Department of Health and Human Services
Public Health Services
Grant Application

Do not exceed 56-character length restrictions, including spaces.

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT
Sleep mechanisms in children: role of metabolism

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION NO x YES
(If "Yes," state number and title)
Number: RFA-HL01-06 Title: Sleep and Sleep Disorders in Children

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR — **New Investigator** ☒ **No** ☐ **Yes**

3a. NAME *(Last, first, middle)*
Gabriel Haddad

3b. DEGREE(S)
MD

3c. POSITION TITLE
Chairman

3d. MAILING ADDRESS *(Street, city, state, zip code)*

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Pediatrics

3f. MAJOR SUBDIVISION

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL:
FAX:

E-MAIL ADDRESS:

4. HUMAN SUBJECTS RESEARCH
No
x Yes

4a. Research Exempt x No ☐ Yes
If "Yes," Exemption No. ____

4b. Human Subjects Assurance No.
Pending

4c. NIH-defined Phase III Clinical Trial
x No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval Date

5b. Animal welfare assurance no
A3312-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
07/01/2002	06/30/2006				

9. APPLICANT ORGANIZATION
Name Albert Einstein College of Medicine
Address

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: ® ☐ Federal ☐ State ☐ Local
Private: ® ☒ Private Nonprofit
For-profit: ® ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
1131624225A2
DUNS NO. *(if available)* 071036636
Congressional District 7

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name
Title
Address

Tel FAX
E-Mail

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name
Title
Address

Tel FAX
E-Mail

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.	SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*	DATE
15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.	SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*	DATE

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT

TABLE OF CONTENTS

(Items A-D: not to exceed 25 pages)*

** SBIR/STTR Phase I: Items A-D limited to 15 pages.*

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

This application entitled "Sleep mechanisms in Children: Role of metabolism" is being transferred from Yale University School of Medicine to Albert Einstein College of Medicine. Since this application is related to NMR studies on adolescent children in sleep, two main issues arise.

PI and the group of investigators: The PI (G.G. Haddad) and one co-Investigator (Dr. L. Kass) have moved from Yale to Einstein and there are no changes for these 2 investigators. Dr. Novotny and Dr. Rothman from Yale are replaced by Dr. H. Hetherington and Dr. J. Pan at Einstein. Both of these investigators at Einstein are major investigators at Einstein and in the area of NMR. Please refer to their respective CVs and to the Appendix (articles) enclosed with the application. It is important to highlight that the two investigators from Yale have collaborated with the two investigators at Einstein and still to date. Indeed, every two weeks one team travels to the other side collaborative process.

Equipment: The NMR facility at Einstein is a first rate one and some of the resources are detailed in the new resource page regarding NMR. Indeed, the 4 Tesla machine had been operational at Einstein for some time and we can proceed with these studies as soon as we wish. In addition, Dr. Kass who used to be the director of the Sleep Center at Yale is here now at Einstein. He has purchased all the needed equipment and has already started to perform studies at the Children's Hospital. He has made provisions to perform sleep studies simultaneously in 2 rooms.

PERFORMANCE SITE(S) *(organization, city, state)*

Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, NY 10461

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Gabriel Haddad, MD	AECOM - Pediatrics	PI
Julie W. Pan, MD	AECOM – Neurology	Co-Investigator
Hoby Hetherington, MD	AECOM – Radiology	Investigator
Lewis Kass, MD	AECOM/MONTE - Pediatrics	Investigator

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ **Yes** ☐ **No**

A. SPECIFIC AIMS

Sleep and the neurobiological mechanisms controlling sleep/wakefulness have been an enigma in spite of important recent advances in the field. Although it is well known now that sleep affects a variety of systems, including the cardio-respiratory, endocrine and autonomic systems, we still do not understand why we sleep and the mechanisms that control sleep. For example, we do not have a good understanding of the mechanisms that induce or maintain sleep or those mechanisms that are activated with sleep deprivation.

One of the potentially important areas that have started to develop is the role of brain metabolism in sleep. Although metabolic studies during sleep have been done in the past few decades, new developments in brain imaging have made it possible only recently to examine the importance of metabolism in sleep research. Positron emission tomography (PET) studies have shown that there are major differences in the activity of certain parts of the brain between slow-wave sleep and REM sleep (Maquet 2000, Braun 1997). In addition, there was a large difference in the cerebral blood flow between the wake state and slow-wave sleep. The link between metabolism and sleep is very well illustrated also in the Benington and Heller's working model in which decreases in glycogen and ATP lead to alterations in released adenosine which, in turn, play an important role in neuronal excitability and sleep induction (Benington 1995). Furthermore, and of major interest, is the growing evidence that glia play a critical role in maintaining neuronal function through metabolic support through glutamate re-cycling and possibly by providing neurons with substrates for glucose oxidation. Glycogen is found only in glia and hence glycogen metabolism, the glutamine-glutamate cycling and glucose oxidation involve links between glia and neurons (Tsacopoulos 1996, Magistretti 2000). Hence, in order to understand sleep and its mechanisms, it becomes important to study the functional integrity and coupling of neurons and glia and their relationship to sleep/wake state.

Although a handful of studies have been done on the adult human brain to decipher the link between metabolism and sleep/wakefulness, there is a paucity of studies on the brain of the child. Clearly, there is considerable evidence that brain maturation indeed continues to take place with age through adolescence and adulthood. Since sleep patterns also continue to change (e.g. consider sleep patterns in the first few months as compared with older children), it is likely therefore that sleep mechanisms also mature with age. In this application, we will focus on adolescent children for practical and conceptual reasons (see below).

It has been clear for many years that sleep, like wakefulness, is not a homogeneous state. Sleep states have their electroencephalographic, autonomic, behavioral and cardio-respiratory signatures. For conceptual reasons that will be clearer in Section B, and in order not to complicate the experimental matrix, we will focus this application on stage III-IV sleep (deep sleep) and will address our questions comparing this sleep stage to a well defined state of wakefulness. Furthermore, since previous studies have taught us about sleep and its mechanisms by studying sleep deprivation, we will, in a subset of our children, address the same questions after sleep deprivation.

Our laboratory has had a long-standing interest in sleep in children, especially as it pertains to the control of respiration and obstructive sleep apnea. For the past 2 decades, we have engaged in clinical and basic research in the control of respiration, consequences of tissue hypoxia and the mechanisms that lead to cell adaptation or injury. In addition, the Nuclear Magnetic Resonance and Imaging Center at this institution is endowed with the talent needed for the proposal and with state-of-the art approaches and techniques that have been developed in the Center and which are essential to the proposal. For example, our ability to measure non-invasively metabolic pathways involved in neuronal-glial functional interactions such as the glutamate-glutamine cycle, neuronal glucose oxidation and glycogen metabolism is of great importance for this application. Hence the integration of our two groups will be exciting and will allow us to

address questions of importance in sleep research in children, an area of research that, at present, is very fertile. Based on previous studies from our laboratory and others, we have formulated the ***general hypothesis*** *that the understanding of sleep mechanisms depends on a detailed understanding of metabolic processes involving the functional coupling between glia and neurons and that the overall down-regulation of synaptic activity during sleep allows for restoration of glial energy stores. Our* ***specific hypotheses*** are as follows:

1. Stage III-IV sleep has a lower metabolic requirement and a lower glutamate turnover rate (tricarboxylic acid cycle rate) in both neurons and glia, as compared to wakefulness.

2. As compared to wakefulness, sleep stage III-IV is characterized by a lower rate of brain neuronal glutamate release and glial glutamate uptake in children; this reduced glutamate/glutamine cycling during this sleep stage in the brain of children is prevented by sleep deprivation.

3. Brain glycogen content increases during the course of sleep in children and sleep deprivation markedly lowers glycogen content.

B. BACKGROUND & SIGNIFICANCE

I. IMPORTANCE

Understanding sleep and sleep mechanisms is important for many reasons. ***First***, about 1/3 of the time in adult life is spent in sleep; more than 70% of the time in young infants is spent in sleep. ***Second***, the length of sleep time, the quality of sleep and the sleep time during the 24 hour of the day can affect in a major way the daily performance of children and adults. ***Third***, there is evidence now that sleep deprivation has remarkable effects on many aspects of physiologic function, including neurocognitive function that impacts on work and school performance and achievement. The link between sleep deprivation and vehicle accidents has now been recognized for many years and has not only cost economically in billions of dollars every year but also in human life due to accidents. Sleep deprivation in the child and adolescent is more appreciated now to be a cause for poor school performance. Indeed, there is reason to believe that obstructive sleep apnea (OSA) and sleep fragmentation and deprivation may be at the basis for attention deficit disorder (ADD) and learning disabilities in some individuals especially since treatment of OSA alleviates ADD (Gozal 2001). ***Fourth***, better understanding of the biochemistry of sleep leads to better understanding of diseases that occur during sleep such as OSA, narcolepsy, parasomnias and insomnia, sleep hypoventilation, and sleep-induced hypoxia and neurodegeneration.

The importance of this particular application stems from a number of aspects. There has been a paucity of sleep studies in children especially as they pertain to brain metabolism and brain physiology during wakefulness and sleep. In fact, to our knowledge, there have been no studies on the relation between sleep and glutamate-glutamine cycling, glutamate turnover rate and the tricarboxylic cycle (TCA) and glycogen measurements. Such an area of investigation is crucial to delve into since a number of hypotheses related to the purpose and function of sleep are based on metabolic variables that at present can be examined non-invasively in the human brain during sleep. These measurements are proposed in our application and these studies will lay the ground for the understanding of metabolism during sleep and wakefulness. Such experiments will not only define sleep but may shed some light on a model, that of Bennington and Heller (Bennington 1995), which will be detailed below. Since sleep deprivation is common, especially in the adolescent age group, we also propose to study brain metabolism in children subjected to

sleep deprivation and compare them with those who were not sleep deprived. Finally, our studies are important since these will constitute the first steps in studying brain metabolism at various ages, including infants and adults, studies that we are planning to perform in the future but are not planned in this application.

II. BACKGROUND

Sleep and sleep research in general. Although sleep research in general has started to move forward in the past few decades or so, especially with the advent of new technology and approaches, research in this area has not moved forward in a major way, in humans and especially in children. There is a general paucity of understanding of sleep mechanisms in general in children, and particularly as it pertains to maturation and development, as well as the epidemiological, biochemical, physiologic and pathophysiologic aspects of sleep. This is particularly important since we know now that a) there are many diseases that are linked to sleep, b) sleep deprivation whether related to behavioral issues or because of disease states can lead to major neurocognitive and cardiovascular ailments, c) we have made great progress in developing newer, non-invasive technologies that can be used in humans including children to make the measurements and perform the studies that are detailed in this application.

Blood Flow, metabolism and Sleep. Since it has been appreciated from clinical and basic studies that the mammalian and human brain needs a constant influx of energy substrates to function adequately, blood flow and metabolic rate became important modalities to study. Indeed, a number of studies have been done in adults (Madsen 1991, Maquet 1990, Madsen 1991, Buchsbaum 1989, Lenzi 1999, Netchiporouk 2001); no studies have been done in children, to our knowledge. These studies used a number of techniques including doppler, the Kety-Schmidt arteriovenous difference technique, 2-FluoroDeoxy-D-glucose and O15 H_20 PET. Madsen and colleagues found that deep sleep was associated with a 25% decrease in cerebral metabolic rate of O_2; REM sleep was much like wakefulness and did not show any decrease. Maquet et al also found that during deep sleep, cerebral glucose metabolic rate was reduced as compared to wakefulness, especially the thalamus (Maquet 2000). Here again, REM sleep was not associated with a decrease. Maquet and colleagues actually hypothesized that one reason for this decrease in glucose utilization rate during deep sleep is that there is a decreased rate of synaptic events, and a decreased need for maintenance of ion gradients and membrane polarity (Maquet 1995). Other studies have also shown that the decrease in metabolic or glucose utilization rate varies between regions and that one of the largest decreases is in the thalamus and specific cortical regions (Maquet 1995, 2000, Braun 1997). Additional studies have also argued that it is debatable whether metabolism is coupled to electrical activity and some studies have actually supported the idea that an uncoupling between electrical activity and cerebral perfusion exists during sleep (Hajak 1994). In summary, although there is evidence that deep sleep seems to be different from REM sleep and wakefulness in terms of blood flow and metabolic rate, little is known about glutamate turnover, TCA cycle rate, glutamate-glutamine cycling, the role of neurons and glia in sleep and how sleep deprivation alters these variables.

Sleep deprivation. The effect of sleep deprivation on many physiologic functions has fascinated many investigators and laboratories for many years. In spite of the fact that there is a large body of literature on sleep deprivation, a considerable number of papers address psychiatric diseases and the sleep deprivation that accompanies such conditions (Wu 1991, Pinna 1998, Patchev 1991). Indeed, besides some animals studies (Mrsulja 1970, Karadzic 1969, Karadzic 1969, Djuricic 1977, Karnovsky 1983), there are very few investigations on metabolism and sleep deprivation in adult normal humans (Wu 1991, Murashita 1999, Thomas 2000) and no studies in adolescent children.

Thomas et al has recently shown that significant decreases in regional cerebral metabolic rate of glucose in several cortical and subcortical structures after sleep deprivation. This was especially the case in the thalamus and prefrontal and posterior parietal cortices. This is an important finding since these regions are involved in alertness and attention or subserve higher order cognitive processes. It was interesting also that no areas of the brain evidenced a significant increase in metabolic rate of glucose. These studies have therefore demonstrated that short term sleep deprivation produces global decreases in brain activity with larger reductions in thalamo-cortical network, a network that is very important to the formation of sleep and sleep states. This is consistent with numerous studies showing that sleep deprivation significantly reduces vigilance, alertness, and maintenance of stable performance and neurocognitive function (Dinges 1995, Dinges 1997, Doran 2001) leading to major accidents (Summala 1994, Philip 1999, Horne 1999, Connor 2001). The studies proposed in this application will be the first to generate data on sleep deprivation, TCA cycle rate and interactions between neurons and glia as measured by the glutamate-glutamine cycling.

Sleep and sleep states in infants and children. There is a large body of literature that has accumulated for more than three decades now on the maturational aspects of sleep and sleep states in infants and children. We and others have established normative data for the first few months of life and subsequently (Haddad 1979, 1980, 1980,1981, 1981, Gopal 1981, Haddad 1982, 1982, 1982, 1993, Schechtman 1993, Gaultier 1995, Louis 1997, Sahni 2000, Horne 2001) . It is very clear now that there are marked maturational changes that take place in the first few years of life and that these continue to occur throughout childhood. Therefore, it is very important to have a protocol, as we have proposed, that includes the study of sleep architecture in the first visit in such a way that we can plan the NMR studies accordingly. As shown above, deep sleep seems to be very distinct from REM sleep and the sleep architecture in adolescent children will be long enough to allow infusion studies as we have proposed.

The Glutamate-Glutamine cycling is important for normal brain function. Glutamate is the major excitatory neurotransmitter in the human cerebral cortex. Neurons that release glutamate lack the enzymes for *de novo* glutamate synthesis. Therefore the synaptic release of glutamate will deplete the nerve terminal cytosolic precursor pool unless compensated by glutamate reuptake or synthesis. This is where glial cells become very critical: glia have a high capacity for transporting glutamate from the synaptic cleft in order to maintain a low ECF concentration of glutamate (Rothstein 1994, Tsacopoulus 1996). In vivo and in vitro studies have indicated that glutamate taken up by glia is converted to glutamine by glutamine synthetase (Meister 1985, Weisinger 1995), an enzyme found exclusively in glia (Martinez-Hernandez 1977). Glutamine is released from the glia to the ECF where it is taken up by neurons and converted back to glutamate through the action of phosphate activated glutaminase (PAG) (Kvamme 1985). The generally accepted model of the glutamate/glutamine neurotransmitter cycle is shown in Fig.1. *No data are available on these measurements during sleep.*

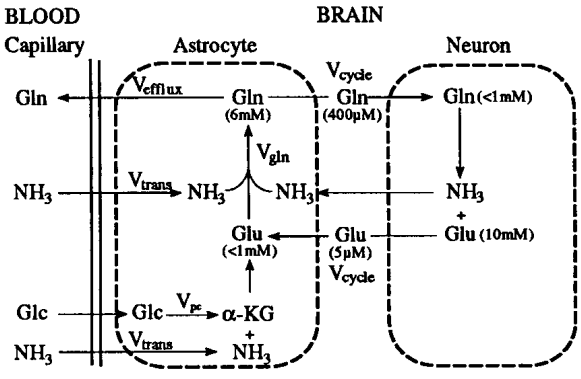


Figure 1. *Model of glutamate/glutamine cycle between neurons and glia. Glutamate released from the neuron is taken up by the glia and is returned to the nerve terminal. Based on extensive data from isotopic labeling studies, immunohistochemical staining of cortical cells for specific enzymes, cell and tissue fractionation studies, it has been proposed that glutamate taken up by the glia is returned to the neuron in the form of*

glutamine.

In vivo coupling between the glutamate/glutamine cycling and neuronal glucose oxidation. MRS and other studies have suggested that the coupling between the glutamate/glutamine cycle is correlated with glial glucose uptake and subsequent neuronal oxidation. The model is based upon work primarily done by Magistretti and coworkers (Magistretti 2000). Recent findings, using ^{13}C MRS in rat cortex, have shown that the glutamate/glutamine cycle i) increases in rate with increasing brain electrical activity in a near 1:1 stoichiometry with neuronal glucose oxidation (Sibson 1998) and ii) is 60- 80% of the rate of total glucose oxidation in the awake non-stimulated cerebral cortex (Sibson 1998,1998, Gruetter 1998, 2000; Shen 1999). Several comprehensive reviews of the evidence from molecular and cellular studies supporting glial localization of glucose uptake related to functional neuroenergetics have been published by Magistretti et al (Tsacopoulos 1996, Pellerin 1998). Such studies on the relation between cycling and glucose oxidation *have not been performed during sleep* and the studies proposed will generate exciting data that would allow us to better understand sleep.

In vivo MRS measurements of the energetic requirements of glia. A long-term controversy in brain metabolism has been the fraction of brain energy production accounted for by glial cells. Early estimates range from 10% to over 50% of oxidative energy production (Siesjo 1978). MRS may be used to measure the rate of glial glucose oxidation based upon the localization of the enzyme glutamine synthetase in the glia (Martinez-Hernandez 1977). This localization allows the rate of the glial TCA cycle to be calculated from the labeling of glutamine from glial glutamate. The most quantitative early findings were by Van den berg and coworkers (1971) who using ^{14}C isotopic labeling strategies assigned a rate to glial Pyruvate dehydrogenase (Pdh), which they referred to as the small glutamate pool, of 15-25% of total Pdh (neuronal + glial) activity. The Pdh rate is equal to the rate of complete glucose oxidation by the TCA cycle plus the rate of net glial anaplerosis. Two recent ^{13}C MRS measurements of human occipital parietal cortex, using a [1-^{13}C] glucose label, have measured glial Pdh as accounting for 8% (Shen 1999) to 21% (Gruetter 2001) of total Pdh activity in the occipital parietal lobe. A limitation of these studies is that the calculated rate of glial Pdh is complicated by label entering via neuronal Pdh and being transferred to the glia by the glutamate/glutamine cycle. As shown originally with ^{14}C methods, a more direct measure of the rate of the glial TCA cycle may be obtained using [2-^{13}C] acetate as a tracer, which is almost exclusively incorporated into glia (Hassel 1997). This is precisely why we have chosen to use acetate infusions in some of our studies, namely, to dissect out the role of the glia from that of neurons, as Rothman and colleagues from our group of investigators have done (Lebon 2001) and found that the glial TCA cycle accounts for approximately 15% of total oxidative energy production in adult human occipital parietal cortex. *These measurements have not been made in sleep or in children* and our proposed investigations promise to open a new avenue in our understanding of sleep brain biochemistry and how it differs from that in wakefulness.

Glycogen and sleep. There has been a considerable debate on the relation between glycogen and sleep and the importance of sleep in the restoration of glycogen in glia. While the idea is provocative, it is speculative and lacks experimental evidence. In addition, there have been no studies on this in children or even adult humans. The argument proposed is that the release of Norepinephrine, 5-HT and histamine that takes place in wakefulness increases the levels of cAMP, which is an important sensor for the increase in glycogenolysis (Magistretti 2000, Chatton 2000). This, in turn, induces the release of adenosine that increases the synchronization of the EEG, which leads to resynthesis of brain glycogen. This is an interesting hypothesis but *human data in sleep and wakefulness* are lacking, which highlights even further the importance of this application. One reason why we are interested in sleep deprivation is to “perturb” the system to

see whether we can detect alterations in glycogen that would be consistent with the hypothesis proposed by Bennington and Heller (Benington 1995).

III. LONG TERM OBJECTIVES

Our long term aims are a) to better understand sleep and b) to be able to better understand diseases that afflict children or adult and which impact on their sleep or diseases that are sleep-related that impact on other functions including neurocognitive, cardiovascular or behavioral functions. As seen from reviewing the state-of-the-art above, there are major gaps in our knowledge and this application focuses on a number of these gaps.

C. PRELIMINARY STUDIES

Sleep and Sleep Studies in the Children's Sleep Center. The Children's Sleep Center at Yale-New Haven Hospital has been fully operational, comprehensive and quite active for over 12 years. The major reasons for referral to our center include suspected obstructive sleep apnea, infant apnea, work-up for apparent life threatening events, Congenital Central Hypoventilation Syndrome, unexplained hypoxemia and difficult to control asthma. Over the past 5 years, we have studied more than 1,000 patients. We have broad ethnic representation since we have ~33% Caucasians, ~39% Afro-Americans and ~27% Hispanic. Most of the patients studied are in pre-school or school age, although we have a substantial adolescent population (~15% of the population studied).

The flow of patients to our laboratory occurs primarily through the pediatric Sleep Disorders Clinic (run by Dr. Lewis Kass). Additional patients are referred directly from other pediatric pulmonologists, otolaryngologists and neurologists from the state of Connecticut and the Northeast. They are then scheduled for a study in our laboratory, which operates 4-5 days per week and is staffed 24 hours a day by trained personnel. The standard polysomnography consists of four electroencephalogram leads, two oculoencephalogram leads, oral-nasal thermistor, end-tidal carbon dioxide measurement with wave flow, pulse oximetry, chin electromyogram, electrocardiography, snore microphone, chest and abdominal inductance plethysmography, limb leads and when clinically indicated, esophageal pH monitoring. The study is videotaped and a technician is on-site to record pertinent observations. Following an initial review by our polysomnography technician, a final review and subsequent report is generated by the medical director of our center (Dr. Lewis Kass). The patient population is available for recruitment into studies.

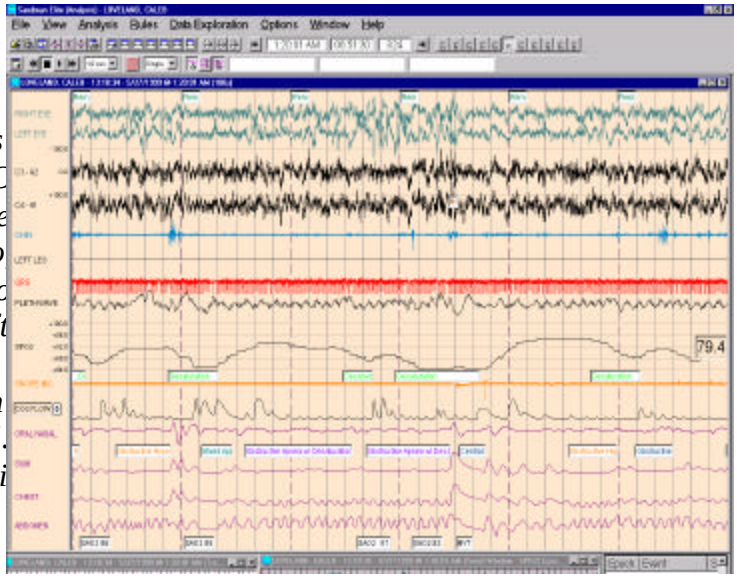


Figure 2: *An example of a "paperless" sleep s*
that we perform in our Sleep Center in the CC
On the left side of the panel, signals are labele
For example, the top 2 waveforms are those o
electrooculogram and the 2 just below are tho
the electroencephalogram. Chin EMG and left
motion transducer follow. The QRS, the O_2
saturation, end-tidal CO_2, nasal thermister an
impedance plethysmography are all recorded.
Programs allow an on-line and off-line analysi

every waveform recording. Each adolescent volunteer will have such a study before embarking on the NMR studies.

Sleep and Sleep Studies in the NMR Center. We have recently obtained preliminary spectral data on an adult human brain during wakefulness and sleep. No glucose or acetate infusion was given since this study was being done to show feasibility of a sleep study in the magnet. As expected, no changes were observed in steady state levels as a result of state change. Indeed, in our proposed work, we study flux rates rather than steady states, as is pointed out in the hypotheses, protocols and the rest of the preliminary data.

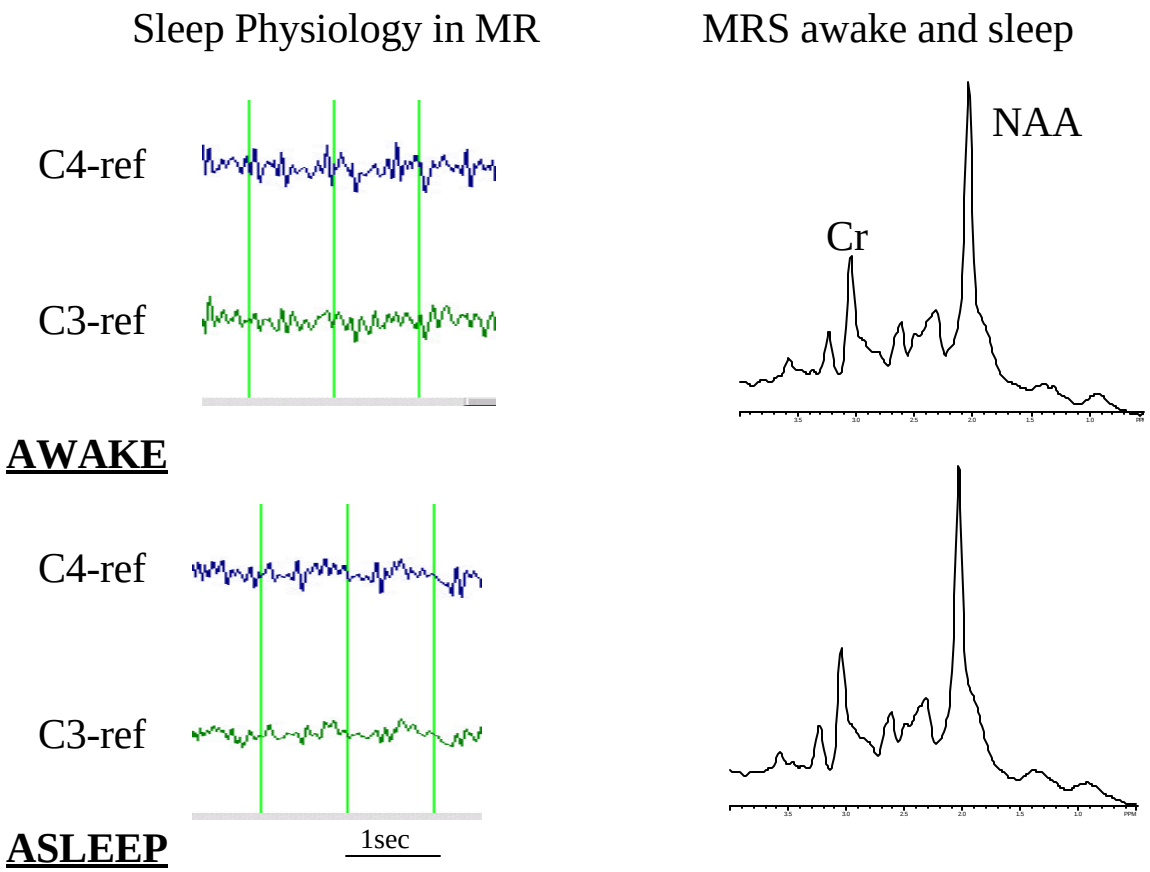


Figure 3. *NMR studies during wakefulness and sleep in the Magnetic Resonance (MR or NMR) Center on an adult volunteer. Each spectrum represents seven min of acquisition using short echo (TE = 16 ms)-proton NMR pulse sequence. Note the presence of N-acetyl aspartate (NAA) and Creatine (Cr), Glutamate and Glutamine in between NAA and Cr. Since these spectra measure steady-state levels, we did not anticipate much difference in concentrations between wake and asleep.*

^{13}C MRS and the relationship between the glutamate/glutamine cycle and neuronal oxidative glucose consumption. Glucose oxidation is the major energy source in the mature brain (Siesjo 1978). Recently Magistretti and coworkers have suggested that the uptake of the neurotransmitter glutamate by the astrocyte may be directly coupled to glucose uptake (Pellerin 1994, 1998). This mechanism may couple glucose uptake to the oxidative needs of the neuron. In order to test whether the glutamate/glutamine cycle is coupled directly to brain glucose metabolism, we measured the rates of glucose oxidation (CMR_{glc}), and the glutamate/glutamine cycle (V_{cycle}) using

in vivo ^{13}C-NMR spectroscopy over a wide range of neuronal activity in rat cortex. We found a close to 1:1 relationship between CMR_{glc} and V_{cycle} over a wide range of electrical activity.

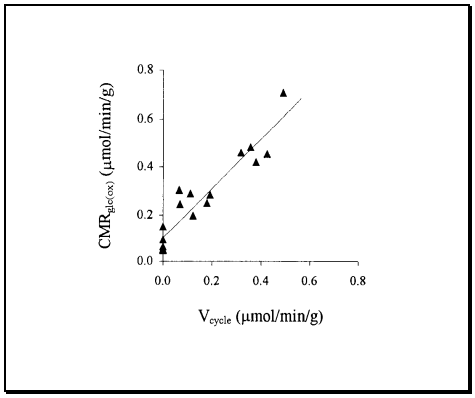
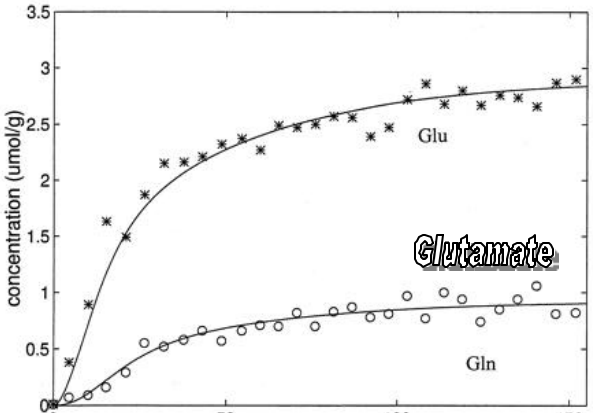


Figure 4. *This graph shows the linear relationship between the rate of oxidative neuronal glucose consumption and the glutamate-glutamine cycle. Linear regression yielded a slope of 1.04 and an intercept of 0.10 with a correlation coefficient of 0.94 ($p < 0.01$, n 17). Oxidative glucose consumption increased in a direct 1:1 proportion with the rate of glutamate-glutamine cycle over the full spectrum of EEG activity from minimal slowing to isoelectric pattern produced by various levels of anesthesia.*

^{13}C Nuclear Magnetic Resonance Spectroscopy studies of Glucose oxidation and the Glutamate-Glutamine Cycling in Human Brain. Due to the sensitivity of the resonance frequency to the chemical environment, ^{13}C-NMR allows the non-invasive measurement of the ^{13}C-isotopic enrichment of specific carbon positions of glutamate, glutamine and many other compounds in living tissue. The natural abundance of the ^{13}C-isotope is 1.1% so that in conjunction with ^{13}C-enriched substrates, rates of isotopic incorporation may be measured. A major research focus of our group at Yale has been the development of MRS methods to measure metabolic flux from ^{13}C-isotopic labeling of metabolites measured *in vivo*. Substrates labeled with the non-radioactive, stable isotope ^{13}C have been employed in vivo to measure metabolic flux, enzyme activity, and metabolic regulation in the living brain of animals and humans (Rothman 1985, Behar 1986, Fitzpatrick 1990, Kunnecke 1993, Gruetter 1992, Rothman 1992, Gruetter 1994, Mason 1992,1995, Manor 1996, Sibson 1997, 1998a, 1998b). Measurement of glutamate and glutamine labeling by ^{13}C-NMR from [1-^{13}C] glucose allows direct measurement of the rate of glucose oxidation and the glutamate/glutamine cycle in the brain.

We have recently used ^{13}C NMR to measure the rates of $CMR_{glc}(ox)$ and V_{cycle} in the occipital/parietal cortex of 6 healthy subjects (Shen 1999). This study showed that, as in the rat, the glutamate/glutamine cycle is 80% of the rate of $CMR_{glc}(ox)$ under non-stimulated awake conditions. The similarity of the ratio between V_{cycle} and $CMR_{glc}(ox)$ supports the presence of a similar tight coupling between these pathways in the human brain (Fig. 5) as demonstrated in the rat brain. *The unique ability of ^{13}C NMR to measure these pathways, and the safety for human*

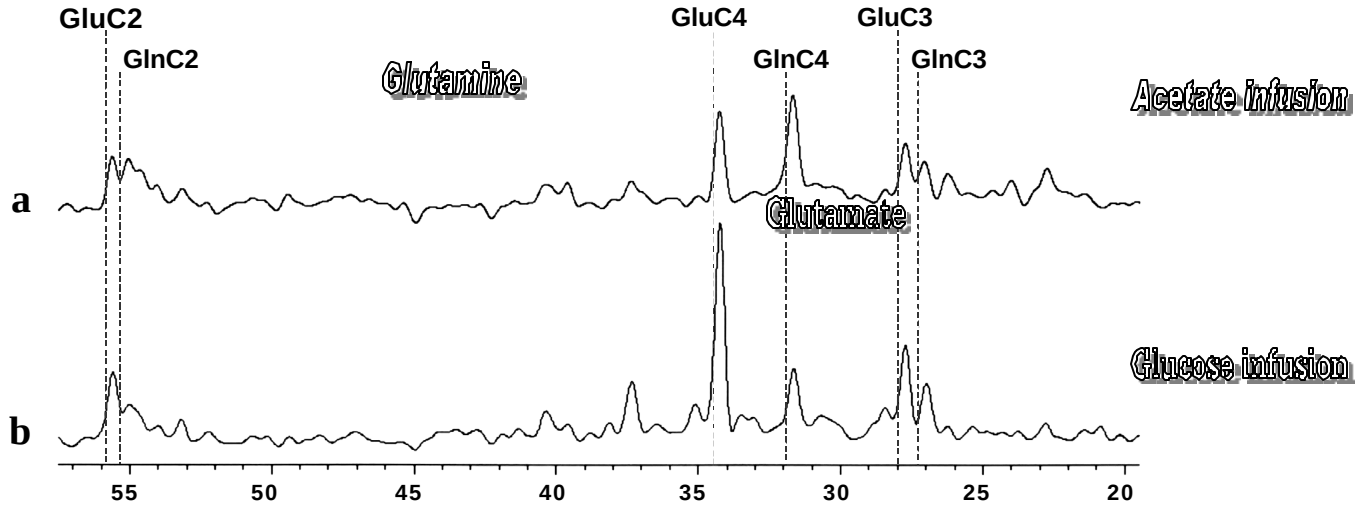


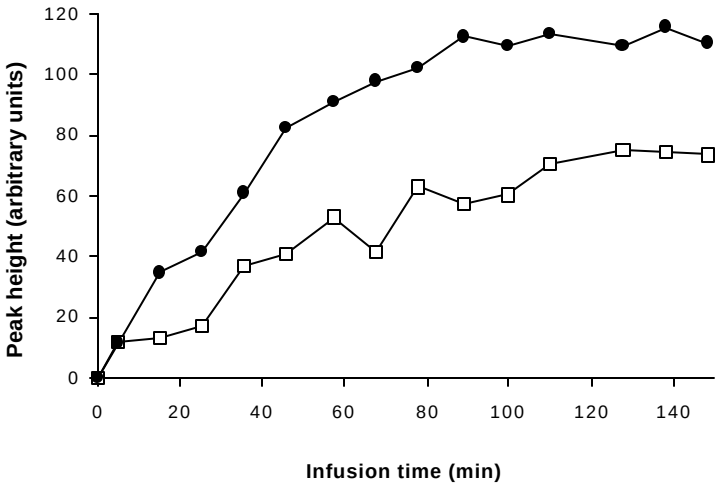
use (no radioactivity or other health risks), makes it an ideal method for studying glucose metabolism in young adults and children. Similarly, this approach will prove useful for the study of wakefulness and sleep.

Figure 5. *Time course of glutamate and glutamine labeling in the occipital lobe of healthy subjects in vivo. Spectra were obtained with a five minute time resolution during a constant infusion of 1-^{13}C-glucose (Shen 1999). Isotopic equilibrium at the [4-^{13}C]-glutamate and [4-^{13}C]-glutamine was reached in less than one hour. Under low light, unstimulated conditions, the mean metabolic rates in human cerebral cortex for CMR_{glc} and V_{cycle}, were 0.80 μmol/g/min (sd 0.03,n=6) and 0.32 μmol/g/min (sd 0.04, n=6).*

^{13}C MRS measurements of the rate of the neuronal TCA cycle , glial TCA cycle, and Glutamine Glutamate Cycle from Acetate. ^{13}C incorporation into brain glutamate and glutamine was measured in 8 volunteers during an intravenous infusion of [2-^{13}C] acetate, which has been shown in animal models to be metabolized specifically in glia. Analysis of the results by mathematical modelling indicates that the glutamate/glutamine neurotransmitter cycle between astroglia and neurons (0.32±0.07 μmol.g^{-1}.min^{-1} mean +/- SD) is the major pathway for neuronal glutamate replenishment and that the astroglial TCA cycle flux (0.14±0.06 μmol.g^{-1}.min^{-1}) accounts for ~15% of brain oxygen consumption. The ratio is similar to that determined using [1-^{13}C] glucose which supports the glutamate/glutamine cycle as accounting for over 80% of the total neuron/glial glutamate trafficking in the human cerebral cortex (Lebon et al. 2001) (Figure 6).

Figure 6. *Top panel shows a spectrum obtained after an acetate infusion in human brain in our*





NMR center (a) compared with a spectrum obtained during a glucose infusion (b). Note the various peaks that are obtained that represent glutamine and glutamate. When 2-^{13}C acetate is the substrate, the glutamine resonance is more intensely labelled than the glutamate resonance, consistent with acetate selectively labelling the glial pool. In contrast 1-^{13}C glucose, which enters primarily through neuronal Pdh, labels glutamate, which is primarily in neurons, and is more intensely labelled. <u>Bottom panel</u> shows the time course of glutamine (black circles) and glutamate (open squares). Note that the glutamine labelling is quicker with the acetate than with the glucose infusion, allowing the rate of the glutamate-glutamine cycle to be determined from approximately 20-30 minutes of data acquisition. The subsequent labeling of glutamate allows the rate of the neuronal TCA cycle to be determined. Compare results in figures 5 and 6.

The development of the acetate method for tracing brain metabolic pathways represents significant progress towards the goal of studying neuronal/glial glutamate trafficking and metabolic rates during sleep. The usage of acetate as a ^{13}C label source instead of glucose allows the rate of the glial TCA cycle to be measured unambiguously and also allows total neurotransmitter trafficking to be measured.

4T Measurements of Human Glycogen 1-^{13}C Labeling. Recently at 9.4T Choi et al have demonstrated the ability to measure the incorporation of 1-^{13}C glucose into the glycogen resonance (Choi 1999)**.** These findings open up the intriguing possibility of studying human glycogen metabolism during sleep. Our laboratory has pioneered the measurement of glycogen in human muscle and liver (Rothman 1991) and we presently are extensively using this methodology to study muscle and liver metabolism. We have recently on our 7T animal system reproduced the findings of Choi, Ugurbil and Gruetter and obtained high sensitivity spectra of glycogen from rat brain (Fig. 7). Assuming similar labeling in the human brain, we anticipate being able to measure the glycogen resonance at 4T with equivalent or better sensitivity. With the anticipated 4T system which we will be using for these studies, we will have a magnetic field strength sufficiently high to perform these measurements on human subjects. In addition, we have recently shown that, with the 4T, glucose transport can be measured at higher resolution in the human brain using ^{1}H NMR spectroscopic techniques to allow differentiation of transport between gray and white matter (deGraff 2001) which represent a significant enhancement of sensitivity from our earlier studies at 2.1T (see Section B above). Based on studies done in animals (Choi 1999), recent ^{13}C NMR studies on amino acid turnover at 4T (Gruetter , 1998, 2000, 2001) and the known concentration of glycogen in the human brain, we expect that glycogen can be measured at 4T with ^{13}C labeling.

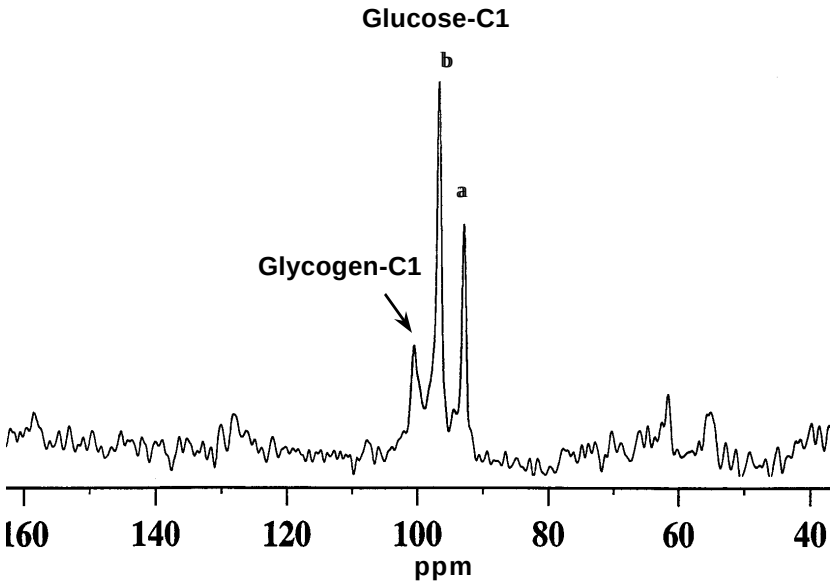


Figure 7. *Spectrum obtained on an adult rat (SD) (tracheotomized and ventilated) (30%O2/70%N2O) under alpha-chloralose anesthesia in our NMR Center. It was infused intravenously with [1-^{13}C]glucose for 5 hrs. The spectrum accumulation time was 11.5 min. This spectrum was acquired at 7 Tesla using ^{1}H-decoupling from a volume-of-interest (VOI) of 8 x 6 x 8 cubic mm.*

Glutamate turnover rate and previous collaborative work. The steady-state rate of glucose oxidation through the mitochondrial TCA cycle (VTCA) was measured in acid extracts of 10-and 30-day-old cerebral cortex of rats receiving [1-13C]glucose intravenously and in neocortical slices superfused in vitro with the same isotope. TCA cycle flux was determined for each age group based on metabolic modeling analysis of the isotopic turnover of cortical glutamate and lactate. The sensitivity of the calculated rates to assumed parameters in the model were also assessed. Between 10 and 30 postnatal days, VTCA increased by 4.3-fold (from 0.46 to 2.0 μmol g-1 min-1) in the cortex in vivo, whereas only a 2-fold (from 0.17 to 0.34 μmol g-1 min-1) increase was observed in neocortical slices (Fig. 8). The much greater increase in glucose oxidative metabolism of the cortex measured in vivo over that measured in vitro as the cortex matures suggests that function-related energy demands increase during development, a process that is deficient in the slice as a result of deafferentiation and other mechanisms. This work as well as others demonstrates that the PI and 2 other investigators on this application have *collaborated before* this application. In addition, these investigators and the PI have collaborated in a Program Project together that they have had for 7-8 years.

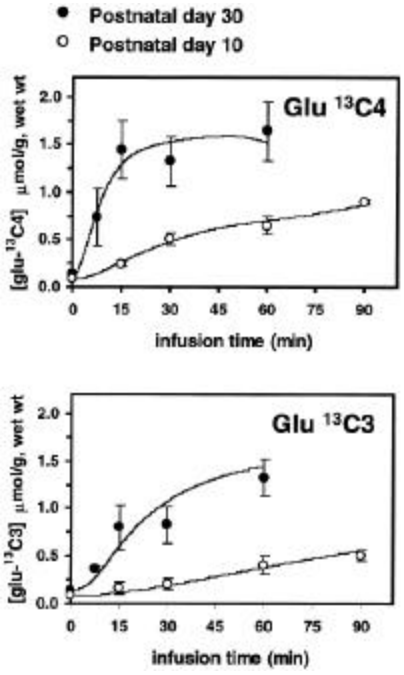


Figure 8. *^{13}C isotopic labeling of neocortical glutamate during an intravenous infusion of [1-^{13}C]glucose in 10- and 30-day-old rats. The ordinate represents the concentration of neocortical glutamate-^{13}C4 (top panel) and glutamate-^{13}C3 (lower panel) at discrete times during the intravenous [1-^{13}C]glucose infusion for each age group. The continuous lines represent the best fit of the metabolic model used to the time course data.*

D. EXPERIMENTAL DESIGN AND METHODS

1. Experimental Protocols

I. Objectives: The objectives of these studies are four-fold: a) To determine glutamate-glutamine cycling between neurons and glia in both wakefulness and quiet sleep in adolescent children. b) To determine the neuronal and glial TCA cycle rate in quiet sleep and wakefulness in these same subjects. c) To determine glycogen content during quiet sleep and wakefulness and d) to study the effect of sleep deprivation on neuronal and glial TCA cycle rate, glutamate-glutamine cycling and glycogen content.

II. Patient population: Subjects will be recruited by Drs. Kass, Novotny and Haddad. Volunteers will be recruited from the Yale-New Haven area by advertisements in local newspapers and posters in the area. Inclusion criteria include the age group of 13-17 years adolescent children and include Tanner Stage II-III. All studies will be performed after a clinic screening visit. *Exclusion criteria* include any known metabolic, endocrine, neurologic, cardiac, GI or respiratory disease, a

hemoglobin <10 g/dl and hematocrit <30%, implanted magnetic material, trauma, history of HIV infection, hepatitis or drug abuse. Venous blood will be drawn during the infusion of labeled glucose and acetate. The maximum blood drawn as described in the Consent form and will be no more than 40 cc during a 9-day period. MR spectra will be obtained at various intervals as described below.

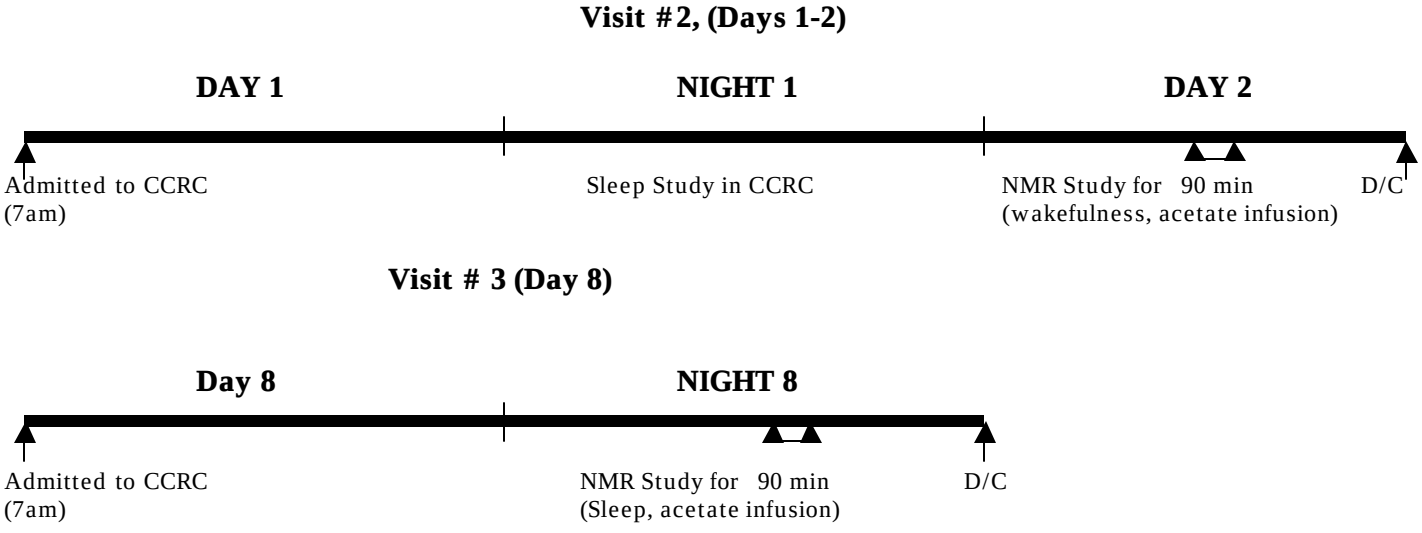
To participate in the MRS study, patients will have a face to face interview with one of the project investigators where the nature of the project, the risks and the benefits of participation in the projects are discussed with the subject. A focused history will be taken and a checklist of hazards will be reviewed with the subject. If following these discussions, the subject continues to be interested in the project, informed written consent will be obtained on the consent form approved by the Yale University Human Investigations Committee (HIC). Both child and parent will sign. Thereafter, the investigators assume clinical responsibility for the subject care.

All measurements will be made using NMR spectroscopy during wakefulness or sleep after patients have been screened and studied in the Sleep center. None of the studies proposed have been done in adults or children. Indeed, only a small part of what is proposed here has been done in animals with the use of invasive techniques (see above in Section B). Our long-term goals include the study of children of various ages, from the very young infant to the adult. For simplicity, for practical and safety reasons, we are starting with this protocol which calls for the study of the older child or adolescent, ie, 13-17 years of age (Tanner II-III). Hence these studies have not been done in the adult or child and the metabolic processes that occur in sleep are very poorly defined. Furthermore, we and others have shown in the past that sleep processes are very different in the child and especially the adolescent, when compared to the adult (Coble 1987, Carskadon 1987).

III. Paradigm and Measurements: The study will involve 3 visits to the Children's Clinical Research Center (CCRC) at the Yale-New Haven Hospital under the observation of Drs. Lewis Kass, Edward J Novotny, Jr. or Gabriel Haddad. Visit # 1 is a screening Specialty Clinic visit during which a physical exam, a history with blood tests will be performed. Blood tests will be taken in the Clinic to test for potential factors that would exclude the volunteer from the study, specifically elevated blood glucose, elevated liver function tests, low hematocrit, and impaired renal function. Visits #2 and 3 will be visits to the CCRC and Nuclear Magnetic Resonance (NMR) visits. ***Screening Clinic Visit (Visit #1) for all children:*** Before the child can be part of the study, the child will come to the Specialty Pediatric Clinic (2^{nd} floor of the Children's Hospital) for a screening visit of approximately 1 hour. During this visit, the PI or co-investigators who will perform a physical examination and ask about the medical history will explain the procedures of the study. During this visit a set of blood (15cc) and urine tests will be done. One week later, after the results of the first screening visit have been evaluated, Drs. Kass, Novotny or Haddad will set up a date for the next visits as described below. ***There are 4 groups of adolescent children*** participating and these will be randomly assigned. Two groups will be studied with an infusion containing ^{13}C-acetate (A1, with normal activities, and A2, with sleep deprivation) and the other two groups with an infusion containing ^{13}C-glucose (G1, with normal activities, and G2, with sleep deprivation). One group receiving the acetate infusion will be studied during wakefulness or sleep after normal daily activities and another group will be studied after sleep deprivation of one whole night. Similarly, each of the two groups receiving the glucose infusion will be studied in the same way as for the groups receiving acetate, that is one group will be studied after normal activities during the previous day and the other after one night without sleep. The protocols of these groups are detailed below.

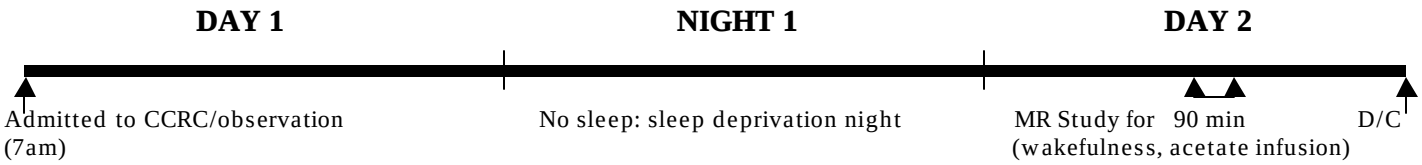
A. GROUPS A1 and A2 (Acetate)

CCRC and NMR Visit (Visit #2), Group Acetate infusion, normal activities (A1): Day 1. The child in this group A1 will be admitted to the CCRC in the morning (7-8am) (see chart below). The child will have normal activities during the day until the evening when the child will have a sleep study in the Sleep laboratory of the CCRC. Each study will involve the recordings of EEG, EOG, EMG, respi-bands for chest and abdominal movements, O_2 saturation, end-tidal CO_2, EKG, leg movements, snoring using a microphone placed on the neck, and a video camera to monitor behavioral aspects during sleep. This will involve placing surface electrodes like those used for the electrocardiogram on the head, forehead and chest. If this study shows that the sleep architecture is normal for the age group, and that there is no medical problem such as snoring, then we will continue with the study, as described below. Otherwise the child will not be eligible for any subsequent part of the study and no additional visits will be required. Day 2. If the child is eligible for the rest of the study, day 2 will be spent in the CCRC until mid-day (at about 12-1 pm) when the child is transported to the NMR Center and studied there. During this study, an intravenous infusion of acetate will be given for over 90 min during which NMR spectra will be collected. The acetate infusion will be given at a rate of 3mg/kg-min. A blood sample (3 cc) will be taken every 15-20 min. This infusion rate will raise blood acetate levels to approximately 2 mM which well within what is tolerated. During the acetate infusion, a blood sample will be taken every 15-20 min and a total of about 15cc will be taken. Furthermore, the child will have to be **awake during the 90-min period of study**. When finished the child can be discharged home (D/C). Every child who is eligible to be studied in the magnet can, for purposes of acclimatization to the magnet, spend a certain time (for a few hours if needed) in a "mock" magnet. This will allow children to acclimate themselves and be prepared for the study the day or night before (see under protocols). ***CCRC and NMR Visit (Visit #3), Group Acetate infusion, normal activities (A1):*** Day 8. This is the last part of the study for this A1 group (see chart below). On Day 8, the child will be admitted to the CCRC in the morning (6-7am) to be observed. In early evening (7-8pm), the child will be taken to the NMR Center to have a study similar to the one performed on Day 2. However, during this study in the NMR center, we will also monitor the sleep stage using EEG and EOG while in the magnet. We will start the same intravenous infusion of acetate and will sample blood (15 cc) over the 90 min period, much like we did on Day 2. The infusion and blood sampling will take place only after the child had **fallen asleep since this study, unlike that on Day 2, is done during sleep**. We will start the infusion only when the child is in the first cycle of Stage III-IV sleep. Sleep state will be monitored continuously during the infusion. After we finish the 90 min NMR study measuring glutamate turnover and glutamate-glutamine cycling (see techniques), the child can be transported to the CCRC and discharged the following morning.

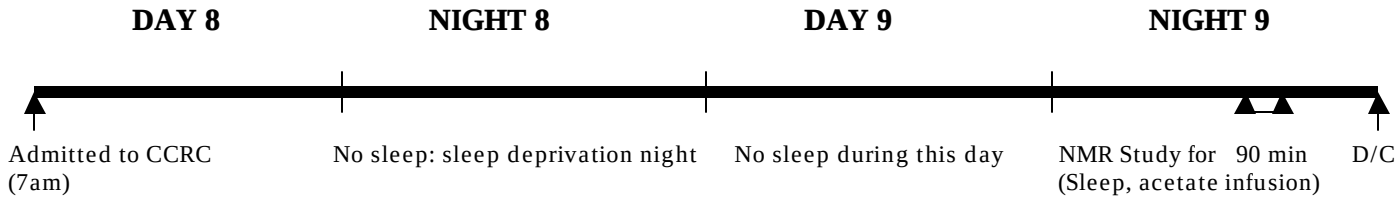


CCRC and NMR Visits, Group Acetate infusion, <u>sleep deprivation</u> (<u>Visits #2,3</u>) (A2). This protocol is exactly the same as for the normal activity group (A1) except that on Day 2 when admitted, the child will not sleep the night that follows the morning of admission (see graph below). The child will be admitted that day before the sleep deprivation night in order to ascertain that the child will have normal activities during the day and that during the night children will be kept awake. They will be able to listen to music, watch TV, read, but they will not be allowed to go to sleep. For adolescent children, this will not, in all likelihood, be difficult. During the following day, children will be studied like the other group on Day 2 (see graph below). Since this a **wakeful study**, the child will be awake in the magnet during the infusion and the blood sampling. The NMR **sleep study** will also, like in the previous group, be done on Day 8. Therefore, the child will be admitted in the morning, stay up that night in the CCRC, stay awake during the day that follows that night and then be studied on the second night when the child will be allowed to go to sleep. The study again will take place in Stage 3 or 4 NREM sleep during the first cycle.

Visit #2, (Days 1-2)



Visit # 3 (Days 8-9)

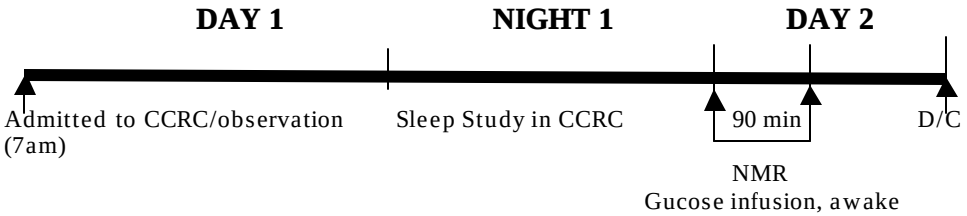


B. GROUPS G1 and G2 (Glucose)

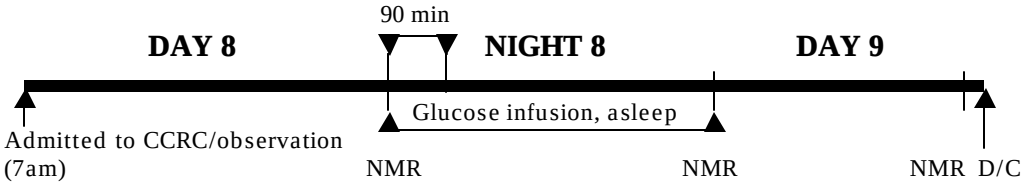
CCRC and NMR Visit <u>(Visit #2)</u>, Group Glucose infusion, <u>Normal Activities</u> (G1): <u>Day 1:</u> This first day will be the same as for the Acetate groups (A1/A2) (see graph below). Essentially, the child will be studied during the first day in the CCRC and Sleep laboratory to determine the sleep architecture. <u>Day 2:</u> During the 12 hours (6am to 6pm) that follow the sleep study in the CCRC, the subject will carry normal activities but will have a glucose infusion for only 90 min during the day, when the **child is awake** (see graph below). ^{13}C glucose will be infused and blood will be sampled during the infusion every 10-15 min to keep plasma glucose at a certain level (see Techniques). The total amount of blood taken will also be about 15 cc. NMR studies will be performed during the infusion in the NMR Center in order to determine glutamate turnover rate. The reason for the use of glucose here is mostly to determine the TCA cycle in a short period. This will complement the acetate infusion since glucose and acetate isotopes label ***<u>first</u>*** the neuronal glutamate and the glial glutamine pools respectively. Hence we will be able to obtain in the first 20-30 min the initial phase of labeling in the neuronal and glial pools respectively. On this second day, the subject will be discharged home after finishing the

glucose infusion. ***CCRC and NMR Visit (<u>Visit #3</u>), Group Glucose infusion, <u>Normal Activities</u> (G1):*** <u>Day 8:</u> On Day 8, the subject will be admitted to the CCRC in the morning and will be asked to maintain daily activities (see graph below). In early evening, an infusion of glucose will be started for 12 hours, **throughout the night, when the child is asleep**. The glucose infusion will be started when the patient is asleep, in the NMR facility. The glucose infusion will be done as described under Techniques for 90 min in order to perform the NMR turnover rate studies that are similar to those done in the wake state on Day 2. However, in order to obtain information for glycogen synthesis and metabolism, we will keep this glucose infusion going for the rest of the night at euglycemia (5mM). The child will spend only the first 90 min in the NMR Center but then will spend the night in the CCRC. NMR spectra will be obtained not only at the beginning, but also at the end and 12 hours after the infusion of glucose had stopped. The reason we will examine glycogen 12 hours after the glucose had stopped is to be able to look at the washout of the label and understand what happens during sleep and wakefulness. Also, like on Day 2, blood will be sampled during the infusion, totaling about 15 cc over the entire infusion. After the NMR studies, the subject can be discharged from the hospital and children in G1 group would have completed the study.

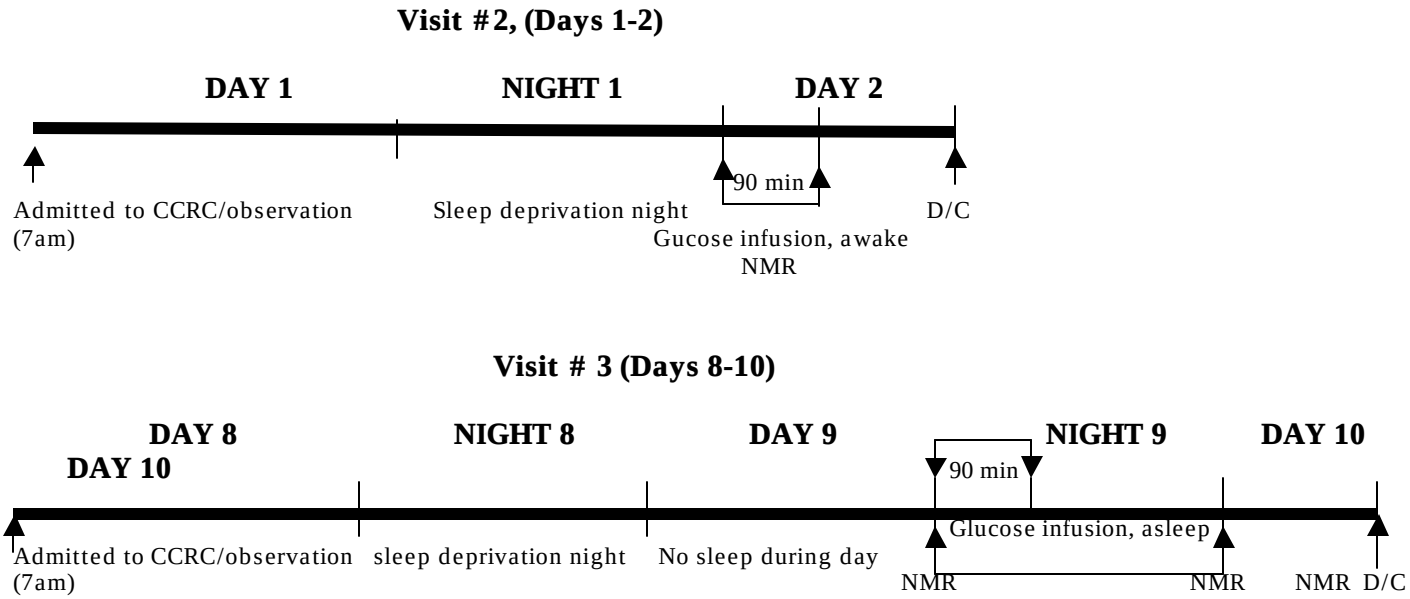
Visit #2, (Days 1-2)



Visit # 3 (Days 8-9)



CCRC and NMR Visits, Group Glucose infusion, <u>Sleep Deprivation</u> (<u>Visits #2, 3</u>) (G2): This protocol is exactly the same as for the G1 group except on Day 2 when admitted, the child will not sleep that night, as we have done with group A2 (see graph below). During the following day, after one night of sleep deprivation, the child will be studied **awake in the magnet** and the protocol is like that of G1 except that the subject has spent the previous night awake in the CCRC. Therefore, an infusion is given after sleep deprivation and the same NMR studies are performed as stated above for the G1 group. The study of Day 8 in this G2 group will be done as follows: The subject will be admitted on Day 8, will stay awake that night on the day of admission in the CCRC, stay awake the following day and then be studied on the second night when the **child is allowed to go to sleep**, in exactly the same way as we did for the G1 group (see graph below). Hence, the subject will have the infusion during the second night when the subject is allowed to sleep, with the NMR studies and protocol (see graph below). Blood sampling is done as on day 8 for the G1 group. The child can be discharged after the NMR studies.



IV. Number of studies: We have the expertise and experience in all facets of the proposed work. Indeed, we are excited about the potential and expected results. With the personnel and resources requested, we will be able to perform the experiments needed over the period requested. From the description above, we have 4 groups of children (A1, A2, G1, G2). All children will have at least 1 visit, that of the Pediatric Specialty Clinic visit. Then those who are chosen will each have 2 visits. The number of patients selected for each protocol is determined from the variance of the measurement and the criteria that the rates of interest be determined with a 95% confidence interval of better than 10%. We also anticipate that 20% of children may not be able to finish the protocol. We estimate therefore that we will need 12 children studied in each of the 4 groups (total of about 48 children). With this in mind, we will have 96 visits and CCRC/NMR studies over the course of the application. Analysis of individual data will be done using rather straightforward parametric (t-testing, ANOVA) or non-parametric statistics (e.g., Wilcoxon Rank Sum) with computations of means and SDs.

V. Statistical Analysis: We estimate below the statistical power for testing our hypotheses. To be conservative, we have calculated the variability in the MRS measurements based upon the measurements we have performed at 2.1T. Due to the low magnetic field strength of this system, the variability was largely determined by measurement sensitivity. At 4T, we anticipate at least 2x greater precision in the MRS measurements. Furthermore many of the comparisons will be performed pair wise on the same subject, which further enhances statistical power. For *Hypothesis 1*, we will determine whether stage III-IV sleep reduces neuronal glucose oxidation the fractional [TCA (sleep)/TCA (awake)] decrease in neuronal TCA cycle. This will be calculated for each subject from the time course of ^{13}C labeling during infusion of 1-^{13}C glucose. In resting, awake human parietal cortex, neuronal glucose oxidation by the TCA cycle accounts for 85% of total glucose oxidation (Shen et al. 1999, Lebon et al. 2001). We therefore anticipate the 25- 30% decrease in cerebral glucose consumption reported in stage III-IV sleep to be primarily due to a decrease in the neuronal TCA cycle. Based upon the variability of the TCA cycle rate measurement determined in awake subjects, we anticipate a mean and standard error of TCA (sleep)/TCA (awake) = 0.70 ±0.07. Because there have been no measurements of glial TCA cycle as a function of brain activity in animal models or humans, we cannot predict the magnitude of the change. Based upon the variability of TCA rates determined in awake subjects, we anticipate that with 12 subjects that we can measure

TCA(sleep)/TCA (awake) in glia with a standard error of approximately ± 0.2 (20%). We believe that if a down regulation of glial functional metabolism is a significant adaptation in stage III-IV sleep that we will see reductions in glial TCA rates larger than this. During sleep deprivation, the ratios of TCA(deprived)/ TCA(non-deprived) for the neuronal compartment will be calculated and compared to that of the glial one [TCA(deprived)/TCA(non-deprived)] for each subject using a t-test.

For *Hypothesis 2*, based upon the relationship found by Sibson et al. (1998) between the functional component of neuronal glucose oxidation and the glutamate/glutamine cycle, we anticipate a similar decrease in the glutamate/glutamine cycle (Vcycle) as with neuronal glucose oxidation. However, the fractional decrease in Vcycle will be greater because approximately 30% of glucose oxidation in humans is due to non-functional processes such as protein synthesis. For both the 1-^{13}C glucose and 2-^{13}C acetate studies we therefore anticipate a ratio of Vcycle(sleep)/Vcycle(awake) of 0.5 with a standard error for 12 subjects of ±0.1. The ratio of Vcycle(deprived)/Vcycle(non-deprived) will also be calculated for each subject. The ratio will then be compared by t test with the group ratios Vcycle(sleep)/Vcycle(awake).

For *Hypothesis 3*, brain glycogen concentration is believed to be on the order of 2-3 μmol/gm wet weight in the cerebral cortex. Although the amount of synthesis during sleep is not known, we anticipate that it will be on the order of 1-2 μmol/gm based on animal models. For an average plasma 1-^{13}C glucose fractional enrichment of 30%, we anticipate based upon measurements in liver and in phantoms simulating the brain that we will have a measurement precision of approximately 0.2 μmol/gm, which is more than sufficient to document any significant increase. Based on studies of muscle and liver glycogen metabolism, depletion leads to rapid recovery of glycogen stores when conditions of synthesis are restored. Therefore we anticipate approximately a two-fold greater glycogen synthesis over night after sleep deprivation. With 12 subjects we anticipate sufficient power for this comparison whether done pair-wise or by group t test.

VI. Interpretation and potential pitfalls:

When considering the expected/anticipated results from these studies, it is important to highlight a number of issues. Our group of investigators has considerable expertise in all facets of the application. For example, Dr. Haddad has been interested in sleep and sleep disorders for many years, since his fellowship when he was at Columbia University working on the Sudden Infant Death syndrome and subsequently at Yale when he and Dr. C. Rosen established the first Sleep Laboratory at Yale-New Haven Hospital (more than 12 years ago). Dr. Kass, more recently, has directed the Sleep Laboratory at this institution and has been interested in sleep since his fellowship as well. Drs. Rothman, Novotny and others in the NMR Center are pioneers in studying children using NMR for a variety of conditions including epilepsy. In addition, they have developed techniques and NMR modeling methods that will be very important in our estimation of glutamate, glutamine flux rates, among other metabolites (see below, under Techniques and in References). Furthermore, state-of-the-art technology will be used to study brain metabolites during sleep. A case in point is the use of the 4-Tesla human magnet that will be used to study glycogen content during wakefulness and sleep in adolescent children.

There are at least two specific aspects that are very exciting in this work. 1) We combine the expertise of both, investigators experts in sleep and those experts in NMR. 2) Sleep has not been subjected to this kind of analysis, neither in children nor in adults. This will be very interesting since not only will we learn a great deal from these studies but we also be able to address certain hypotheses and models such as Magistretti's hypothesis and working model regarding neuronal and glial interactions and importance of glia in sleep metabolism.

Our interpretations of how the results of the study will support or refute our hypotheses as well as specific models of the role of neuronal/glial interactions in sleep are described. ***Below, we***

detail our potential interpretations and possible pitfalls for each of the 3 hypotheses proposed.

Hypothesis 1: Stage III-IV sleep has a lower metabolic requirement and a lower glutamate turnover or tricarboxylic acid cycle rate (TCA) in both neurons and glia, as compared to wakefulness. There is a considerable amount of data from PET and other methods (see Section B) which indicate that a decrease in regional brain metabolism occurs in Stage III-IV sleep. This reduction in metabolism is consistent with the decrease in electrical activity associated with this state. It is unknown what fraction of this reduction is due to neurons versus glia. The use of MRS in combination with the acetate infusions proposed specifically introduces label into the glia and will allow this question to be addressed. We anticipate during sleep deprivation the rate of the neuronal and glial TCA cycle will be similar to the awake state. This finding would fit in with our hypothesis that stage III-IV sleep is required for a reduction of functional neuroenergetics in order to allow metabolic restoration. Alternatively the finding of a reduced TCA cycle would imply that the brain can not sustain the high metabolic activity needed for normal awake function without sleep. This latter finding may explain the loss of cognitive performance in individuals with extended sleep deprivation. The question of whether functional or non-functional metabolic processes are down regulated would be answered by the measurements of the glutamate/glutamine cycle.

Hypothesis 2: As compared to wakefulness, sleep III- IV is characterized by a lower rate of brain neuronal glutamate release and glial glutamate uptake in adolescent children; this reduced glutamate/glutamine cycling during this sleep stage in brain of children is prevented by sleep deprivation. We anticipate, based upon the relationship established in animal models (Sibson 1998, 1999) that the reduced metabolic rate in sleep stage III-IV will be associated with a reduction in glutamatergic neuronal activity as indicated by a lower rate of the glutamate-glutamine cycle. Based on the literature, we anticipate a greater than 25% reduction in the neuronal TCA cycle rate and an even greater (40%) reduction in the glutamate-glutamine cycle (the larger reduction reflecting there being an approximately 30% component of human brain energy metabolism not related to functional or synaptic activity). Both of these reductions are well within the sensitivity of the methods we will use. In contrast, if we find a reduction in the TCA cycle rate but little or no reduction in the glutamate-glutamine cycle, we would interpret this as indicating that, rather than reducing synaptic activity during sleep, the brain is reducing the energy cost of "housekeeping" functions such as protein and DNA synthesis. Little is known about the effect of sleep on glial energy requirements. However, if the glutamate-glutamine cycle rate is decreased, we would anticipate finding reduced glial energy consumption, possibly due to both a) the reduction in the energy cost of maintaining the glutamate glutamine cycle and b) the reduced ion pumping needs. Indeed, studies in isolated cells and slices suggest the glial membrane depolarizes during synaptic activity, when glutamate and Na^+ are being taken up by glia (Magistretti 2000). Therefore, if functional activity is reduced we would anticipate finding a reduction in the glial TCA cycle due to reduced energy needs for ionic pump activity. We anticipate that during sleep deprivation the rate of the glutamate/glutamine cycle will be similar to the awake state. This finding would fit in with our hypothesis that stage III-IV sleep is required for a reduction of functional neuro-energetics in order to allow metabolic restoration. Alternatively the finding of a reduced glutamate/glutamine cycle would imply that the brain can not sustain the high metabolic activity needed for normal awake function without sleep. This latter finding may explain the loss of cognitive performance in individuals with extended sleep deprivation.

Hypothesis 3: Brain glycogen content increases during the course of sleep in children and sleep deprivation markedly lowers glycogen content. In muscle, glycogen is well established to have

a key role in energy metabolism by providing a rapidly available source of ATP during high power demand which cannot be responded to by an increase in oxygen consumption. Glycogen may play a similar role in glial energy metabolism by providing a rapid source of ATP during bursts of electrical activity associated with normal function. As described above, we anticipate a reduction in the energy demands on the glia during stage III-IV sleep due to both a reduction in glutamate-glutamine cycling and other energy demands on the glial cell associated with function. An appealing hypothesis is that during stage III-IV sleep the glia can replenish its glycogen stores, similar to what occurs in the muscle during rest after a meal (Shulman et al. 1990). In this aim, we will use NMR, which has the unique capability of non-invasively measuring brain glycogen, to determine whether glycogen synthesis occurs during sleep. Furthermore, we will test if sleep deprivation markedly lowers glycogen content. If we find that our hypothesis is correct we would interpret the result as supporting an important role for stage III-IV sleep for the replenishment of brain energy stores as theorized by a number of investigators including Bennington and Heller (Bennington 1995). The finding of little change in glycogen would suggest that glycogen in the brain is primarily an energy store for emergency situations like hypoxia and hypoglycemia. The role of reducing the functional demands on the glia during sleep may then be to allow "repairs and modifications" to cellular structures, with repletion of energy stores being of secondary importance.

Effect of Stage III-IV Sleep Duration on MRS results. A limitation of the MRS method is that it takes on the order of 90 minutes to complete a study. We recognize that in some cases children will not remain in stage III-IV sleep for that duration. However in the 1-^{13}C glucose measurement the neuronal TCA cycle may be calculated with 25 minutes of data, because of the rapid entry of label into the neuronal glutamate pool. Similarly during the 2-^{13}C acetate infusion the rates of the glutamate/glutamine cycle and the glial TCA cycle rate may be calculated with 25 minutes of data due to rapid entry of label into the glial cells. By performing both glucose and acetate studies on all subjects we insure that on all subjects we will be able to measure all three rates. In subjects who are able to sustain sleep throughout the infusion period the performance of both studies provides us with an additional validation of the rates.

General potential pitfalls. There are, however, certain aspects of this work that we will need to be very cautious about. First, we will need to make sure that the period of sleep or wakefulness is long enough for the isotopic measurements. The longest measurement is the acetate measurement, which requires 60-90 minutes to complete. In addition, we will use also glucose infusions in some studies for a short period such as 90 min. To insure that we can achieve this duration of sleep in the magnet we will pre-screen subjects in the sleep lab, and in some cases in the magnet room to reach steady states. If periods of sleep get interrupted by short periods of wakefulness (as could happen during sleep), this information will be included in the metabolic modeling to insure that these periods were not sufficiently long to influence the results. Second, we believe, as we detailed in Section B, that flux rates in quiet sleep will be different from those in wakefulness, based on the literature. However, we do not know what the differences might be between these 2 stages when measuring variables outlined above. Third, one potential problem with sleep studies in the magnet is the noise elicited with the generation of spectra. This noise can be attenuated markedly and hence this problem can be alleviated. In addition, because of the rhythmicity of the noise, subjects generally adapt and can fall asleep in the magnet as demonstrated in our Preliminary data. Finally, we do have a "mock" magnet to which we can invite children to accustom themselves to the noise produced in the magnet, whether just before the study or the night before during which they can spend a few hours in the magnet to acclimate themselves.

2. Techniques

Infusion Protocol and Plasma Measurements.

Glucose infusion. Plasma glucose concentration and fractional enrichment will be controlled by using the glucose clamp technique (Shulman 1990). This will require placement of intravenous catheters in the antecubital veins and 1-^{13}C glucose will be infused. The fractional enrichment of glucose will be raised by an initial pulse of 99% enriched D-[1-^{13}C] glucose in a 20% wt/vol solution which typically requires 0.2g of glucose/kg. This will be followed by infusion of a 60% enriched D-[1^{13}C] glucose solution to maintain the plasma fractional enrichment constant during the NMRS experiment. Plasma glucose will be controlled by adjusting the infusion rate of glucose based on blood samples obtained from the opposite arm every 5 to 10 minutes using a Beckman glucose analyzer (Beckman, Fullerton, CA). The plasma glucose will be initially hyperglycemic (10 mM) for the first 10 to 20 minutes and then allowed to drop to around 7 mM for the remainder of the infusion. With this protocol we expect to maintain the fractional enrichment of the plasma glucose constant. The fractional enrichment of glucose will be determined from blood drawn every 15 to 20 minutes throughout the infusion and later measured by GCMS.

For the infusions during stage III-IV sleep, the plasma glucose will be allowed to drop to 5.0 mM and then sustained there over night with a continuous drip IV containing 99% enriched [1-^{13}C] glucose at 1 mg/kg-min body weight into an antecubital vein. During the infusion the plasma glucose fractional enrichment will drop to approximately 30% (1/2 the anticipated peak enrichment during the magnet study and be sustained there until the next morning). Plasma samples will be obtained every 15 minutes to check the plasma glucose level and for later fractional enrichment analysis.

Acetate infusion

^{13}C MRS spectra will be obtained during a 120-min [2-^{13}C] acetate infusion (350 mmol·l^{-1} sodium salt 99% ^{13}C enriched, Isotec, Miamisburg, Ohio) at an infusion rate of 3 mg·kg^{-1}· min^{-1} into an antecubital vein. Plasma samples for acetate analysis will be collected every 10-15 minutes.

Plasma measurements

Fractional enrichments and plasma acetate concentrations will be measured from blood samples collected at 10-15 minute intervals and analyzed on a Hewlett-Packard 5890 gas chromatograph (HP-1 capillary column, 12m × 0.2mm × 0.33mm film thickness) interfaced to a Hewlett-Packard 5971A mass selective detector operating in the electron impact ionization mode). Additional deproteinized samples will be analyzed by ^{1}H MRS at 360 MHz in D_2O to determine the degree of enrichment at the C1 position of glucose due to hepatic scrambling.

[1-^{13}C] glucose plasma isotopic fraction will be measured in samples obtained every 15 min by gas chromatography-mass spectrometry of the pentacetate derivatives of plasma glucose after deproteinization and deionization (Shulman et al 1990).

In vivo ^{13}C MRS during 1-^{13}C glucose and 2-^{13}C acetate infusion. The subjects will be laid supine in a 4.0 Tesla whole body magnet (Bruker, Billerica, MA, USA) equipped with active-shielded gradients . A volume of 48 ml (4x3x4 cm3) will be localized within the mesial parietal lobe (as it has one of the biggest decrease in metabolic rate in stage III-IV sleep) of the brain

that excludes major blood vessels and ventricles, based on inversion recovery 1H magnetic resonance images (MRI) obtained just before the start of the infusion (repetition time TR = 2500ms, inversion time T1 = 800ms, echo time TE = 10ms. Following volume selection the Bo homogeneity in the volume will be optimized using an adiabatic automated field mapping routine. ^{13}C MRS spectra will be obtained using a adiabatic polarization transfer sequence we have implemented on a 2.1T whole-body magnet (Shen 1999). The RF coil assembly used consists of two circular ^{1}H coil loops (13 cm diameter each) arranged spatially to generate a quadrature field and a 8.5-cm diameter circular surface coil for ^{13}C detection. The localization is based on the ISIS technique. Hyperbolic secant pulse (u = 7) of 7 ms duration with a bandwidth of 3850 Hz were used to localize a volume of 48 ml. For adiabatic polarization transfer ^{1}H magnetization is excited and polarized with a segmented BIR4 90^{o} pulse and a simultaneous adiabatic full passage (AFP) pulse on ^{13}C to defocus ^{1}H-^{13}C one-bond J evolution. An adiabatic half-passage pulse (AHP) is applied simultaneously with the 3rd segment of the BIR4 90^{o} pulse to obtain polarization transfer. The transferred magnetization (I_zS_y) is then refocused by a BIREF-1 pulse on ^{13}C and a simultaneous AFP pulse on ^{1}H. The duration of each AHP segment is 0.4 ms. WALTZ-16 decoupling with its 90^{o} pulse length set to 700 μs is applied during acquisition of 202 ms. Hartman-Hahn leakage during simultaneous pulses is minimized by setting the frequency sweep width ratio of the ^{1}H and ^{13}C adiabatic pulses to 1.6. The peak RF power for polarization transfer is set to ca. 830 Watts and 230 Watts for ^{13}C and ^{1}H, respectively based on calibrations in phantom samples. The RF power for decoupling is calibrated using an adiabatic double spin echo sequence generating a *y* profile through the gradient isocenter to null the signal from water at volume center.

^{13}C MRS measurements of glycogen. Measurements of glycogen will be performed using the sequence of Choi et al. (1999) adapted to usage in human subjects at 4T. Localization in the sequence is achieved primarily through outer volume suppression, which minimizes the loss of signal from brain glycogen due to its short T2 (Rothman 1991). Prior to implementation of this sequence in human studies localization will be tested in two compartment phantoms designed to simulate the brain and surrounding muscle.

Data Analysis. Data analysis will be performed off line on a Dell 1.7 GHz PC using home written software based on the MATLAB program. Spectra will be zero-filled to 32K, multiplied with a Gaussian function corresponding to 2 Hz line broadening, Fourrier-transformed, and phase-corrected. Due to the T_2 weighing of the polarization transfer sequence, broad background signals from brain proteins and lipids are eliminated leaving a flat baseline throughout the spectrum. The time courses of resonance intensities will be obtained using an LC model with input functions determined from solutions containing pure compounds measured using the same pulse sequence and coil. Resonances to be fit in the [2^{13}C]acetate infusion studies will be the glutamate C4, C3, C2, glutamine C4, C3, C2, aspartate C4, C3, C2 and acetate C2 resonances.

Quantification of ^{13}C concentrations The time course of ^{13}C NMR signal intensities of glutamine and glutamate will be quantitated as described above. The resonance intensities will be converted to ^{13}C concentrations by comparison to the signal intensity obtained from a solution of 50 mM glutamate and 50 mM glutamine at pH 7.0 at the same position relative to the surface coil. Differences in loading will be corrected for by comparison with the signal obtained from a 2-cm sphere containing ^{13}C labeled formic acid at coil center (Gruetter 1994). We have shown that concentrations obtained using this procedure agree well with values obtained from surgical biopsy of human brain (Gruetter 1994). The concentration of [1-^{13}C] glycogen will be determined by integration across a 300 Hz bandwidth and comparison with glycogen in solution (Rothman 1991).

Metabolic modeling and labeling strategies. Time courses of the ^{13}C isotopic turnover of C4, C3 and C2 glutamate and glutamine will be analyzed using a mathematical description of labeling in the neuron and astrocyte. For each carbon of glial and neuronal glutamate and glutamine mass and isotope balance equations are written with the metabolic flow between positions represented by the fluxes in the model (see Mason 1995 for details of the procedure). Rates are then calculated as described in Shen 1999 and Lebon 2001.

E. HUMAN SUBJECTS

A. Subject Population

This study will include adolescent children, between the ages of 13 to 17 years. This population will be composed of males and females and of various ethnicities as documented in Section C and patient selection is independent of race and gender. Subjects will be recruited by Drs. Kass, Novotny and Haddad. Volunteers will be recruited from the Yale-New Haven area by advertisements in local newspapers and posters in the area. Inclusion criteria include the age group of 13-17 years adolescent children and include Tanner Stage II-III. All studies will be performed after a clinic screening visit. *Exclusion criteria* include: any known metabolic, endocrine, neurologic, cardiac, GI or respiratory disease, and

1. Hemoglobin <10 g/dl and hematocrit <30%
2. Trauma
3. History of HIV infection or Hepatitis, history of drug abuse
4. Subjects will be questioned about metallic objects, electrodes, rods and screws, colored contact lens, dental prostheses, limb prostheses, eye prostheses, shrapnel, metal in head, eye, or skin, and embolization coils. If they cannot be removed safely, the subject will be excluded from the study. Subjects with claustrophobia will also be excluded from the study.

B. Risks

1. Physical Risks

The risks of this study are small. The approximate blood loss will be 40cc maximum over 9 day period. Intravenous catheters used during the glucose or acetate infusion are associated with a mild to moderate degree of pain upon insertion, and a small risk of localized bruise, hematoma and/or infection. Other than the needle stick for the local numbing (anesthesia) of your skin before the infusion is started, this is a painless procedure. On rare occasions a bruise may occur at the infusion site. On very rare occasions inflammation of the vein may occur at the same site. Such complications usually disappear spontaneously or with local heat.

These risks will be minimized by the use of the smallest possible catheter and sterile technique. In addition, because we will only raise the glucose level minimally, there will be no danger of hypoglycemia at the end of the infusion. Acetate will increase the acidity (lactate) and the Na^+ load in the blood but at the concentration and duration we are giving the acetate, the amount of lactate and salt given will be a small fraction of the amount of Na^+ and acidity present in the lactated Ringers solution given to children with dehydration.

Patient discomfort /passing out in magnet –During each study the subject will be monitored by EEG, EOG and EKG and by the nurse who will be in the magnet room with the patient at all times. If at any point the patient complains of discomfort the study will be halted and the subject removed from the magnet. If the subject passes out during the procedure, the subject will be removed from the magnet on the mobile patient bed and taken out of the room. Once outside the

room the patient will be assessed by an MD who will be present throughout the procedure. If necessary resuscitation procedures will be performed and an ambulance called to the MR Center to take the patient to the emergency room of Yale New Haven Hospital.

Patient injured in magnet – In the very unlikely event of a patient being injured in the magnet the patient will immediately be removed and brought to the hallway in the mobile patient bed. The MD present will evaluate the severity of the injury. Depending on the severity the patient will either be wheeled to the emergency room on the patient bed or an ambulance will be called to the MRC. If necessary first aid will be applied to stop any bleeding. If the patient is pinned to the magnet by a metal object the spectroscopist running the scan will enter the room and assess whether the object can be safely removed. If it can, the object will be removed from the patient and the procedures outlined above followed. If the object cannot be removed the magnet will be de-energized in order to eliminate the magnetic field. The de-energization procedure will take on the order of 1 minute.

Magnetic resonance spectroscopy will be performed at Yale Medical School Magnetic Resonance Center on a 4 Tesla magnet with a 72 cm clear bore and Bruker Avance electronics (purchased already). Ionizing radiation is not used, and there are no known side effects of the procedure. Some subjects who undergo MRS feel anxious from being in an enclosed space. If this occurs, the procedure will be stopped. The most significant hazard concerns magnetic objects. If brought in the magnet room they may be drawn forcefully in to the magnet and many cause injury. Metallic conductor and electronic circuits can become thermally heated when exposed to fluctuating magnetic fields. ^{13}C MRS spectroscopy will be performed within the FDA guidelines for regional specific absorption rate of rf power (4.0 W/kg locally) and gradient switching rate (400mT/m-sec). Adherence to these guidelines are insured by the safety circuitry of the spectrometer which will shut down the system if either the power deposition or gradient switching rate exceeds safe levels. In addition the gradient coil/amplifier is physically incapable of exceeding FDA guidelines.

Magnetic Resonance Spectroscopy methods are thought to be among the safest techniques available for medical research and diagnosis. The United States Food and Drug Administration has set guidelines for safe exposure of humans undergoing magnetic resonance spectroscopy. The proposed study will operate within these guidelines. A few people experience claustrophobia while lying in the magnet. The most significant hazard concerns magnetic objects. If brought into the magnet room they may be drawn forcefully into the magnet and may cause injury. Metallic conductor and electronic circuits may become thermally hot when exposed to fluctuating magnetic fields. The MRS studies described in this proposal will be used for research into the basic mechanisms related to sleep and sleep deprivation and will not benefit the subjects directly.

2. Risk of Discrimination

We feel that the risks to the study participant will be small due to the encoding protection and the restricted access to data files that could link a subject to a code number, as will be described in Part D below.

C. Consent Procedures

Participation will only be voluntary, by informed, written consent, and subjects will be carefully informed of the voluntary nature of the study and that they can withdraw at anytime. All subjects with whom we have direct contact will be given the consent form attached to this protocol. A

copy of the signed Yale consent form will accompany all samples sent to the PI's laboratory or his collaborators for analysis.

No relatives will be interviewed without the informed consent of the adolescent and informed consent will be obtained from all participants. Refusal to participate will in no way influence the receipt of treatment.

For all children we will first obtain the written consent of the parents (or guardian). We then explain the procedure to the child and ask the child's permission. The child may have, in fact, already witnessed the collection of a blood sample from the parents or older siblings. The child will also need to assent in writing. In families and individuals who are studied directly by us, a member of our staff contacts each individual, explains the research proposal, obtains written consent, and then proceeds to draw the blood sample or arranges to have the sample drawn. Should a study participant choose to withdraw from the study, the data derived up to that point from the participant's medical or family history will remain intact in the database.

D. Protection of Subjects

1. Conveyance of Information Back to Subjects

The purpose of this research protocol is to study the metabolic profile in the brain and try to understand the differences between wakefulness and sleep. Since the studies that we are proposing are novel and we do not have any such data in the literature, most likely, we will not be able to provide information for use to the parents. However, if we find some important information, we will convey the information to the relevant members of the family.

In the event that information arises that the PI deems important to convey back to a study subject, an independent oversight committee approved by the Yale Human Investigation Committee will first review it. Permission to convey information back to a study subject will be dependent on obtaining approval from this independent oversight committee.

2. Protection from physical injury

Please refer to section above. In addition, we emphasize that in the magnet room, subjects will be observed by one member of the two to three person team. Visual and verbal contact will be maintained throughout the spectroscopy if the study calls for wakefulness. Subjects who become claustrophobic in the magnet will be removed immediately from the magnet room. Patients who experience distress in the magnetic room for any reason will be removed from the magnet room in accordance with well established procedures developed for the Magnetic Resonance Center. All members will be thoroughly familiar with the established emergency plan. A non-magnetic stretcher will be on standby next to the entrance to the magnet. Non-magnetic oral airways, Ambubag, and stethoscope are stored with the emergency stretcher in the magnet room. Should an emergency arise, one member of the spectroscopy team will telephone the emergency operator to notify the hospital's resuscitation team of the emergency in the Magnetic Resonance Center. The other members will remove the patient via stretcher from the magnet room taking the patient to the part of the Magnetic Resonance Center designated for cardiopulmonary resuscitation.

3. Confidentiality

A major concern in this study is protection of confidentiality. Of course no names or identifying information will be used in any publications. In addition, labeling of tubes and all identification of individuals during data analysis will be by code number, not name. All data will be stored in

computer files by numbers. Individuals names will appear *only* on the initial forms and primary laboratory log sheets, which are available only to senior lab personnel.

Blood samples will be stored in the PI's laboratory, or in the laboratory of the collaborating investigator who enrolls the participant into the study, for an indefinite period of time. Access to blood will be restricted to members of the PI's/or collaborating investigators laboratory's only. Under no circumstance will patient information accompany any sample outside of the laboratory, except as non-traceable encoded information.

Any data files that could link a code number to an individual study subject will be password protected and stored on removable magnetic Zip® disks. These disks will be kept in a locked drawer in the PI's office. Magnetic tape back-ups of these disks will be stored off-site in a similarly secured place. Data files that could link a code number to an individual study subject will never by copied onto a networked hard drive, or e-mailed, or sent through the internet. The source of the research material will be the analysis of the patient's spectra and historical data obtained from the patient. Data on identifiable persons are kept locked in the investigator's office. The data from an individual patient are catalogued by number only. Subjects are not individually identified in publications or oral research reports

4. Data and Safety Monitoring Plan

Patients undergoing these studies will be monitored very carefully. First, during the studies, patients will be monitored in the Magnet using electrophysiologic measures (EEG and EOG) and EKG. All data will be acquired using computerized techniques and will be saved on disc files. No personnel other than those listed will be involved with the data at any time and data will be stored in databases that are password-protected, only known to this group of investigators. It is also important to realize that the studies proposed with glucose or acetate infusions have been done in the MR Center and no new drugs or agents are used in this study. All isotopes used are stable and have been used numerously as part of studies done in the MR Center previously. One member of the two to three person team will watch the subjects while in the magnet room. Patients who experience distress in the magnetic room for any reason including claustrophobia will be removed from the magnet room in accordance with well-established procedures developed for the Magnetic Resonance Center. All members will be thoroughly familiar with the established emergency plan (see above). The assessment of risk is as follows:

1. Attribution of **adverse effects**: Unrelated to the investigational agents. It is possible however that a hematoma or a bruise occurs as a result of an IV.
2. Plan for **grading** adverse events: No adverse effects or mild adverse effects anticipated.
3. Plans for **reporting** unanticipated and anticipated **adverse** events: Serious unanticipated adverse effects will be reported immediately to the HIC and any appropriate funding and regulatory agencies.
4. Plans for **reviewing and reporting non-serious** anticipated or non-anticipated adverse events: The PI will conduct a review of all adverse effects at least quarterly. The PI will evaluate the frequency and severity of these effects and determine if modifications to the protocol or consent form are required. A chart accompanying the quarterly summary will be sent to the HIC.
5. **Safety review**: The PI and HIC are responsible for monitoring the data and conducting performance of safety reviews every 3 months. Either the PI or the HIC has the authority to stop or modify the study. The HIC will review all safety data at least once a year.

Although the type of studies proposed using the CRC or the NMR center are performed every day at Yale New haven Hospital and the Yale School of Medicine, **teaching** of key personnel about equipment used in the CRC, in the NMR center will be continued and emphasized. Also, teaching everyone involved with the children in this study about subject confidentiality will be very important.

5. Minor Children

On all young children every effort will be made to collect a minimum amount of blood necessary.

E. Potential Benefits

There are no likely benefits to the individual child. It is hoped that eventually this research will lead to the discovery of novel and specific therapeutic agents that are effective in ameliorating the disease conditions as pertaining to sleep or sleep deprivation. Any such benefit, however, would be many years into the future. The individual subject will only benefit in as much as the study will advance understanding of the basic mechanisms related to sleep and sleep deprivation.

F. Risk-Benefit Ratio

No immediate or direct benefits to subject. Potentially there will be significant benefits to society and to the subjects and members of the subjects' families should important insight into factors influencing sleep and sleep deprivation. There is only minimal risk to children and adults, essentially in the area of confidentiality and potential stigmatization. The potential benefits are also more likely to be present for subjects and their families.

F. VERTEBRATE ANIMALS- None

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I. CONSORTIUM/CONTRACTUAL ARRANGEMENTS : None