MPE)
8
7
6
5
4
3
2
1
0
-2
-1
0
1
2
3
4
5
6
Infusion time (hrs)
Hyper AA
Hypo AA

Table 4.1 Arterial plasma and cerebral tissue leucine enrichment. Data presented as mean ± SEM. *Plasma/cerebral tissue values; significance within groups determined by paired *t* test, **$p<0.05$; MPE = mole percent excess, P = plasma, IF = intracellular free, PB = protein-bound

	Primene Infusion (10 / 8)*	Insulin/Dextrose Infusion (8 / 8)*
MPE_P	5.09 ± .45	4.88 ± .54
MPE_{IF}		
Cerebral Cortex	4.21 ± .67	3.11 ± .32
Cerebellum	3.74 ± .66	2.97 ± .33
MPE_{PB}		
Cerebral Cortex	0.52 ± .06	0.43 ± .04
Cerebellum	0.29 ± .03**	0.31 ± .04**

brain and showed no significant differences between the two animal groups, with mean content values of ~ 0.29 μmol/g dry weight for the Hyper AA and ~ 0.24 μmol/g dry weight for the Hypo AA animals (Table 4.2). Protein-bound leucine content was likewise similar for the brain regions and animal groups with mean content values of ~ 3.55 μmol/g dry weight for the Hyper AA and ~ 3.42 μmol/g dry weight for the Hypo AA animals (Table 4.2). Fractional synthetic rates for the cerebral cortex and cerebellum were calculated using both the intracellular-free and plasma enrichment values for the precursor pool measurements providing for maximal and minimal FSR values, respectively, as previously discussed by Schaefer and Krishnamurti (1). While there were no significant differences between the Hyper and Hypo AA groups when comparing FSR values, cortical tissue values were significantly higher than respective cerebellar tissue values (all $p<.05$) (Table 4.2).

4.3.3 Cerebral amino acid flux

Leucine and leucine enrichment or MPE fractional extraction values for both animal groups averaged 2 to 4% and showed no significant change as measured over the course of the study (Table 4.3).

4.3.4 Fetal disposal rate

The leucine disposal rate (DR) as a measure of fetal utilization of leucine for protein synthesis and oxidation as well as loss across the placenta was determined from the plasma enrichment values at steady state as measured at 2 to 6 hours of experimental study. Mean DR values were significantly higher in the Hypo AA group animals receiving the insulin/dextrose infusion at 12.8 ± .3

Table 4.2. Cerebral tissue leucine content and protein synthesis. Data presented as mean ± SEM. Significance within groups determined by paired *t* test, *p<0.05; IF = intracellular free, PB = protein-bound, FSR = fractional synthetic rate; max values calculated using MPE_{IF}, min values calculated using MPE_p as precursor pool

	Primene Infusion (n=8)	Insulin/Dextrose Infusion (n=8)
Leucine $_{IF}$ (μmol/g)		
Cerebral Cortex	0.29 ± .03	0.24 ± .02
Cerebellum	0.28 ± .03	0.23 ± .02
Leucine $_{PB}$ (μmol/g)		
Cerebral Cortex	3.52 ± .10	3.39 ± .17
Cerebellum	3.58 ± .11	3.44 ± .13
FSR max (%/day)		
Cerebral Cortex	58 ± 3	59 ± 4
Cerebellum	31 ± 3*	41 ± 3*
FSR min (%/day)		
Cerebral Cortex	41 ± 4	38 ± 4
Cerebellum	24 ± 3*	26 ± 2*

Table 4.3 Cerebral substrate metabolism. Data presented as mean ± SEM.

	Control Period	Infusion Period 2 – 6 hrs
Primene infusion, n = 7		
leucine fractional extraction	0.02 ± .01	0.01 ± .01
^{13}C-leu MPE fractional extraction		0.06 ± .04
Insulin / Dextrose infusion, n = 7		
leucine fractional extraction	0.03 ± .03	0.06 ± .02
^{13}C-leu MPE fractional extraction		0.04 + .02

μmol/kg/min when compared to that of the Hyper AA group animals receiving the Primene infusion at 11.5 ± .2 μmol/kg/min (P<.01).

4.4 DISCUSSION

In the present study we have determined the effects of increased and decreased systemic amino acid levels on cerebral protein synthesis in the near term ovine fetus using a continuous tracer infusion of the stable isotope L-[1-^{13}C]-leucine thereby avoiding the use of radioactive materials. The L-[1-^{13}C]-leucine infusion rate used was slightly higher than that previously used by Loy et al (21) and calculated to give an infusion of isotopically labeled leucine similar to that we have previously reported using [^{14}C]-leucine (3). Steady-state plasma enrichment values were attained by ~40 minutes, which is somewhat earlier than that we (3) and others (21) have previously reported and can be attributed to the initial bolus of [^{13}C]-leucine given prior to the onset of the continuous infusion.

MPE_P values for both groups of animals as studied averaged 5%, which is higher than that reported by Loy et al (21) from the umbilical artery at ~3.2%, but consistent with the correspondingly higher infusion rates for [^{13}C]-leucine in the present study. MPE_{IF} values averaged 3.5% and while somewhat higher in the cerebral cortex tissues and the Hyper AA group animals, these differences were not significant. Of interest, Loy et al (21) report plasma [^{13}C]-KIC MPE values of ~2.1%, α-ketoisocaproic acid or α-KIC being interconvertible with leucine within the cell through reversible transamination and moving freely between the intra- and extracellular pools. To the extent that [^{13}C]-KIC MPE_P values then reflect

[^{13}C]-leucine MPE_{IF} values (22) and taking into account the higher infusion rate of [^{13}C]-leucine in the present study, the MPE_{IF} values herein reported are correspondingly higher than that reported by Loy et al (21). MPE_{PB} values averaged 0.4% and while little different between the two groups of animals, these values were significantly higher in the cerebral cortex tissues reflecting the higher rates of cerebral protein synthesis herein shown.

The measurement of tissue MPE_{PB} leucine along with that of the precursor pool at a defined time from the start of the tracer infusion provides a means of estimating the fractional synthetic rate of brain proteins, ie., the percentage of brain proteins newly synthesized per unit time. We utilized both plasma leucine enrichment (MPE_P) and tissue intracellular free leucine enrichment (MPE_{IF}) as the leucine precursor pools to protein synthesis. However, the protein synthetic rate that is calculated by using the plasma leucine enrichment defines only the unidirectional flux of plasma leucine into protein synthesis and will underestimate total unidirectional flux, since some of the leucine released by protein degradation within cells can be utilized without first recycling via the circulating blood. Conversely, the protein synthetic rate that is calculated by using the intracellular free leucine enrichment will overestimate total unidirectional flux to the extent that protein breakdown mainly releases unlabeled leucine thereby diluting the intracellular free leucine enrichment and with any loss of leucine enrichment from the intracellular free pool due to the unavoidable time-delay between cessation of the isotope infusion and the freezing of tissues.

FSR_{max} values for the cerebral cortex using MPE_{IF} as the precursor pool differed very little between the Hyper and Hypo AA animal groups and averaged 58% and 59%/day respectively, which is somewhat higher than that reported by Czikk et al (3) for animals at ~135 days gestation at ~20%/day and by Schaefer and Krishnamurti (1) for animals at ~128 days gestation at ~37%/day. FSR_{min} values for the cerebral cortex using MPE_P as the precursor pool were determined for both the Hyper and Hypo AA group at 41% and 38%/day respectively, which is again somewhat higher than that reported by Schaefer and Krishnamurti (1) at ~14%/day and calculated from the data of Czikk et al (3) at ~16%/day. As such, we found no evidence for a differential effect of modest increases versus decreases in circulating amino acids as variably maintained over 2-4 hours and reported in the previous chapter (21), on cerebral protein synthesis in the near term ovine fetus.

The transport of amino acids across the BBB is an important control point for the regulation of cerebral protein synthesis, and in turn is affected by the transport capacity of the various amino acid BBB transporters, amino acid affinity for these transporters, and amino acid plasma concentrations (6). While the increases and decreases in plasma amino acids as studied would be expected to have corresponding effects on cerebral protein synthesis as seen in other organ tissues (7, 8, 11, 12, 18), it is possible that the amino acid changes were of insufficient duration, or degree, given the low k_m (high affinity) values reported for BBB amino acid transport such that the neutral and basic amino acid transporters are normally highly saturated at physiologic concentrations of plasma amino

acids (6). In this regard the intracellular free leucine content within the brain, while somewhat higher in the Hyper versus the Hypo AA group animals, was not significantly different as the probable key amino acid determinant for protein synthesis (12), and would indicate a relatively wide "safety range" before changes in circulating amino acids from the norm impact on levels in the brain sufficiently to effect cerebral protein synthesis.

However, it is also possible that the associated increase in fetal insulin in the Hyper and Hypo AA groups reported by others (14, 23) has similarly increased cerebral protein synthesis, given the elevated values from that previously reported, which could negate any effect from the induced changes in plasma amino acids. Insulin crosses the BBB through a receptor-mediated transport mechanism which is saturable although varying considerably among species (24), which might account for the similar FSR values for the Hyper and Hypo AA groups despite the higher plasma insulin levels with the insulin infusion (14, 23). Any increases in cerebral protein synthesis that have occurred could then be mediated through glucose conversion to non-essential amino acids (25), amino acid sparing from catabolic to anabolic processes (3), and/or the direct stimulation of protein synthesis by insulin, as seen in other tissues (26, 27).

While cerebral FSR values showed little difference between the Hyper and Hypo AA groups, those for the cerebral cortex were significantly higher than respective values for the cerebellum. This differs from our previous study in the near term ovine where there were no regional differences in the measures of protein synthesis from the cerebral cortex, cerebellum, or brain stem (3), which

one might expect given the heterogeneous makeup of these structures and random tissue sampling. It is thus of interest that the insulin receptor is not uniformly distributed throughout the BBB (28), and to the extent that cerebral protein synthesis is indeed increased and insulin mediated in the animals herein studied, might then contribute to the regional differences noted.

Cerebral fractional extraction values for leucine and leucine enrichment at 2 to 4% for both animal groups are similar to that we have previously reported (3) and consistent with a net flux for leucine into the brain for protein accretion and/or oxidative needs. However, these values for the most part were not significantly different from zero reflecting the methodologic variance inherent in measuring small arterio-venous differences and offered little insight for predicting changes in cerebral leucine uptake and thereby in protein synthesis.

With knowledge of the rate of isotopically labeled leucine infusion into the fetal circulation and the leucine enrichment in the plasma at steady-state, ie., MPE_P, a fetal disposal rate for leucine can be calculated representing the rate at which leucine is utilized for protein synthesis and oxidation as well as loss across the placenta (29). These rates again present minimal estimates of protein synthesis and oxidation by the whole fetal organism, since the leucine carried by the fetal plasma mixes intracellularly with the unlabeled leucine that is derived from protein degradation, thereby producing a level of isotopic enrichment at the sites of cellular metabolism that is less than that of arterial plasma (30). DR values were significantly higher in the Hypo AA group animals at 12.8 μmol/kg/min when compared to that of the Hyper AA group animals at 11.5

μmol/kg/min, which in turn is somewhat higher than that previously reported for the ovine fetus both preterm (21) and near term (29). These DR values are consistent with the findings of others in the ovine fetus whereby amino acid utilization (15-17), protein synthesis (13, 14), and oxidation (13, 17), are variably increased in a dose dependent manner in relation to insulin levels and with these expected to be stepwise increased in the Hyper and the Hypo AA group animals as herein studied (14, 23).

In the present study we have utilized a mixed amino acid infusate and an insulin euglycemic clamp technique to determine the effects of induced hyper and hypo aminoacidemia on cerebral protein synthesis during early brain development. There was no evidence for a differential effect of modest increases versus decreases in circulating amino acids on cerebral protein synthesis as studied, which may be attributed to the saturable nature of the BBB transporters for amino acids (6), although this has not been well studied for the ovine fetus. This would indicate that protein metabolism in the brain is less sensitive to systemic amino acid change than is seen for other organ tissues (11, 13, 14, 18), which would be important for the developing brain when the relatively high rates of protein synthesis/degradation with tissue growth and remodeling would be more vulnerable to changes in amino acid availability. However, it is possible that the associated increase in fetal insulin with the amino acid and insulin/dextrose infusions (14, 23) has increased cerebral protein synthesis to a similar degree. To the extent that fetal cerebral protein synthesis is increased, and for the amino acid infusate animals with endogenous insulin levels well within

the range seen with glucose loading (14, 31), then growth and developmental processes within the brain may well be impacted and are deserving of further study.

4.5 REFERENCES

1. Schaefer AL, Krishnamurti CR. Whole body and tissue fractional protein synthesis in the ovine fetus in utero. Br J Nutr. 1984 Sep;52(2):359-69.

2. Abrams RM, Burchfield DJ, Sun Y, Smith CB. Rates of local cerebral protein synthesis in fetal and neonatal sheep. Am J Physiol. 1997 Apr;272(4 Pt 2):R1235-44.

3. Czikk MJ, Sweeley JC, Homan JH, Milley JR, Richardson BS. Cerebral leucine uptake and protein synthesis in the near-term ovine fetus: Relation to fetal behavioral state. Am J Physiol Regul Integr Comp Physiol. 2003 Jan;284(1):R200-7.

4. Sun Y, Deibler GE, Jehle J, Macedonia J, Dumont I, Dang T, et al. Rates of local cerebral protein synthesis in the rat during normal postnatal development. Am J Physiol. 1995 Feb;268(2 Pt 2):R549-61.

5. Dunlop DS, Elden WV, Lajtha A. Protein degradation rates in regions of the central nervous system in vivo during development. Biochem J. 1978 Mar 15;170(3):637-42.

6. Pardridge WM. Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. Neurochem Res. 1998 May;23(5):635-44.

7. Watt PW, Corbett ME, Rennie MJ. Stimulation of protein synthesis in pig skeletal muscle by infusion of amino acids during constant insulin availability. Am J Physiol. 1992 Sep;263(3 Pt 1):E453-60.

8. Jurasinski C, Gray K, Vary TC. Modulation of skeletal muscle protein synthesis by amino acids and insulin during sepsis. Metabolism. 1995 Sep;44(9):1130-8.

9. Tessari P, Barazzoni R, Zanetti M, Kiwanuka E, Tiengo A. The role of substrates in the regulation of protein metabolism. Baillieres Clin Endocrinol Metab. 1996 Oct;10(4):511-32.

10. Giordano M, Castellino P. Correlation between amino acid induced changes in energy expenditure and protein metabolism in humans. Nutrition. 1997 Apr;13(4):309-12.

11. Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC. Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats. Am J Physiol Endocrinol Metab. 2002 Oct;283(4):E824-35.

12. Kimball SR, Jefferson LS. Regulation of global and specific mRNA translation by oral administration of branched-chain amino acids. Biochem Biophys Res Commun. 2004 Jan 9;313(2):423-7.

13. Liechty EA, Boyle DW, Moorehead H, Auble L, Denne SC. Aromatic amino acids are utilized and protein synthesis is stimulated during amino acid infusion in the ovine fetus. J Nutr. 1999 Jun;129(6):1161-6.

14. de Boo HA, van Zijl PL, Smith DE, Kulik W, Lafeber HN, Harding JE. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr Res. 2005 Aug;58(2):270-7.

15. Milley JR. Effects of insulin on ovine fetal leucine kinetics and protein metabolism. J Clin Invest. 1994 Apr;93(4):1616-24.

16. Thureen PJ, Scheer B, Anderson SM, Tooze JA, Young DA, Hay WW,Jr. Effect of hyperinsulinemia on amino acid utilization in the ovine fetus. Am J Physiol Endocrinol Metab. 2000 Dec;279(6):E1294-304.

17. Brown LD, Hay WW,Jr. Effect of hyperinsulinemia on amino acid utilization and oxidation independent of glucose metabolism in the ovine fetus. Am J Physiol Endocrinol Metab. 2006 Dec;291(6):E1333-40.

18. Kobayashi H, Borsheim E, Anthony TG, Traber DL, Badalamenti J, Kimball SR, et al. Reduced amino acid availability inhibits muscle protein synthesis and decreases activity of initiation factor eIF2B. Am J Physiol Endocrinol Metab. 2003 Mar;284(3):E488-98.

19. Gressens P, Muaku SM, Besse L, Nsegbe E, Gallego J, Delpech B, et al. Maternal protein restriction early in rat pregnancy alters brain development in the progeny. Brain Res Dev Brain Res. 1997 Oct 20;103(1):21-35.

20. MacLachlan JN, Hemstreet S, Matushewski B, McCallum JD, Richardson BS. Induced hyper and hypo amino acidemia in the ovine fetus near term: Effects on electrocortical activity. Reprod Sci. 2008 Sep;15(7):710-9.

21. Loy GL, Quick AN,Jr, Teng CC, Hay WW,Jr, Fennessey PV. Versatile stable isotope technique for the measurement of amino acids and keto acids: Comparison with radioactive isotope and its use in measuring in vivo disposal rates. Anal Biochem. 1990 Feb 15;185(1):1-9.

22. Horber FF, Horber-Feyder CM, Krayer S, Schwenk WF, Haymond MW. Plasma reciprocal pool specific activity predicts that of intracellular free leucine for protein synthesis. Am J Physiol. 1989 Sep;257(3 Pt 1):E385-99.

23. Wilkening RB, Boyle DW, Teng C, Meschia G, Battaglia FC. Amino acid uptake by the fetal ovine hindlimb under normal and euglycemic hyperinsulinemic states. Am J Physiol. 1994 Jan;266(1 Pt 1):E72-8.

24. Plum L, Schubert M, Bruning JC. The role of insulin receptor signaling in the brain. Trends Endocrinol Metab. 2005 Mar;16(2):59-65.

25. Davis TA, Burrin DG, Fiorotto ML, Reeds PJ, Jahoor F. Roles of insulin and amino acids in the regulation of protein synthesis in the neonate. J Nutr. 1998 Feb;128(2 Suppl):347S-50S.

26. Clark CM,Jr. The stimulation by insulin of amino acid uptake and protein synthesis in the isolated fetal rat heart. Biol Neonate. 1971;19(4):379-88.

27. Cahill GF, Aoki TT, Marliss EB. Insulin and muscle protein. In: Handbook of Physiology. Endocrinology. Washington DC: Am Physiol Soc; 1972. p. 563-77.

28. Banks WA. Blood-brain barrier and energy balance. Obesity (Silver Spring). 2006 Aug;14 Suppl 5:234S-7S.

29. van Veen LC, Teng C, Hay WW,Jr, Meschia G, Battaglia FC. Leucine disposal and oxidation rates in the fetal lamb. Metabolism. 1987 Jan;36(1):48-53.

30. Smith CB, Deibler GE, Eng N, Schmidt K, Sokoloff L. Measurement of local cerebral protein synthesis in vivo: Influence of recycling of amino acids derived from protein degradation. Proc Natl Acad Sci U S A. 1988 Dec;85(23):9341-5.

31. Philipps AF, Porte PJ, Stabinsky S, Rosenkrantz TS, Raye JR. Effects of chronic fetal hyperglycemia upon oxygen consumption in the ovine uterus and conceptus. J Clin Invest. 1984 Jul;74(1):279-86.

CHAPTER FIVE

GENERAL DISCUSSION

5.1 GENERAL DISCUSSION

Fetal behavioural states, akin to postnatal sleep states, are present in the ovine fetus near term. Well-differentiated electrocortical (ECOG) patterns are evident from 120 days gestation (term = 145 days), with a temporal relationship to episodic muscle and breathing activity, indicative of behavioural states (1). There is initially a high proportion of time in the low-voltage ECOG state with rapid eye movements (REM) at >50%, with approximately 40% of the time spent in the high-voltage ECOG NREM state and only brief periods of wakefulness. A behavioural state effect on cerebral metabolic rate is evident for the near term ovine fetus with an increase during the low-voltage REM state which likely reflects increased neuronal activity (2). Conversely, cerebral protein synthesis and degradation, i.e. turnover, appear to be increased during the high-voltage NREM state (3) as seen in adult animals (4, 5), indicating that the decrease in the brain's metabolic demand during NREM sleep does not result from a decrease in biosynthetic activity and may, in fact, favour the synthesis of new proteins. These findings support the restorative theory of sleep (6) whereby energy conservation during NREM sleep favours the anabolic restoration of tissue components. Moreover, peak rates of protein synthesis within the brain, either globally or selectively, may participate in mechanisms that trigger subsequent REM periods, conditions for stimulating the peaks having been generated during the preceding NREM period when neuronal firing was lower (6).

Protein synthesis during brain growth and development has been studied both pre- and postnatally in sheep (3, 7, 8) and postnatally in rats (9), since

proteins are fundamental components of all tissue elements and with protein metabolism directly related to maturational events (9). Initial study in the near-term ovine fetus by Schaefer and Krishnamurti (8) using a tyrosine isotopic-dilution technique reported high rates of cerebral protein synthesis with a protein fractional synthetic rate (FSR) between 14 and 37%/day, ie., the percentage of brain protein that is newly synthesized per day. In a more recent study using a continuous tracer infusion of L-[1-^{14}C]-leucine, we (3) showed a cerebral protein FSR of ~20%/day for the near-term ovine fetal brain, which was similar when measured regionally in the cerebral cortex and cerebellum. Postnatal studies in rats have also shown high rates of brain protein synthesis during early development, with peak values occurring shortly after birth and gradually decreasing thereafter (10, 11). While high rates of cerebral protein synthesis are thus evident during early life in both sheep and rats in support of the brain's growth and development, these rates are considerably greater than anticipated for protein accretion, indicating that protein degradation must also play an important role in the growth and development of the brain (3, 10).

The precursor availability of amino acids within the brain might then impact on protein synthesis and/or degradation, ie., protein turnover, thereby impacting growth processes. While the effects of increasing and decreasing amino acid availability on protein turnover in the developing brain have yet to be determined, postnatal studies as well as that in the whole fetus would suggest that alterations in protein synthesis and/or degradation should be considered. Studies in both animals and humans with acute or chronic amino acid supplementation report a

protein-anabolic effect at the whole-body level and for select organ tissues (11-15). Similarly, studies in the ovine fetus with a mixed amino acid infusion report protein accretion to be increased at the whole-body level (16, 17). These protein effects can be attributed in part to related increases in insulin (17) which promotes protein accretion when given to the ovine fetus (18-20), although this effect has not been studied for the brain. Furthermore, maternal protein restriction during pregnancy in the rat and a presumed reduction in essential amino acid availability also alters growth processes in the brain during early development (21).

Utilizing the chronically catheterized ovine fetal model, the purpose of the current study was three fold: Firstly, to characterize the change in circulating amino acid levels utilizing both insulin infusion to decrease and amino acid infusion to increase amino acid concentrations; second, to determine the effect of altered substrate availability on fetal behavioural state activity and metabolism. The third aim was to determine what impact these induced changes in substrate availability may have upon the rate of protein synthesis in the developing fetal brain.

In our current study, the amino acid infusion utilized in the Hyper AA group produced a 33% increase in fetal amino acid concentration, while the insulin/dextrose infusion decreased amino acid levels an average of 33% from control values. Both results are in keeping with previous studies using both insulin to decrease (22) and amino acid infusion to increase (16) circulating amino acid levels in the ovine fetal hindlimb. However, despite these moderate

increases and decreases in circulating amino acid levels, there was no significant change in the mean percent time or duration of fetal ECOG activities for either study group during the experimental period. Previous studies using a similar, acute insulin infusion with resulting hypoglycemia found no changes in ECOG activity (23).

A constant tracer amino acid infusion was used to determine the effects of Hyper and Hypo AA on cerebral protein synthesis in the preterm ovine fetus. While previous studies have utilized [^{14}C]-leucine, our current study utilized [^{13}C]-leucine as an amino acid tracer to look at synthetic rates in the ovine fetal brain, with the expectation that a non-radioactive isotope would provide a safer alternative (24) to the radioactive [^{14}C]-leucine tracer (3).

Despite the use of both a mixed amino acid infusate and an insulin euglycemic clamp technique to induce hyper and hypo amino acidemia respectively, there was no effect on cerebral protein synthesis as measured from cerebral cortex and cerebellar tissues. The absence of any differences between the two groups may be attributed to the saturable nature of the BBB transporters for amino acids (25), although this has not been well studied for the ovine fetus.

While there were no differences in the rate of protein synthesis when compared between the hyper and hypo amino acid groups, it is interesting to note both treatments result in a fractional synthetic rate much higher than that previously reported for the ovine fetus during spontaneous rates of synthesis (3). It is likely that fetal insulin levels are increased with both the amino acid and insulin/dextrose infusion (17, 22). The impact of insulin upon cerebral protein

synthesis may occur to a similar degree in both groups and is certainly deserving of further study.

5.2 FUTURE STUDIES

The current study utilized acute induced changes in amino acid concentrations to examine the short term effects of both hyper and hypo amino academia. Alternatively, previous studies have utilized dietary restriction to induce long term changes in precursor amino acid availability. Maternal protein restriction during pregnancy in the rat and a presumed reduction in essential amino acid availability alters growth processes in the brain during early development (21). A study involving chronic maternal dietary restriction in the pregnant sheep would provide further insight into the mechanisms by which amino acids affect fetal cerebral protein synthesis. In contrast, Insulin-like growth factor 1 (IGF-1) is known to promote growth by stimulating protein synthesis during fetal development (26), not only in the brain (27) but other tissues as well (28). Infusion of IGF-1 to the brain and the subsequent changes in behavioural state and protein synthesis would be of great interest for future study.

The transport of amino acids across the BBB is an important control point for the regulation of cerebral protein synthesis. It is affected by the transport capacity of the various amino acid BBB transporters, amino acid affinity for these transporters, and amino acid plasma concentrations (25). While the increases and decreases in plasma amino acids here studied might be expected to have corresponding effects on cerebral protein synthesis as seen in other organ

tissues (11, 12, 15), it is possible that the amino acid changes were of insufficient duration, or degree, given the low k_m (high affinity) values reported for BBB amino acid transport such that the neutral and basic amino acid transporters are normally highly saturated at physiologic concentrations of plasma amino acids (25). It is likely the ovine fetus exhibits a wide 'safety range' before changes in circulating amino acids from the norm impact on levels in the brain sufficiently to affect cerebral protein synthesis. Moreover, given the low k_m values for the BBB transporters, a selective increase or decrease in the plasma concentration of a single amino acid is more likely to affect the brain availability of that amino acid as well as the other amino acids competing for the shared BBB carrier system, than a generalized increase or decrease in all amino acids (25) as in the present study. Thus future studies utilizing a infusion of a single amino acid for a longer time period may further demonstrate the interrelationship between amino acid availability and fetal cerebral protein synthesis.

A limitation to the present study is that fetal insulin levels will be increased as reported by others for animals similarly studied, by ~25% for the Hyper AA group (17) and by more than 40-fold for the Hypo AA group (22). Insulin promotes whole-body protein synthesis in the ovine fetus (18-20), however the effect of insulin on fetal cerebral protein synthesis remains to be determined. It is possible that the associated increase in fetal insulin in the Hyper and Hypo AA groups has similarly increased cerebral protein synthesis, given the elevated values from that previously reported, which might then negate any effect from the induced changes in plasma amino acids. Insulin can cross the BBB through a

receptor-mediated transport mechanism which is saturable although varying considerably among species (29). Utilizing the euglycemic clamp technique and a concomitant insulin infusion, future studies focusing on the effects of varying insulin concentrations upon fetal brain metabolism and disposal rates would allow for a more complete examination of the relationship between insulin availability and fetal brain development.

5.3 CONCLUSIONS

1. The amino acid infusion in the Hyper AA group and insulin infusion in the Hypo AA group produced a 33% increase and decrease in circulating amino acid concentrations respectively, and is a reliable technique for the induction of acute changes in the fetal nutritional milieu.
2. Acute changes in substrate availability do not effect electrocortical expression in the near term ovine fetus.
3. Despite a 33% increase/decrease in circulating amino acids for the Hyper and Hypo AA groups respectively, there were no changes observed in the rate of cerebral protein synthesis between groups. However, these fractional synthetic rates are higher than previously reported spontaneous rates of cerebral protein synthesis, indicating a possible role for insulin in the control of cerebral protein synthesis within the developing brain.

5.4 REFERENCES

1. Szeto HH, Hinman DJ. Prenatal development of sleep-wake patterns in sheep. Sleep. 1985 Dec;8(4):347-55.

2. Richardson BS, Carmichael L, Homan J, Gagnon R. Cerebral oxidative metabolism in lambs during perinatal period: Relationship to electrocortical state. Am J Physiol. 1989 Nov;257(5 Pt 2):R1251-7.

3. Czikk MJ, Sweeley JC, Homan JH, Milley JR, Richardson BS. Cerebral leucine uptake and protein synthesis in the near-term ovine fetus: Relation to fetal behavioral state. Am J Physiol Regul Integr Comp Physiol. 2003 Jan;284(1):R200-7.

4. Ramm P, Smith CT. Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. Physiol Behav. 1990 Nov;48(5):749-53.

5. Nakanishi H, Sun Y, Nakamura RK, Mori K, Ito M, Suda S, et al. Positive correlations between cerebral protein synthesis rates and deep sleep in macaca mulatta. Eur J Neurosci. 1997 Feb;9(2):271-9.

6. Adam K. Sleep as a restorative process and a theory to explain why. Prog Brain Res. 1980;53:289-305.

7. Abrams RM, Burchfield DJ, Sun Y, Smith CB. Rates of local cerebral protein synthesis in fetal and neonatal sheep. Am J Physiol. 1997 Apr;272(4 Pt 2):R1235-44.

8. Schaefer AL, Krishnamurti CR. Whole body and tissue fractional protein synthesis in the ovine fetus in utero. Br J Nutr. 1984 Sep;52(2):359-69.

9. Sun Y, Deibler GE, Jehle J, Macedonia J, Dumont I, Dang T, et al. Rates of local cerebral protein synthesis in the rat during normal postnatal development. Am J Physiol. 1995 Feb;268(2 Pt 2):R549-61.

10. Dunlop DS, Elden WV, Lajtha A. Protein degradation rates in regions of the central nervous system in vivo during development. Biochem J. 1978 Mar 15;170(3):637-42.

11. Watt PW, Corbett ME, Rennie MJ. Stimulation of protein synthesis in pig skeletal muscle by infusion of amino acids during constant insulin availability. Am J Physiol. 1992 Sep;263(3 Pt 1):E453-60.

12. Jurasinski C, Gray K, Vary TC. Modulation of skeletal muscle protein synthesis by amino acids and insulin during sepsis. Metabolism. 1995 Sep;44(9):1130-8.

13. Tessari P, Barazzoni R, Zanetti M, Kiwanuka E, Tiengo A. The role of substrates in the regulation of protein metabolism. Baillieres Clin Endocrinol Metab. 1996 Oct;10(4):511-32.

14. Giordano M, Castellino P. Correlation between amino acid induced changes in energy expenditure and protein metabolism in humans. Nutrition. 1997 Apr;13(4):309-12.

15. Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC. Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats. Am J Physiol Endocrinol Metab. 2002 Oct;283(4):E824-35.

16. Liechty EA, Boyle DW, Moorehead H, Auble L, Denne SC. Aromatic amino acids are utilized and protein synthesis is stimulated during amino acid infusion in the ovine fetus. J Nutr. 1999 Jun;129(6):1161-6.

17. de Boo HA, van Zijl PL, Smith DE, Kulik W, Lafeber HN, Harding JE. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr Res. 2005 Aug;58(2):270-7.

18. Milley JR. Effects of insulin on ovine fetal leucine kinetics and protein metabolism. J Clin Invest. 1994 Apr;93(4):1616-24.

19. Thureen PJ, Scheer B, Anderson SM, Tooze JA, Young DA, Hay WW,Jr. Effect of hyperinsulinemia on amino acid utilization in the ovine fetus. Am J Physiol Endocrinol Metab. 2000 Dec;279(6):E1294-304.

20. Brown LD, Hay WW,Jr. Effect of hyperinsulinemia on amino acid utilization and oxidation independent of glucose metabolism in the ovine fetus. Am J Physiol Endocrinol Metab. 2006 Dec;291(6):E1333-40.

21. Gressens P, Muaku SM, Besse L, Nsegbe E, Gallego J, Delpech B, et al. Maternal protein restriction early in rat pregnancy alters brain development in the progeny. Brain Res Dev Brain Res. 1997 Oct 20;103(1):21-35.

22. Wilkening RB, Boyle DW, Teng C, Meschia G, Battaglia FC. Amino acid uptake by the fetal ovine hindlimb under normal and euglycemic hyperinsulinemic states. Am J Physiol. 1994 Jan;266(1 Pt 1):E72-8.

23. Richardson BS, Hohimer AR, Bissonnette JM, Machida CM. Insulin hypoglycemia, cerebral metabolism, and neural function in fetal lambs. Am J Physiol. 1985 Jan;248(1 Pt 2):R72-7.

24. Loy GL, Quick AN,Jr, Teng CC, Hay WW,Jr, Fennessey PV. Versatile stable isotope technique for the measurement of amino acids and keto acids: Comparison with radioactive isotope and its use in measuring in vivo disposal rates. Anal Biochem. 1990 Feb 15;185(1):1-9.

25. Pardridge WM. Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. Neurochem Res. 1998 May;23(5):635-44.

26. Giacobini MM, Olson L, Hoffer BJ, Sara VR. Truncated IGF-1 exerts trophic effects on fetal brain tissue grafts. Exp Neurol. 1990 Apr;108(1):33-7.

27. D'Costa AP, Xu X, Ingram RL, Sonntag WE. Insulin-like growth factor-1 stimulation of protein synthesis is attenuated in cerebral cortex of aging rats. Neuroscience. 1995 Apr;65(3):805-13.

28. Douglas RG, Gluckman PD, Ball K, Breier B, Shaw JH. The effects of infusion of insulinlike growth factor (IGF) I, IGF-II, and insulin on glucose and protein metabolism in fasted lambs. J Clin Invest. 1991 Aug;88(2):614-22.

29. Plum L, Schubert M, Bruning JC. The role of insulin receptor signaling in the brain. Trends Endocrinol Metab. 2005 Mar;16(2):59-65.